Atypical Multiple Drug Resistance in a Human Leukemic Cell Line Selected for Resistance to Teniposide (VM-26)

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ABSTRACT

Resistance to the cytotoxic effects of many natural product drugs after exposure to a single agent is a common observation. The classes of drugs included in the “classic” multiple drug resistance phenotype are Vinca alkaloids, anthracyclines, epipodophyllotoxins, and antibiotics. We report here the characterization of a human leukemic cell line (CEM/VM-1) with “atypical” multiple drug resistance: despite resistance and cross-resistance to etoposide, anthracyclines, mitoxantrone, and 4’-[(9-acridinyl)amino]methanesulphon-m-anisidide (mAMSA), these cells retain sensitivity to the Vinca alkaloids. Further, even though this even cell line is ≈40-fold cross-resistant to the cytotoxic effect of etoposide (VP-16), it is similar to drug-sensitive CEM cells in the cellular pharmacology of [1H]VP-16 as determined by zero time binding, initial influx rate, steady state drug concentration, and unidirectional efflux. Our studies suggest that the resistance of CEM/VM-1 cells to epipodophyllotoxins is due to an altered interaction between drug and its cellular target(s) by a mechanism unrelated to the decreased cellular concentration of drug associated with the “classic” multiple drug resistance phenotype.

INTRODUCTION

The simultaneous resistance to several classes of “natural product” drugs in vivo and in vitro after exposure to a chemically dissimilar agent is termed MDR. This is most frequently seen with cells selected for resistance to alkaloids or to anthracyclines. Compared to drug-sensitive cells, MDR cells usually have a decreased steady state drug level (1–5), decreased retention of drug (1, 6, 7), and specific changes in plasma membrane glycoproteins (8–11). The relationship between the pharmacological and biochemical changes is unclear. The mechanism by which MDR cells accumulate and/or retain less drug than sensitive cells is also unknown.

Resistance or cross-resistance to the epipodophyllotoxins (semisynthetic derivatives of the “natural product” podophyllotoxin) and its relationship to MDR has been studied in cell lines developed in several laboratories (12–20). While most of these lines showed decreased drug accumulation and cross-resistance to the Vinca alkaloids as is typical of the MDR phenotype, there are reports of a teniposide-resistant Chinese hamster ovary line with minimal cross-resistance to VLB (17), and an mAMSA-resistant P388 line (12) and HL-60 line (20) that are cross-resistant to the epipodophyllotoxins and sensitive to the Vinca alkaloids.

We report here the development and characterization of a teniposide-resistant human leukemic lymphoblastic cell line that expresses “atypical” MDR. This line (CEM/VM-1) has a pattern of cross-resistance similar to cells having the “classic” MDR phenotype with the exception that it is completely sensitive to the Vinca alkaloids. Also, in contrast to the “classic” MDR phenotype, the CEM/VM-1 cells accumulate nearly as much drug as do drug-sensitive CEM cells, suggesting a mechanism of resistance other than decreased drug retention. Comparison of the CEM/VM-1 line and our well-characterized “classic” MDR cell line CEM/VLB100 is also reported. A preliminary account of this work has been presented (21).

MATERIALS AND METHODS

Chemicals and Supplies. Eagle’s minimal essential medium, with Earl’s spinner salts, was purchased from Grand Island Biological Co. (Grand Island, NY) and fetal bovine serum was from HyClone Laboratories (Sterile-Systems, Inc., Logan, UT). VCR and VLB were generous gifts of Eli Lilly and Co. (Indianapolis, IN). DOX was from Adria Laboratories (Wilmington, DE), and bleomycin, VM-26, and VP-16 were provided by Bristol-Myers Laboratories (Syracuse, NY); dimethyl sulfoxide was from Fisher Scientific Co. (Fair Lawn, NJ). The drug solutions were prepared as reported previously (22); mAMSA, obtained from the Drug Synthesis and Chemistry Branch, DTP, DCT, National Cancer Institute, was dissolved in 100% dimethyl sulfoxide and then diluted with 0.9% sodium chloride to a concentration of 1 mg/ml before its dilution to final concentration with 0.9% sodium chloride. Methanol and acetonitrile were purchased from Burdick and Jackson Laboratories (Muskegon, MI). [1H]VP-16 was purchased from Moravek Biochemicals, Brea, CA; and [carboxyl-14C]Julin was from New England Nuclear Products, Boston, MA. Propidium iodide was obtained from Calbiochem (San Diego, CA). All other chemicals and supplies were obtained from commercial sources.

Cells and Culture Conditions. Teniposide-resistant cells were selected from the original parental CCRF-CEM cell line by growth in the intermittent presence of sublethal concentrations of drug as described for our VCR-resistant cells (22). The CEM/VM-1 line was cloned by limiting dilution (23) and retained its resistance to VM-26 for at least 2 months in the absence of drug. All cells used for experiments were grown in drug-free media for at least 1 week. We have described the selection and maintenance conditions for the CEM/VLB100 cell line elsewhere (11).

Growth Inhibition Assay. The concentration of drug required to inhibit growth of cells by 50% (IC50) in 48 hours was determined for each cell line, as described previously (22). Fold resistance is calculated by dividing the IC50 of the resistant line by the IC50 of that drug in the CEM parent line.

DNA Content. CEM, CEM/VLB100 and CEM/VM-1 cells were analyzed by flow cytometry for DNA content and cell cycle distribution as described previously (24).

Cellular Pharmacology of VP-16. Influx, efflux, accumulation, and retention of [1H]VP-16 by the cell lines were determined by the cold buffer wash method, as previously described (25). Intracellular water volume was determined from the difference between the wet and dry weights of cell pellets, taking into account the extracellular volume, as determined with [14C]Julin. Intracellular water (ml/g dry weight) was

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2 Leukemia Society of America, Special Fellow; Recipient of Biomedical Research Support Grant RR05584-21 from NIH.

3 To whom requests for reprints should be addressed, at St. Jude Children’s Research Hospital, Department of Biochemical and Clinical Pharmacology, 332 North Lauderdale, P.O. Box 318, Memphis, TN 38101.

4 The abbreviations used are: MDR, multiple drug resistance; CEM, parent CCRF-CEM human lymphoblast cell line; CEM/VLB100, CEM subline selected for resistance to vinblastine; VLB; vinblastine; CEM/VM-1, cloned CEM subline selected for resistance to VM-26; VM-26, teniposide; 4’-demethylepipodophyllotoxin 9-(4,6-O-2-thienylideine-β-D-glucopyranoside); VP-16:213, VP-16, etoposide; 4’-demethylepipodophyllotoxin 9-(4,6-O-2-thienylideine-β-D-glucopyranoside); mAMSA, 4’-[(9-acridinyl)amino]methanesulphon-m-anisidide; DOX, doxorubicin; VCR, vincristine; HPLC, high pressure liquid chromatography.
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determined to be 4.3, 4.5, and 3.9 for CEM, CEM/VM-1, and CEM/VLB,oo cells, respectively. Data are expressed either as nmol VP-16/g dry weight or μmol VP-16/liter cell water as previously reported (25). Some drug accumulation studies were also done using a standard silicone oil technique (26) for separating the cells from the extracellular medium. At indicated times, aliquots of cell suspension were layered over 0.5 ml Nyosil 50 oil (William F. Nye, Inc., New Bedford, MA) in a microcentrifuge tube and centrifuged for 5 min at room temperature. The [3H]VP-16 used in the experiments was 93-97% pure by HPLC (27).

HPLC Analysis for Possible Cellular Metabolism of [3H]VP-16. Cell pellets were prepared for drug metabolism studies after a 60-min incubation at 37°C using an initial extracellular VP-16 concentration of 170 μM. After incubation with drug, cell pellets containing [3H]VP-16 were treated twice with 100 μl of 100% methanol; this treatment extracted 100% of the radiolabel. Analysis for potential metabolites of [3H]VP-16 was by the HPLC method of Sinkule and Evans (27).

Statistics. Comparison of rates of influx of VP-16 in the three cell lines was done with a Wald χ² analysis (28). P values for simple comparisons of drug accumulation, retention, and t₀ were obtained by Student’s t test (28).

RESULTS

Drug Cytotoxicity. Forty-eight-h IC₅₀ values of several drugs for the parent drug-sensitive (CEM), VLB-resistant (CEM/VLB,oo), and VM-resistant (CEM/VM-1) cell lines are shown in Table 1. Each of the resistant lines is equally resistant or cross-resistant to VM-26 and VP-16, permitting direct comparison of degrees of cross-resistance and cellular pharmacology data. They are also each ≈20-fold cross-resistant to mitoxantrone and 80- to 150-fold cross-resistant to DOX. The CEM/VM-1 line is resistant to mAMSA, but sensitive to the Vinca alkaloids VLB and VCR. Both resistant lines are sensitive to bleomycin.

Flow Cytometric Findings. Since similarities or differences in degrees of resistance to the drugs in Table 1 could be due to differences in growth rates or cell cycle distribution, we determined doubling times for each line and analyzed, with cells in log phase growth, the DNA content of each line. Results of these experiments are shown in Table 2. The similarity of both these measurements in all three lines suggests that growth kinetics do not account for contrasting cross-resistance patterns in these lines, and lends further support to the comparability of the other data presented in this paper.

Influx, Efflux, Steady State Accumulation, and Retention of [3H]VP-16. The uptake of [3H]VP-16 was measured from 0.5 to 10 s to determine initial influx rates in the three cell lines (Fig. 1). Using the first three time points (0.5-2.0 s), comparison of the rates of drug influx (slopes) showed only a 2-fold difference between CEM and CEM/VM-1 cells, but a greater than 10-fold difference between the CEM and CEM/VLB,oo cells. These initial influx rates are given in Table 3. There is no significant difference (P = 0.91) in zero-time binding (Y intercept value) among the cell lines (Fig. 1).

Despite the fact that the two drug-resistant lines are equally cross-resistant to VP-16, they accumulated different amounts of drug at steady state, as shown in Fig. 2. CEM/VM-1 cells accumulated 70% as much [3H]VP-16 as the CEM cells, but the CEM/VLB,oo cells accumulated less than 10% as much [3H]VP-16 as the drug-sensitive controls. These steady state values are listed in Table 3.

Removal of [3H]VP-16 from the extracellular compartment results in the rapid loss of drug from all cell lines to a plateau (Fig. 2). Statistically, these levels of nonexchangeable drug (Table 3) were significantly different only when comparing the two resistant cell lines. Unidirectional efflux of [3H]VP-16 was determined in these cell lines after “loading” for 30 min with 5 μM [3H]VP-16 to achieve steady state drug levels (Fig. 2). As observed for influx, there was a similar rate of efflux of VP-16 from the parental CEM and CEM/VM-1 cells (Table 3). (Because of the small standard error, these rates are statistically different; but the difference is of doubtful biological significance.) However, ac-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cross-resistance of CCRF-CEM sublines selected for resistance to VM-26 or VLB</th>
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<tbody>
<tr>
<td>Drugs</td>
<td>CCRF-CEM IC₅₀ (μM)</td>
</tr>
<tr>
<td>VM-26</td>
<td>48.0 ± 39.0</td>
</tr>
<tr>
<td>VP-16</td>
<td>416 ± 200</td>
</tr>
<tr>
<td>VLB</td>
<td>2.76 ± 2.50</td>
</tr>
<tr>
<td>VCR</td>
<td>2.40 ± 2.10</td>
</tr>
<tr>
<td>DOX</td>
<td>29.0 ± 24.0</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>12.2 ± 14.7</td>
</tr>
<tr>
<td>mAMSA</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>1.26 ± 0.84'</td>
</tr>
</tbody>
</table>

a) 50% inhibitory concentration in a 48-h growth inhibition assay ± SD. Values shown for CEM are means of 14 to 18 separate experiments. For CEM/VM-1 and CEM/VLB,oo cells, values are means of 3 to 12 separate experiments.

b) IC₅₀ of the resistant cell line divided by that of the parent CCRF-CEM line.
c) Units/liter.
d) Degree of resistance significantly different (P < 0.001) from CEM/VM-1 cells.

e) Degree of resistance significantly different (P < 0.02) from CEM/VLB,oo cells.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Doubling times and cell cycle distribution of drug-sensitive and -resistant cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Doubling time (h)</td>
</tr>
<tr>
<td></td>
<td>G₁</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>CEM/VM-1</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>CEM/VLB,oo</td>
<td>32 ± 7</td>
</tr>
</tbody>
</table>

* Determined graphically and defined as the number of hours required for cells in log phase growth to double in number. Values are the mean ± SD of four to five experiments.

* Determined by DNA content, as indicated in "Materials and Methods."

* Percentages do not equal 100 when added, since these are mean values of three separate experiments.

Fig. 1. Initial uptake of [3H]VP-16 by CEM, CEM/VM-1, and CEM/ VL B,oo cells. External concentration of [3H]VP-16 was 5 μM. Each point is the mean of triplicate determinations from three experiments run on separate days. Bars, mean ± SE. See "Materials and Methods" for experimental details.

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Table 3 Analysis of VP-16 influx, efflux, accumulation, and retention in CEM, CEM/VM-1, and CEM/VLB100 cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Influx rate (nmol/g dry weight/s)</th>
<th>Efflux (nmol/s)</th>
<th>VP-16 concentration (nmol/g dry weight)</th>
<th>Nonexchangeable VP-16 concentration (nmol/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>0.230 ± 0.084</td>
<td>102 ± 1</td>
<td>21.39 ± 0.80</td>
<td>0.73 ± 0.21</td>
</tr>
<tr>
<td>CEM/VM-1</td>
<td>0.123 ± 0.026</td>
<td>94 ± 1</td>
<td>14.63 ± 0.94</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>CEM/VLB100</td>
<td>0.021 ± 0.046</td>
<td>256 ± 33</td>
<td>1.79 ± 0.18</td>
<td>0.20 ± 0.03†</td>
</tr>
</tbody>
</table>

* Includes first three points from 0.5-2.0 s. Data are presented as mean ± SE from three separate experiments run on different days. Three to four measurements at each of five different times were taken as previously described (25).

# Cells were incubated with 5 μM [3H]VP-16 for 30 min, after which they were washed twice in ice-cold buffer. Total VP-16 was measured in triplicate and the cells were resuspended in two to three times the original volume of VP-16-free buffer at 37°C. Over the next 300 s, at least 12 measurements were made of cellular VP-16 concentration. The log of the fraction of exchangeable VP-16 remaining in the cell as a function of time after resuspension was plotted (not shown) and the resulting linear efflux rate was used to calculate the t½ values shown. Nonexchangeable VP-16 was determined from measurement of VP-16 concentration taken 30-45 min after resuspension of cells in VP-16-free buffer. Data are presented as the mean ± SE from three experiments run on different days.

† Significantly different from CEM (P < 0.01).
‡ Significantly different from CEM (P < 0.05).
# Significantly different from CEM/VM-1 (P < 0.01).
$ Significantly different from CEM/VM-1 (P < 0.001).

Fig. 2. Accumulation and retention of VP-16 in CEM, CEM/VM-1, and CEM/VLB100 cells. Cells were incubated with 3.6 μM [3H]VP-16 and intracellular VP-16 was determined at the times indicated. After 25 min, cells were harvested, washed twice in ice-cold 0.85% sodium chloride, and resuspended at 37°C in two to three times the original volume of drug-free incubation buffer. Over the next 20 min the cell-associated epipodophyllotoxin concentration was measured.

Fig. 3. Determination of [3H]VP-16 accumulation by CEM, CEM/VM-1, CEM/VLB100 cells using the cold buffer wash method (Fig. 2). The two CEM sublines in this report are resistant to at least five of the eight drugs studied; therefore, each line could be said to be "multiply drug resistant." All the drugs are natural products or their semisynthetic derivatives, and all except bleomycin have been reported to be involved in MDR (2, 8, 12, 14). The proposed mechanisms of cytotoxicity of the drugs in Table 1 include DNA damage (29–32) (DOX, mitoxantrone, bleomycin, and mAMSA), tubulin binding (33) (VLB and VCR), and alteration of topoisomerase activity (34–37) (mAMSA, DOX, VM-26, and VP-16). However, the CEM/VLB100 line is resistant to the Vinca alkaloids, while the reverse is true of the CEM/VM-1 line. These contrasting cross-resistance data suggest that even though the lines are equally resistant to the epipodophyllotoxins, the mechanisms of resistance differ. In support of this conclusion, the resistance of the CEM/VLB100 line to VP-16 was likely due to the characteristic decrease in steady state drug concentration associated with the "classic" MDR phenotype. This was probably not the case for the CEM/VM-1 line. Therefore, despite resistance to several classes of natural product drugs, the MDR of the CEM/VM-1 cells is "atypical" because the cellular pharmacology of VP-16 in these cells is similar to that in the CEM cells and also because the CEM/VM-1 cells are sensitive to the Vinca alkaloids.

Our observations with the CEM/VLB100 line (Table 1) are consistent with those in the literature in that in each MDR line showing decreased accumulation of natural drug products, there is cross-resistance to both the Vinca alkaloids and anthracyclines (4–6, 38–40). Comparable studies with lines similar to the CEM/VM-1 line are limited: of the three lines reported conditions intracellular accumulation was approximately 3.6 μM in each cell line.

VP-16 Metabolism in CEM/VM-1 Cells. Since the CEM/VLB100 cells and the CEM/VM-1 cells were equally resistant to VP-16, but the cellular drug concentration in the CEM/VM-1 cells was ~5-fold higher than in the CEM/VLB100 cells, the question of drug metabolism was addressed. If the parent drug were metabolized to an inactive or less active form in the CEM/VM-1 cells, the effective drug concentration in the two lines might be similar. We therefore examined our CEM/VM-1 cells for metabolites of VP-16. No metabolites were found by HPLC analysis (data not shown), suggesting that the mechanism of resistance in the CEM/VM-1 cells is not due to increased metabolism of the parent compound.

DISCUSSION

The two CEM sublines in this report are resistant to at least five of the eight drugs studied; therefore, each line could be said to be "multiply drug resistant." All the drugs are natural products or their semisynthetic derivatives, and all except bleomycin have been reported to be involved in MDR (2, 8, 12, 14). The proposed mechanisms of cytotoxicity of the drugs in Table 1 include DNA damage (29–32) (DOX, mitoxantrone, bleomycin, and mAMSA), tubulin binding (33) (VLB and VCR), and alteration of topoisomerase activity (34–37) (mAMSA, DOX, VM-26, and VP-16). However, the CEM/VLB100 line is resistant to the Vinca alkaloids, while the reverse is true of the CEM/VM-1 line. These contrasting cross-resistance data suggest that even though the lines are equally resistant to the epipodophyllotoxins, the mechanisms of resistance differ. In support of this conclusion, the resistance of the CEM/VLB100 line to VP-16 was likely due to the characteristic decrease in steady state drug concentration associated with the "classic" MDR phenotype. This was probably not the case for the CEM/VM-1 line. Therefore, despite resistance to several classes of natural product drugs, the MDR of the CEM/VM-1 cells is "atypical" because the cellular pharmacology of VP-16 in these cells is similar to that in the CEM cells and also because the CEM/VM-1 cells are sensitive to the Vinca alkaloids.

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with an “atypical MDR” phenotype (12, 17, 20), single point drug accumulation studies have been done on one line (12, 41). Consistent with our results, Kessel et al. (41) showed no change in accumulation of mAMSA or daunorubicin in the P388/AMSA line.

Comparison of results shown in Figs. 2 and 3 suggest that there is a fraction of accumulated VP-16 in both resistant lines (but not in the parent line) that is lost from the cell by washing at 0°C; this fraction may be intracellular free drug (i.e., osmotically active) or drug loosely bound in or on the cell. These results are consistent with another study reporting loss of VM-26 during cold buffer washing of L1210 cells resistant to VM-26 (40).

Our findings are unique in that the data suggest a mechanism(s) of epipodophyllotoxin resistance in CEM/VLM-1 cells different from that in the CEM/VLBioo cells. Resistance to VP-16 in the CEM/VLBioo cells probably involves a “classic” MDR mechanism with decreased drug accumulation and retention associated with characteristic membrane changes (1). The CEM/VLM-1 cells, in contrast, show only a slight decrease in control cell-associated drug and no detectable metabolism of parent compound that might result in a lower effective drug concentration. Other data indicate that these cells do not overexpress the MDR “marker” glycoprotein (gp180), or its mRNA.

Kinetic data (Table 3) in this paper present the first evidence that, in contrast to cells with the “classic” MDR phenotype (CEM/VLBioo), alterations in transport and accumulation of VP-16 most likely do not account for epipodophyllotoxin resistance in our human leukemic cell line (CEM/VLM-1). Preliminary experiments with VM-26 (data not shown) and VCR (21) produced similar results. Further, we showed that resistance to VP-16 in the CEM/VLM-1 cell line is likely not due to drug metabolism. We suggest, therefore, that the mechanism of epipodophyllotoxin resistance in the CEM/VLM-1 line is alteration of a cellular drug target(s). The compounds to which this line is cross-resistant (epipodophyllotoxins, anthracyclines, mAMSA) implicates changes in topoisomerase II (34–37) or in other factors involved in topoisomerase II activity (42, 43); but this remains to be determined.

We conclude that the CEM/VLM-1 line is resistant to the cytotoxic action of multiple natural drug products, but because of its sensitivity to the Vinca alkaloids and minimal alteration in drug accumulation, this line expresses an “atypical” MDR phenotype. Studies are currently in progress in our laboratories to define the mechanism of this “atypical” MDR.

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