Increased Rate of Adenosine Triphosphate-dependent Etoposide (VP-16) Efflux in a Murine Leukemia Cell Line Overexpressing the Multidrug Resistance-associated Protein (MRP) Gene

Aurelio Lorico, Germana Rappa, Srinivasan Srimatkandada, Carlo V. Catapano, Daniel J. Fernandes, Joseph F. Germino, and Alan C. Sartorelli

Department of Pharmacology and Developmental Therapeutics Program [A. L. G. R. S. S., A. C. S.] and Department of Internal Medicine (Oncology) [J. F. G.], Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520, and Department of Experimental Oncology, Hollings Oncology Center, Medical University of South Carolina, Charleston, South Carolina 29425 [C. V. C., D. J. F.]

ABSTRACT

WEHI-3B/NOVO is a cloned murine leukemia cell line selected for resistance to novobiocin that is cross-resistant to the cytotoxic action of etoposide (VP-16) and to a lesser extent to a variety of other topoisomerase II (topo II)-reactive drugs. We have reported previously (Cancer Res. 52: 2782-2790, 1992) that WEHI-3B/NOVO cells exhibit a pronounced decrease in VP-16 induced DNA-topo II cross-link formation compared to the parental WEHI-3B/S cell line in intact cells, in the absence of a significant difference in the F4 unknotting activity of topo II assayed in nuclear extracts. Because the pattern of cross-resistance was suggestive of a topo II-mediated mechanism, we have ascertained whether a change in topo II can account for the multidrug-resistant phenotype of WEHI-3B/NOVO cells. No differences existed between WEHI-3B/S and WEHI-3B/NOVO cells in topo II mRNA and protein levels, as well as in the amount of topo II associated with the nuclear matrix.

Neither sensitive nor resistant cells expressed detectable levels of the MDR1 gene; however, VP-16 accumulation in WEHI-3B/NOVO cells was 3-4-fold less than that present in WEHI-3B/S cells, whereas doxorubicin accumulation was the same in both cell lines. Overexpression was associated with a greater extent in parental sensitive cells. Thus, an increased rate of efflux of VP-16 was responsible for the lower steady-state concentration of the drug in resistant cells. The efflux $K_m$ for VP-16 in WEHI-3B/NOVO cells was 254.7 $\mu$M and the $V_{max}$ was 10.4 pmol/s/10^7 cells. In the presence of the inhibitors of energy metabolism, sodium azide and deoxyglucose, the efflux of VP-16 was markedly inhibited; readdition of glucose restored the original efflux rate.

Northern blot analyses using the human 10.1 probe for the 3'-terminal region of the multidrug-resistance protein (MRP) cDNA revealed a mRNA species of approximately 6 kb in WEHI-3B/NOVO cells but not in WEHI-3B/S cells. Overexpression was associated with amplification of the cognate gene. To ascertain whether the overexpressed gene in WEHI-3B/NOVO cells was the murine MRP or a different member of the same superfamily of ATP-binding ABC cassette transporters, a 341-bp MRP cDNA probe was generated from a murine genomic library. The murine probe spanned a region corresponding to nucleotides 4367-4708 of the human cDNA sequence, with which it shared 86% nucleotide sequence homology. Using this probe, Northern blot analyses demonstrated that WEHI-3B/NOVO cells overexpressed the MRP gene relative to WEHI-3B/S cells. It has been shown clearly by others that transfection of the human MRP cDNA is sufficient to induce VP-16 resistance in several tumor cell lines. Thus, although the characteristics of the efflux mechanism in WEHI-3B/NOVO cells differ from that reported for the MRP, it is conceivable that overexpression of the murine MRP gene in WEHI-3B/NOVO cells is responsible for the increased rate of VP-16 efflux that results in decreased accumulation of drug, decreased formation of potentially lethal topo II-DNA covalent complexes and, ultimately, reduced cytotoxicity.

INTRODUCTION

The epipodophyllotoxins VP-16 and VM-26 are among the most useful of the available antineoplastic agents, with clinical activity being demonstrated both in hematological malignancies and in solid tumors (1). The anticancer activity of these agents is presumably due to their ability to stabilize the topo II enzyme intermediate covalently bound to DNA in a step that precedes DNA religation (2). This mechanism of action is shared by other antitumor agents such as the anthracyclines, aminoacridines, and ellipticines (2-4). As is the case for most chemotherapeutic agents, the efficacy of the epipodophyllotoxins in cancer therapy is limited by the occurrence of drug resistance in the targeted tumor cell population. The cellular insensitivity to drugs that act at the level of topo II is usually expressed as MDR (5). In several cell lines, MDR has been related to the overexpression of the mdr1 gene that codes for a membrane-associated glycoprotein, the P-glycoprotein, which causes a decrease in the intracellular accumulation of a variety of cytotoxic drugs, including those that inhibit topo II (5, 6).

Several cell lines display an atypical pattern of MDR; the predominant mechanism of resistance appears to be due to qualitative and/or quantitative changes in topo II rather than to alterations in drug export (7-19). The clinical relevance of these mechanisms of multidrug resistance is still uncertain. Recently, however, transport-related but P-glycoprotein-independent mechanisms of resistance have been reported (20, 21), especially for VP-16, which appears to have a lower affinity for the P-glycoprotein compared to other MDR1-related drugs (22). Cole et al. (20) have identified the MRP gene, which encodes a Mr 190,000 membrane-bound glycoprotein (23). This gene, which is overexpressed in several non-P-glycoprotein multidrug-resistant cell lines (23-25), has been cloned and the nucleotide sequence determined (20). Because it shares sequence homology with ATP-binding cassette transmembrane transporters, it has been included in this superfamily (20), which also includes the mdr1 gene. The amino acid identity between the MRP and the P-glycoprotein, however, is only 15%. A higher homology exists between MRP and other members of this superfamily, including the Leishmania cystic fibrosis transmembrane conductance regulator LtpgpA, which confers resistance to arsenite, and Y-CF1, which confers resistance to cadmium in yeast. Transfection of human MRP cDNA was sufficient to confer a multidrug-resistant phenotype in several tumor cell lines (26, 27).

Because the antineoplastic agents frequently used in the selection of...
MDR cell lines (i.e., VP-16 and doxorubicin) target topo II, are extruded from cells by the P-glycoprotein, and appear to be substrates for the MRP protein, many cell lines selected for resistance to these agents exhibit at least two different alterations capable of conferring insensitivity (10, 11, 16, 25). Such multiple changes complicate the detailed analysis of individual mechanisms in resistant cell lines.

Novobiocin is a topo II-targeted drug that is not extruded from cells through the P-glycoprotein efflux system (28); for this reason, we used this antibiotic to develop and characterize a novobiocin-resistant subline of the WEHI-3B murine monomyelocytic leukemia (29). In contrast to a relatively low level of resistance to novobiocin (1.7-fold), WEHI-3B/NOVO cells exhibit a high level of resistance to VP-16 (11-fold). Although WEHI-3B/NOVO cells displayed cross-resistance to a wide variety of anti-topo II inhibitors, namely VP-16, VM-26, doxorubicin, aclacinomycin A, m-AMSA, and elsamycin, and although the resistant phenotype seemed to result from a decrease in the drug-induced formation of covalent complexes between topo II and DNA (29), no differences in topo II enzymatic activity were detected between resistant and sensitive sublines. Furthermore, WEHI-3B/NOVO cells were not cross-resistant to vincristine (29), which is a good substrate for the P-glycoprotein.

In all non-P-glycoprotein-expressing MDR cell lines reported thus far, resistance has been associated with a reduction in drug-induced topo II-DNA covalent complexes or, upon protein denaturation, in drug-induced topo II-mediated DNA cleavage. Recent studies suggest that the functional topo II in proliferating cells is the form that is incorporated into the salt-insoluble nuclear matrix (30–32). Thus, the effects of both m-AMSA and VM-26 in CEM leukemia cells have been found to be localized in the nuclear matrix (33); moreover, in a VM-26-resistant cell line, insensitivity was related to the decreased activity of nuclear matrix topo II (34). In the present report, we have determined the potential role of topo II, in particular the importance of its association with the nuclear matrix and the potential role of the P-glycoprotein and the MRP genes in the MDR phenotype of WEHI-3B/NOVO cells.

**MATERIALS AND METHODS**

**Cell Growth Characteristics.** WEHI-3B/S and WEHI-3B/NOVO cell clones were maintained in suspension culture in McCoy’s 5A medium (GIBCO, Grand Island, NY) supplemented with 15% fetal bovine serum at 37°C in an atmosphere of 5% CO₂ in air as described previously (29).

**Immunoblotting.** WEHI-3B/S and WEHI-3B/NOVO cells in exponential growth were washed with cold PBS containing a mixture of five protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of soybean trypsin inhibitor, 1 mM benzamidine, 2 μg/ml of aprotinin, and 50 μg/ml of leupeptin). The cell pellet was then lysed with 2% SDS in PBS containing the five protease inhibitors. Although WEHI-3B/NOVO cells displayed cross-resistance to a wide variety of anti-topo II inhibitors, namely VP-16, VM-26, doxorubicin, aclacinomycin A, m-AMSA, and elsamycin, and although the resistant phenotype seemed to result from a decrease in the drug-induced formation of covalent complexes between topo II and DNA (29), no differences in topo II enzymatic activity were detected between resistant and sensitive sublines. Furthermore, WEHI-3B/NOVO cells were not cross-resistant to vincristine (29), which is a good substrate for the P-glycoprotein.

**In vitro studies.** pleiotropy. The relative amounts of specific proteins present on Western blots of WEHI-3B/S and WEHI-3B/NOVO cells were quantified using a Visage 2000 image analysis system (Biorad, Ann Arbor, MI).

**Isolation of the Nuclear Matrix.** For the preparation of cell membranes, nuclei were centrifuged at 1,000 × g for 10 min at 4°C, and the supernatants were layered over 5 ml of a buffer containing 35% sucrose, 0.01 M Tris-HCl (pH 7.5), and 1 mM EDTA and centrifuged at 16,300 × g for 30 min at 4°C. The interface was diluted 1:5 with 0.25 M sucrose containing 0.01 M Tris-HCl (pH 7.5) and subsequently centrifuged at 100,000 × g for 45 min at 4°C. The pellet was resuspended in 0.25 M sucrose containing 0.01 M Tris-HCl (pH 7.5), passed several times through a 25-gauge needle, and used immediately or stored at −70°C for later use.

Nuclear matrices were prepared by a modification of the procedure described earlier (35). Briefly, 8 × 10⁶ cells in exponential growth were harvested and washed once in serum-free medium. The cell pellets were resuspended in homogenization buffer (10 mM Tris-HCl (pH 7)-2 mM MgCl₂-1 mM phenylmethylsulfonyl fluoride-10 μg/ml of soybean trypsin inhibitor-1 mM benzamidine-2 μg/ml of aprotinin-50 μg/ml of leupeptin) and allowed to swell for 15 min on ice. The cells were then disrupted by 25 strokes in a Dounce homogenizer. The cell lysates were layered over a solution of 45% sucrose (w/v) at 4°C and centrifuged at 1900 × g for 30 min at 4°C. The nuclear pellets were resuspended in 1 ml of low salt buffer (10 mM Tris-HCl (pH 7)-1 mM MgCl₂-10 mM NaCl-1 mM phenylmethylsulfonyl fluoride-10 μg/ml of soybean trypsin inhibitor-1 mM benzamidine-2 μg/ml of aprotinin-50 μg/ml of leupeptin). One ml of high salt buffer (10 mM Tris-HCl (pH 7)-0.2 mM MgCl₂-1 mM phenylmethylsulfonyl fluoride-10 μg/ml of soybean trypsin inhibitor-1 mM benzamidine-2 μg/ml of aprotinin-50 μg/ml of leupeptin, containing either 2 mM NaCl or 0.5 mM ammonium sulfate) was then added over a period of 1 h with gentle shaking. After an additional 30 min of incubation on ice, DNase I (2000 units) was added, and the samples were incubated for 30 min at 37°C. The resulting pellets were resuspended in 10 ml of high salt buffer containing 1.0 mM NaCl or 0.25 mM ammonium sulfate, centrifuged at 7000 × g for 15 min at 4°C, and then washed an additional time with 10 ml of low salt buffer at 4°C. The final nuclear matrix pellets were resuspended in 50 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM DTT, and the above listed protease inhibitors.

**Transport of [³H]VP-16.** The cellular fluxes of radiolabeled VP-16 were measured according to methodology described earlier (28). The viability of cells after exposure to investigational drugs was always assessed by the exclusion of trypan blue. At the indicated times after the onset of drug treatment, 100 μl of cell suspension were added to a tube containing 10 ml of ice-cold PBS, and the cells were collected by centrifugation. An aliquot of the supernatant was saved for measurement of extracellular radioactivity, and the pellet was resuspended in 60 μl of ice-cold PBS and placed in an “oil stop” tube consisting of a 400-μl Eppendorf microfuge tube containing 125 μl of oil (16% Fisher 0121 light paraffin oil and 84% Dow Corning 550 silicon fluid, with a final specific gravity of 1.04 g/ml) layered over 30 μl of 15% trichloroacetic acid and immediately centrifuged for 30 s at 10,000 g using a Beckman Model B microfuge. Microfuge tubes were cut through the oil layer, and the lower parts placed in glass mini scintillation vials. After the addition of 200 μl of distilled water, vials were vortexed vigorously, 5 ml of scintillation cocktail were added, and radioactivity therein was measured by scintillation spectrometry. Zero-time binding, measured by adding radiolabeled VP-16 to the cells prechilled on ice, was always less than 1%. The intracellular volume of WEHI-3B/S cells was calculated for untreated cells and for cells exposed to different concentrations of drugs using 3H₂O to determine the total water space and [¹⁴C]inulin to calculate extracellular space (36).

**Construction and Screening of Genomic DNA Library.** Genomic DNA was extracted from WEHI-3B/S and WEHI-3B/NOVO cells by standard procedures (35), subsequently cleaved by BamHI, and ligated to BamHI-digested ZAP Express vector (Stratagene, La Jolla, CA). Packaging and amplification of the library were accomplished by standard procedures (35). Total plaques (2 × 10⁸) were screened under conditions of low stringency of hybridization with the human 10.1 MRP cDNA probe. The inserts from the positive plaques were recovered by PCR using T3 and T7 as primers. The inserts were sequenced by the dye termination method (35), and the sequence information was compared to the sequence of human MRP cDNA by computer analysis using the University of Wisconsin Genetics software package to determine the degree of homology.
Preparation of the Murine MRP Probe. One µg of total RNA, isolated by the method of Chomczynski and Sacchi (37) from WEHI-3B/NOVO 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, dCTP, dGTP, and dTTP) containing 2 units/µl of RNase inhibitor (Promega, Madison, WI), 0.003 A₂₆₀ random hexanucleotides (Boehringer Mannheim, Indianapolis, IN), and 0.4 unit/µl of Moloney murine leukemia virus reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT). After incubation for 10 min at 25°C and for 15 min at 42°C, the reaction was terminated by incubation for 5 min at 99°C. A 341-bp fragment of murine MRP cDNA was subsequently amplified from the resulting DNA mixture by PCR with the following specific primers: 5′-primer, TGC-AGT-CAG-TGT-CCT-GAT-GA; 3′-primer, TTC-TCC-TTT-GTC-CAG-GAC. The fragment was cloned in the PCR vector (Invitrogen, San Diego, CA), cut with EcoRI, separated on a 2% agarose gel, and recovered and purified from the gel by the Gene Clean II kit procedure (Biol 101, Inc., Vista, CA). The fidelity of the murine MRP sequence was confirmed by DNA sequence analysis.

Northern Blotting. Northern blotting was performed using standard techniques (35). The human topo II cDNA probe pBS-hTop2 (38) and the human topo I DNA probe (39) were gifts from Dr. J. C. Wang (Harvard University, Boston, MA). The human MRP probe, a 1.0-kb EcoRI cDNA fragment (20), was donated by Dr. S. Cole (Queen’s University, Kingston, Canada). The human MDR, cDNA probe, phaMDR/A (40), was a gift from Dr. M. G. Gottesman (National Cancer Institute, Bethesda, MD). Radiolabeling of these probes was performed using the Amersham Multiprime labeling system (Amersham, Arlington Heights, IL). The blots were hybridized at 42°C overnight with the radiolabeled probe in 50% formamide. After washings, the membranes were exposed to X-ray film at −70°C with intensifying screens.

Southern Blotting. Genomic DNA (15 µg) from WEHI-3B/S and WEHI-3B/NOVO cells was completely digested with EcoRI, HindIII, BamHI, PstI, or BglII, subjected to electrophoresis on a 0.7% agarose gel, and blotted onto a nitrocellulose membrane. The DNA was hybridized at 42°C overnight in 50% formamide with the radiolabeled probe and washed at 42°C. The membranes were exposed to X-ray film at −70°C with intensifying screens.

RESULTS

Patterns of Cross-Resistance. We have reported previously that, in addition to an approximately 2-fold level of resistance to novobiocin, the WEHI-3B/NOVO subline exhibited 7- and 11-fold levels of cross-resistance to the topo II-targeted drugs VM-26 and VP-16, respectively, and about 2-fold resistance to the intercalating topo II-reactive drugs doxorubicin, m-AMSA, and aclacinomycin A, whereas sensitivity to the cytotoxic action of the non-topo II-acting agents camptothecin and vincristine was not altered (29). We have now extended the analysis of the pattern of cross-resistance to include the antimitabolite 1β-d-arabinofuranosylcytosine, the VP-16 analogue W-68 (the substitution of the glycosidic moiety of which by an arylamino group has made it more cytotoxic toward cells that overexpress the P-glycoprotein while leaving its topo II-inhibitory activity unchanged; Ref. 41), sodium arsenite, and colchicine, which variability was less than 10%.

Expression of Topo II. In several non-P-glycoprotein-mediated multidrug-resistant cell lines, drug insensitivity has been associated with a decreased level and/or activity of topo II. Measurement of the catalytic activity of topo II by assaying the ATP-dependent unknotted line P4 DNA did not demonstrate differences in enzyme activity between the two cell lines (29). Similar levels of the M₇, 170,000 topo II were observed in cell lysates from WEHI-3B/S and WEHI-3B/NOVO cells (Fig. 1, left panel) using monoclonal antibodies raised against a synthetic peptide from the human M₇, 170,000 topo II sequence (45). An antiserum raised in rabbits against the native purified p170 form of topo II from the murine P388 leukemia (44, 46) also gave analogous results (data not shown). To determine whether the equivalent levels of topo II in the sensitive and resistant clones corresponded to an equal expression of the topo II gene, Northern analyses were conducted using a band-depletion immunoblotting assay to verify that the forma

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VP-16 concentration (µM)</th>
<th>Percent of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI-3B/S</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>74.7</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>49.4</td>
<td></td>
</tr>
<tr>
<td>WEHI-3B/NOVO</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25</td>
<td>98.3</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*Cells (10⁶ cells/ml) in logarithmic growth were incubated with 25 or 50 µM VP-16 for 30 min at 37°C. Cells were washed, lysed as described in “Materials and Methods,” and 25 mg of lyase protein were subjected to 7.5% SDS-PAGE, after which the proteins were immunoblotted using an antisera against the M₇, 170,000 isoform of topo II. The membranes were analyzed by densitometry using a V手法的成像分析系统。Controls were normalized to 100%. Results are the mean of two separate experiments in which variability was less than 10%.*

Expression of MDR. We have shown that WEHI-3B/NOVO cells are not cross-resistant to the MDR-related drugs vincristine and colchicine (29), which are substrates for the P-glycoprotein. Furthermore, the P-glycoprotein was not detectable on the surface of WEHI-3B/S and WEHI-3B/NOVO cells by flow cytometric analyses of immunostained cells (28). To obtain further evidence that the P-glycoprotein was not expressed in WEHI-3B cells, both WEHI-3B/S and WEHI-3B/NOVO cells were analyzed for the expression of MDR₁ mRNA. Northern blotting, using a human cDNA probe known to cross-hybridize well with murine MDR₁ and MDR₂ (40), did not detect MDR₁ mRNA in either cell line (results not shown).

Band Depletion Immunoblotting. Because the resistant phenotype of the WEHI-3B/NOVO leukemic clone appears to result from a decrease in the drug-induced formation of DNA-protein cross-links, as measured by the potassium-SDS precipitation assay (29), we have used a band-depletion immunoblotting assay to verify that the formation of VP-16-stabilized covalent complexes between the M₇, 170,000 isoform of topo II and DNA was decreased in the resistant subline compared to parental cells. Several groups have shown that topoisomerases can be prevented from entering polyacrylamide gels by their drug-induced cognate binding to DNA (42, 43). When intact WEHI-3B/S cells were exposed to different concentrations of VP-16 for 30 min at 37°C and then lysed with an SDS-containing solution, the endogenous complexes between the M₇, 170,000 isoform of topo II and DNA, stabilized at the time of the addition of SDS, were covalently linked, and the DNA-bound M₇, 170,000 form of topo II was partially prevented from entering the gel. However, VP-16 treatment only minimally depleted the bands from WEHI-3B/NOVO cells. Thus, densitometric analyses showed that 50.6% of the M₇, 170,000 topo II band was depleted in WEHI-3B/S cells by a 30-min period of exposure to 50 µM VP-16, whereas no depletion occurred in WEHI-3B/NOVO cells (Table 1). The same experimental approach was used to analyze the effects of VP-16 on the M₇, 170,000 isoform of topo II. No band depletions were observed up to 100 µM VP-16 in either WEHI-3B/S or WEHI-3B/NOVO cells (data not shown), presumably reflecting a lower affinity of VP-16 for the M₇, 180,000 form of the enzyme (44).
duted with human top II cDNA. Consistent with previous reports (38, 39), the top II-specific probe hybridized to an RNA species of approximately 6.2 kb (Fig. 2A), whereas the top I-specific probe hybridized to an RNA species of approximately 4.1 kb (Fig. 2B). Both top I and II mRNA levels were very similar in the two cell lines. In these studies, γ-actin was used as an internal standard.

**Nuclear Matrix-associated Topo II.** One of our laboratories has shown previously that the amount of immunoreactive top II in the nuclear matrices of multidrug-resistant CEM/VM-1 cells was decreased more than 3-fold relative to that of parental CEM cells, whereas no significant difference occurred in the amount of enzyme present in the nonmatrix fraction of nuclei from these cell lines. Therefore, we have ascertained whether this was also the case for WEHI-3B/NOVO cells. In Fig. 1, right panel, is shown a representative Western blot that was obtained after electrophoresis of 50 μg of matrix protein isolated from each cell line with either a NaCl- or an ammonium sulfate-containing solution. Quantitation of the relative amounts of immuno reactive top II in 7 immunoblots of 5 different matrix preparations was obtained by scanning the positive films of the Western blots using a Visage 2000 scanner; these measurements demonstrated that the amounts of immunoreactive top II in the nuclear matrices of WEHI-3B/NOVO cells was equivalent to that present in WEHI-3B/S cells.

**Drug Transport Studies.** Recently, P-glycoprotein-independent transport-related mechanisms of resistance have been proposed (21, 47), especially for VP-16, which appears to have a lower affinity for the P-glycoprotein than that of other MDR1-related drugs (22). The few reports of the membrane transport of VP-16 suggest that the drug enters cells by passive diffusion and is actively extruded by the P-glycoprotein in cells in which this protein is expressed (48, 49). To ascertain whether a difference in drug transport might be responsible for the resistant phenotype of WEHI-3B/NOVO cells, we measured the cellular uptake of VP-16, demonstrating that in WEHI-3B/NOVO cells the steady-state levels of [3H]VP-16 were 3--4 times lower than in parental WEHI-3B/S cells (Fig. 3a). In contrast, no difference was observed between WEHI-3B/S and WEHI-3B/NOVO cells in the accumulation of doxorubicin, as measured by flow cytometric analysis of cells exposed to doxorubicin for 2 h (Fig. 3b). Furthermore, no difference occurred between parental and resistant sublines in the accumulation of the antimetabolite [3H]5-fluoro-2′-deoxyuridine (data not shown).

In both sensitive and resistant WEHI-3B cells, intracellular VP-16 binding did not appear to play an important role in the accumulation of the epipodophyllotoxin because the noneffluxable pool of VP-16 was found to be extremely small (50); therefore, differences in the binding of this agent to cellular components do not appear to play a role in the phenomenon of resistance to VP-16 in WEHI-3B/NOVO cells. Measurement of the bidirectional fluxes of [3H]VP-16 were conducted to ascertain whether a decrease in the rate of uptake or an increase in the rate of efflux, or both, were responsible for the decrease in the steady-state accumulation of VP-16. No difference existed in the rate of uptake of VP-16 between parental and resistant cells over the first 60 s (data not shown). Beyond the first 60 s of incubation, however, [3H]VP-16 began to accumulate to a greater extent in parental cells, reaching a plateau at 20 min (Fig. 4). These findings implied that a change in the rate of efflux of VP-16 was responsible for the marked decrease in the steady-state levels of this agent in WEHI-3B/NOVO cells. To determine whether this was indeed the case, WEHI-3B/S and WEHI-3B/NOVO cells were loaded to the same intracellular concentration of [3H]VP-16 by incubation for 30 min with 3.3 and 10 μM VP-16, respectively. Cells were then washed free of excess drug at 0°C and resuspended in VP-16-free medium at 37°C. The rate of efflux of VP-16 in WEHI-3B/NOVO cells was considerably faster than in WEHI-3B/S cells (Fig. 5), indicating that in WEHI-3B/NOVO cells a membrane protein responsible for the export of VP-16 was operative.

Although the P-glycoprotein is not expressed in WEHI-3B/NOVO cells, the possibility that an alternative ATP-dependent pump analogous to the P-glycoprotein was present in WEHI-3B/NOVO cells was examined. To determine whether VP-16 transport in WEHI-3B/NOVO cells was ATP dependent, we inhibited cellular energy metabolism by incubation of cells in medium without glucose that contained 10 mM sodium azide and 1 mg/ml of 2-deoxy-d-glucose. The intracellular concentration of ATP was measured by HPLC on a Whatman Partisil 10 SAX anion-exchange column and was found to be decreased by greater than 90% after incubation for 20 min in the presence of sodium azide. Under these conditions of energy deprivation, the rates of VP-16 efflux from WEHI-3B/NOVO and WEHI-3B/S cells were measured (Fig. 5). Cells were loaded with radiolabeled VP-16 for 30 min by exposure to the epipodophyllotoxin in the presence of sodium azide and 2-deoxy-d-glucose, subsequently washed with ice-cold PBS, and resuspended in drug-free medium at 37°C in the presence or absence of sodium azide and 2-deoxy-d-glucose.
MECHANISM OF VP-16 RESISTANCE

A. 

Topo II - 28S -

18S -  γ-Actin

B. 

28S -  Topo I -

18S - γ-Actin

Fig. 2. Northern blot analyses of topo I and II mRNAs from WEHI-3B/NOVO and WEHI-3B/S cells. Twenty μg of total RNA from WEHI-3B/NOVO and WEHI-3B/S cells were hybridized with cDNAs for topo II (A), topo I (B), and γ-actin as an internal control. Topo II, topo II (approximately 6.2 kb) mRNA; Topo I, topo I (approximately 4.1 kb) mRNA; 28S and 18S, positions of rRNA species. Similar results were obtained in several replicate experiments.

STATE-STATE CELLULAR LEVELS OF [3H]VP-16 (A) AND DOXORUBICIN (B) IN WEHI-3B/S (O) AND WEHI-3B/NOVO (D) CELLS EXPOSED TO VARIOUS EXTRACELLULAR CONCENTRATIONS OF DRUG. Exposures to [3H]VP-16 were for 30 min, which resulted in the attainment of steady-state levels of VP-16, and intracellular radioactivity was measured as described in "Materials and Methods." Exposure to doxorubicin was for 2 h, and doxorubicin fluorescence was measured by flow cytometry, and the results expressed in arbitrary units of fluorescence. Points, mean of two separate experiments in which the variability was less than 10%.

Fig. 3. Time course of the influx of VP-16 by WEHI-3B/S (C) and WEHI-3B/NOVO (D) cells. Cells were incubated with 4 μM [3H]VP-16 as described in "Materials and Methods," and intracellular radioactivity was measured at different times thereafter. Points, mean of 2–3 experiments in which the variability was less than 10%.

VP-16 in WEHI-3B/NOVO cells was found to be 254.7 μM, whereas the V_max was 10.4 pmol/s/10^7 cells (Fig. 6).

Many well known ATP-dependent membrane carriers, including active nucleoside transporters, require the presence of extracellular sodium for their function (36). To ascertain whether the transport of VP-16 in WEHI-3B/NOVO cells was sodium dependent, VP-16 accumulation was measured in the absence of extracellular sodium after washing cells twice with Na^+-free HBSS containing 0.15 M choline chloride as the counterion instead of 0.15 M NaCl, and conducting the measurements in HBSS containing choline chloride. This procedure is sufficient to reduce the extracellular Na^+ concentration to less than 5% of its normal concentration (36). Under these conditions, the steady-state concentrations of [3H]VP-16 were 1.3 ± 0.16 (mean ± SD) μM in the presence of sodium and 1.1 ± 0.07 μM in sodium-depleted medium (difference not statistically significant by Student's t test), indicating that the VP-16 transporter operative in WEHI-3B/NOVO cells is not sodium dependent.

Expression of MRP. The probe 10.1, complementary to a 1-kb region of the 3' end of the human MRP cDNA, hybridized to an mRNA species of approximately 6 kb present in WEHI-3B/NOVO cells but not in parental WEHI-3B/S cells (Fig. 7A). Southern blot analyses were used to ascertain whether the selective expression of this gene in the resistant cell line was attributable to amplification of the MRP gene (Fig. 8). Genomic DNA (15 μg) from WEHI-3B/S and WEHI-3B/NOVO cells was completely digested with EcoRI, HindIII, BamHI, PstI, or BglII. After electrophoresis through a 0.7% agarose gel and blotting, the DNA was hybridized with probe 10.1 or with the pBS-hTop2 probe. The probes were subsequently stripped from the
overexpressed in WEHI-3B/NOVO cells compared to the parental strain, which it shared 86% nucleotide sequence homology. A representative Northern blot hybridizations. The probe spanned a region corresponding to nucleotides 4367-4708 of the human cDNA sequence, with murine regions of homology with the human MRP cDNA. The 341-bp fragment was cloned as described in "Materials and Methods," sequenced by the dye termination method (35) and used as a probe in Southern analysis with WEHI-3B/NOVO DNA (Fig. 8). Partial sequence analysis revealed that the two inserts were identical. Two regions of 100 and 150 nucleotides were 80-85% homologous to a corresponding region of the terminal part of the human MRP cDNA and 100% homologous to the murine MRP cDNA. A 341-bp fragment of the murine MRP cDNA was then amplified by reverse transcriptase-PCR from the total RNA of WEHI-3B/NOVO cells, using as specific primers two oligonucleotides based on the nucleotide sequence of the murine regions of homology with the human MRP cDNA. The 341-bp fragment was cloned as described in "Materials and Methods," sequenced by the dye termination method (35) and used as a probe in Northern blot hybridizations. The probe spanned a region corresponding to nucleotides 4367-4708 of the human cDNA sequence, with which it shared 86% nucleotide sequence homology. A representative experiment shown in Fig. 7B illustrates that the murine MRP gene was overexpressed in WEHI-3B/NOVO cells compared to the parental strain.

filters, which were then rehybridized with a GAPDH probe as an internal control. A comparison of the intensity of the bands, normalized for the GAPDH signal, revealed that the gene recognized by the anti-MRP probe was amplified 2.5 ± 0.3-fold (mean ± SE) in WEHI-3B/NOVO cells compared to parental WEHI-3B/S cells, whereas no change was detected in the copy number of the top II gene. For the restriction enzymes used, no qualitative differences were noted in the hybridization pattern between resistant and parental cells (Fig. 8).

To ascertain whether the overexpressed gene in WEHI-3B/NOVO cells was the murine MRP or a different member of the same superfamily of ATP-binding ABC cassette transporters, we adopted the following strategy. A BamHI-digested genomic library was constructed from WEHI-3B/NOVO cells in the ZAP Express vector, and the library was screened with the human 10.1 MRP cDNA probe under conditions of low stringency of hybridization. The screening of the WEHI-3B/NOVO genomic library resulted in the identification of two plaques. The insert size in both cases was 1.7 kb, corresponding to the size of the lower band obtained by Southern analysis with BamHI-digested WEHI-3B/NOVO DNA (Fig. 8). Partial sequence analysis revealed that the two inserts were identical. Two regions of 100 and 150 nucleotides were 80-85% homologous to a corresponding region of the terminal part of the human MRP cDNA and 100% homologous to the murine MRP cDNA. A 341-bp fragment of the murine MRP cDNA was then amplified by reverse transcriptase-PCR from the total RNA of WEHI-3B/NOVO cells, using as specific primers two oligonucleotides based on the nucleotide sequence of the murine regions of homology with the human MRP cDNA. The 341-bp fragment was cloned as described in "Materials and Methods," sequenced by the dye termination method (35) and used as a probe in Northern blot hybridizations. The probe spanned a region corresponding to nucleotides 4367-4708 of the human cDNA sequence, with which it shared 86% nucleotide sequence homology. A representative experiment shown in Fig. 7B illustrates that the murine MRP gene was overexpressed in WEHI-3B/NOVO cells compared to the parental strain.

### DISCUSSION

Many cell lines, in addition to the WEHI-3B/NOVO cell line described in this report, that have a non-P-glycoprotein-mediated MDR phenotype have been characterized recently (7-25). In these cell lines, resistance was generally induced by exposure of cells to compounds that are mutagenic in mammalian cells (i.e., VP-16, VM-26, doxorubicin, m-AMSA, or an ellipticine; Refs. 51-54). Thus, most non-P-glycoprotein-overexpressing MDR cell lines described thus far appear to have mixed phenotypes with more than one of the following changes: (a) a low level and/or activity of top II; (b) an increased expression of MRP; (c) an increased content of glutathione and/or glutathione S-transferase activity; and/or (d) a change in the level of drug-metabolizing enzymes. In contrast, WEHI-3B/NOVO cells were developed by exposure to novobiocin, which is presumed to be nonmutagenic (55). Therefore, the risk of genotypic changes unrelated to the resistant phenotype, or of mixed mechanisms of resistance should be very low. In fact, novobiocin-sensitive and novobiocin-resistant clones of WEHI-3B displayed the same growth rate, cell cycle distribution, cell size, DNA and protein contents, cloning efficiency, and intracellular ATP levels.

In virtually all non-P-glycoprotein MDR cell lines, a decrease in the formation of drug-induced top II-DNA covalent complexes or DNA cleavage has been reported, generally with a good correlation being exhibited between the degree of resistance to the formation of these complexes and the level of insensitivity to the cytotoxic properties of the drug under study. We have reported previously that WEHI-3B/NOVO cells exhibit a pronounced decrease in VP-16 induced DNA-topo II cross-links in intact cells, compared to parental sensitive WEHI-3B/S cells (29). In the present report, we extend these findings by showing that the amount of VP-16-stabilized covalent complexes between the Mr 170,000 isoform of top II and chromatin DNA is less in WEHI-3B/NOVO compared to WEHI-3B/S cells. Although these observations, together with the pattern of cross-resistance that was exhibited only by topo II-targeted drugs, were suggestive of an alteration in topo II as the predominant cause of resistance, we did not detect any difference in the P4 unknotted activity of topo II from WEHI-3B/S and WEHI-3B/NOVO cells (29). Furthermore, the amount of VP-16-induced cleavable complexes in nuclear extracts from the two cell lines was equivalent, as were the ATP requirements of the top II extracted from the two cell lines (29). We now report that WEHI-3B/S and WEHI-3B/NOVO cells have equal intracellular levels of the enzyme, as well as the same steady-state levels of top II mRNA.

Fernandes et al. (34) have reported that the level and activity of top II in isolated nuclear matrices from VM-26-resistant CEM/V1 leukemia cells were decreased relative to the sensitive parental line, 

---

4 R. G. Deeley, personal communication.
MECHANISM OF VP-16 RESISTANCE

A.

Fig. 7. Northern blot analyses of MRP mRNA from WEHI-3B/NOVO and WEHI-3B/S cells. A, 15 (Lanes 1 and 3) or 30 (Lanes 2 and 4) µg of total RNA were hybridized with a 1.0-kb EcoRI fragment of the human MRP cDNA. B, 15 µg of total RNA were hybridized with a 341-bp probe from the murine MRP cDNA. GAPDH was used as an internal control. Similar results were obtained in several replicate experiments.

whereas the amount of extractable nuclear topo II was unchanged. It was suggested that a mutation in topo II may have altered the association of this enzyme with the newly synthesized DNA of the nuclear matrix, leading to a decrease in the interaction between topo II-targeted drugs and matrix-bound enzyme. Our finding that WEHI-3B/NOVO cells are highly resistant to the formation of covalent complexes induced by VP-16 (29) in relatively newly synthesized DNA (30 min labeling in the presence of VP-16) suggested that in these cells, as in CEM/VM-1 cells, an alteration in the association of topo II with the nuclear matrix was the basis of the resistant phenotype. Preparations of nuclear matrices from WEHI-3B/S and WEHI-3B/NOVO cells contained equal amounts of immunoreactive topo II. The state of phosphorylation of topo II has been shown to be associated with drug resistance, with phosphorylation of topo II resulting in a reduction in the levels of VP-16-induced topo II-DNA covalent complexes (56–57). Moreover, a mutation in the topo II α gene in the absence of an enzymatic defect has been reported to be associated with VP-16 resistance in the human FEM-X melanoma cell line (58). However, our observation that cleavable complex formation is essentially the same in nuclear extracts prepared from WEHI-3B/S and WEHI-3B/NOVO cells (29), rules against the presence of differences in the state of phosphorylation or mutations of the enzyme in the two cell lines. Therefore, the two most studied mechanisms of multidrug resistance (i.e., overexpression of the P-glycoprotein and alteration of the activity of topo II) are most likely not operative in WEHI-3B/NOVO cells. We have also found that neither the parental nor the resistant WEHI-3B clones express detectable levels of the p110 protein (data not shown), which has also been implicated in non-P-

b

Fig. 8. Southern blot analyses of genomic DNA from WEHI-3B/NOVO (R) and WEHI-3B/S (S) cells digested with EcoRI (E), HindIII (H), BamHI (B), PstI (P), or BglII (Bg). The DNA was hybridized with the pBS-8Top2 anti-topo II probe (a) or with the 1.0-kb EcoRI fragment of the human MRP cDNA (b) as described in “Materials and Methods.” Molecular size standards are shown on the left. Exposure was for 12 days at −70°C using an intensifying screen.
glycoprotein-mediated multidrug resistance (21). Moreover, WEHI-3B/NOVO cells are not cross-resistant to vincristine, which is one of the agents to which p110-overexpressing cell lines exhibit cross-resistance, and which was used as the selecting agent for the resistant cell line to which an antibody to the \( M_2 \), 110,000 protein was developed.

The possibility that an alteration in drug transport was responsible for the resistant phenotype of WEHI-3B/NOVO cells was then investigated. We reported previously (29) that only a small decrease in the accumulation of \([\text{H}]\text{VP-16}\) was detectable in WEHI-3B/NOVO cells compared to the parental strain. Subsequently, however, we have found that a high zero-time binding of \([\text{H}]\text{VP-16}\) associated with the "cold" method (50) used interfered with the assay. The methodology that we have used in the present study to measure the intracellular transport of \([\text{H}]\text{VP-16}\) in WEHI-3B/S and WEHI-3B/NOVO cells combines the cold stop (36) with the cold method (50), a procedure that reduces the zero-time binding of \([\text{H}]\text{VP-16}\) to less than 1% of its steady-state concentration, allowing accurate measurement of this transport activity in WEHI-3B/NOVO cells, which accumulate low levels of \([\text{H}]\text{VP-16}\). We have found that an increase in the efflux rate of VP-16 in WEHI-3B/NOVO cells was responsible for a 3-4-fold decrease in the steady-state levels of VP-16 in these resistant cells compared to the parental sensitive subline. The efflux of VP-16 appears to be mediated by an energy-dependent, non-sodium-dependent saturable transporter with a relatively low affinity for VP-16, as evidenced by a relatively high \( K_m \).

Recently, by analyzing the intracellular accumulation of daunorubicin in MRP-transfected cells, Zaman et al. (59) have concluded that MRP is a plasma membrane drug-efflux pump. Northern blot analyses using both human and murine probes for the 3'-terminal portion of MRP cDNA revealed a mRNA species of approximately 6 kb in WEHI-3B/NOVO cells but not in the parental WEHI-3B/S cell line. Overexpression of this gene is at least partially due to amplification of the cognate gene. To prove that this was indeed the murine MRP, we screened a genomic DNA library from WEHI-3B/NOVO cells with the human MRP probe and isolated a clone containing two regions of 100–150 nucleotides with 80% nucleotide homology to the human MRP cDNA and 100% homology to the murine MRP cDNA. On the basis of this sequence information, we amplified by reverse transcriptase-PCR a 341-bp fragment from total RNA of WEHI-3B/NOVO cells, cloned the fragment in an appropriate vector, and used it as a probe in Northern blotting analyses, confirming that the MRP gene was indeed overexpressed in WEHI-3B/NOVO cells, relative to parental WEHI-3B/S cells.

Our findings that WEHI-3B/NOVO cells are not cross-resistant to vincristine, colchicine, and arsenite, and that the low level of resistance to doxorubicin is not associated with an alteration in the intracellular accumulation of this anthracycline, differ from the pattern of cross-resistance reported for most human MRP-overexpressing cell lines, as well as for tumor cell lines transfected with the human MRP cDNA, which become resistant to Vinca alkaloids, colchicine, anthracyclines, and the epipodophyllotoxin derivatives VP-16 and VM-26.

Two explanations can be considered for this discrepancy: (a) it is possible that the native murine MRP gene preferentially encodes for epipodophyllotoxin, rather than Vinca alkaloid and anthracycline, resistance. A lack of significant cross-resistance to doxorubicin has actually been reported by Slapak et al. (60) in vincristine-selected murine erythroleukemia cells that overexpress the MRP gene. Moreover, because the level of primary resistance is low in WEHI-3B/NOVO cells, cross-resistance to vinblastine and colchicine may only be detectable at higher levels of primary resistance and/or after higher levels of MRP expression; or (b) another gene(s), possibly coamplified with the MRP gene, might be responsible for the primary resistance to VP-16 and VM-26 and for the low level of primary resistance to novobiocin. Preliminary studies from our laboratory on an MRP-transfected cell line suggest that novobiocin is not a substrate for the MRP pump, strengthening the possibility that another not yet discovered gene is at least partially responsible for the pattern of resistance of WEHI-3B/NOVO cells.

In conclusion, we have shown that the predominant mechanism of resistance to VP-16 in WEHI-3B/NOVO leukemia cells consists of an increase in the rate of ATP-dependent VP-16 efflux, which results in a lower intracellular accumulation of VP-16, that in turn results in a lower number of potentially lethal VP-16 stabilized covalent complexes between the \( M_2 \), 170,000 isoform of top II and DNA, and that this phenotype is associated with the overexpression of the MRP gene. Transfection of full-length murine MRP cDNA in the parental WEHI-3B/S clone, currently ongoing in our laboratory, should clarify whether the overexpression of the murine MRP gene is able to confer a multidrug-resistant phenotype analogous to that of WEHI-3B/NOVO cells.

ACKNOWLEDGMENTS

The authors thank Drs. F. Drake and G. Astaldi-Ricotti for the anti-topo II antibodies, Dr. S. P. C. Cole for the MRP probe, Dr. R. E. Handschumacher for helpful discussions, Dr. R. G. Decle for sharing with us data on the murine sequence of the MRP cDNA, and V. Vellucci for technical advice.

REFERENCES


Increased Rate of Adenosine Triphosphate-dependent Etoposide (VP-16) Efflux in a Murine Leukemia Cell Line Overexpressing the Multidrug Resistance-associated Protein (MRP) Gene

Aurelio Lorico, Germana Rappa, Srinivasan Srimatkandada, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/55/19/4352

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.