Modulation of Drug Sensitivity by Dipyridamole in Multidrug Resistant Tumor Cells in Vitro

David R. Shalinsky, Michael Andreeff, and Stephen B. Howell

Department of Medicine and the Cancer Center, University of California, San Diego, La Jolla, California 92093 [D. R. S., S. B. H.], and Memorial-Sloan Kettering Cancer Center, New York, New York 10021 [M. A.]

ABSTRACT

The concept of overcoming multidrug resistance using modulators is based on the hypothesis that there will be a synergistic interaction between the modulator and the cytotoxic agent. We examined the ability of dipyridamole (DPM) to synergistically enhance drug sensitivity in drug-sensitive KB-3-1 cells and their drug-resistant variants, KB-GRC1 and KBV1 cells, using median effect analysis to produce a quantitative measure of the extent of synergy. The drug-resistant variants were resistant to vinblastine (VBL), colchicine (COL), and etoposide (VP-16) in the order VBL > COL > VP-16 on the basis of 50% inhibitory concentration values obtained by clonogenic assay with continuous drug exposure. The extent of staining with the monoclonal antibody HYB-241, directed at a 180,000 form of the mdr1 gene product, correlated with drug resistance for all three drugs (r ≥ 0.92). DPM and verapamil elevated the steady state content (C₅₀) of VBL, but there was no correlation between elevation of C₅₀ and the extent of synergy observed. DPM enhanced the cytotoxicity of VBL and COL in a synergistic manner in KB-GRC1 cells, and in KBV1 cells DPM interacted synergistically with VBL. VPL was synergistic with VBL only in KB-GRC1 cells. No synergy was observed in the parental KB-3-1 line. These data indicate that, although both DPM and verapamil can increase C₅₀ in cells not expressing P-glycoprotein, such an increase was not associated with synergy. In cells expressing mdr1, synergy was observed, and it was greatest for the cytotoxic agent for which expression of mdr1 produced the greatest fold-resistance and enhancement of C₅₀. However, neither the level of resistance, the level of expression of mdr1, nor the ability of the modulator to alter C₅₀ accurately predicted whether the interaction would be truly synergistic. We conclude that additional factors determine the nature of the drug interaction.

INTRODUCTION

Multidrug resistance has been related to the overexpression of the mdr1 gene and consequent production of a M, 170,000 membrane-associated surface P-glycoprotein. As a prominent member of a superfamily of membrane-associated transport proteins, P-glycoprotein is often present in increased amounts in drug-resistant cells. P-glycoprotein is believed to function as an efflux pump for various lipophilic xenobiotics, leading to reduced accumulation of chemotherapeutic agents, thus rendering cells resistant. Although many agents that can enhance sensitivity of multidrug-resistant cells have been identified, the nature of this interaction (synergistic, additive, antagonistic) has not been formally determined in most cases. The concept of overcoming multidrug resistance with modulating agents is based on there being a synergistic interaction between the modulator and the cytotoxic agent. Although the potency of the modulating agents thus far identified is far less than that of chemotherapeutic agents at concentrations effective in reversing drug resistance, most produce some degree of cell kill themselves. This complicates determination of the nature of the interaction and requires the use of some form of isobologram or median effect analysis.

DPM inhibits tumor cell growth in vitro (3–5), potentiates the activity of a variety of anticancer agents in vitro (6–10), and also has the potential for potentiating anticancer activity in vivo (11, 12). We recently reported that DPM synergistically modulated the sensitivity of human ovarian carcinoma 2008 cells to VP-16, doxorubicin, and VBL in a manner that was highly correlated with its ability to increase cellular drug content (13, 14). Based on the facts that these drugs participate in the multidrug-resistant phenotype, that DPM can inhibit azidopine binding to P-glycoprotein in membrane vesicles from drug-resistant KB cells (15), and that DPM can selectively modulate cytotoxicity in drug-resistant P388 cells (6), we sought to examine the effect of mdr1 expression on the modulatory abilities of DPM.

In this study, we examined the nature of the interaction between DPM and drugs to which multidrug-resistant cells are commonly resistant using the technique of median effect analysis. We determined the ability of DPM to synergistically enhance drug sensitivity to VP-16, COL, and VBL using the drug-sensitive epidermoid carcinoma cell line KB-3-1 and a drug-resistant variant, KB-GRC1 (16), derived from KB-3-1 by transfection of the mdr1 gene. Theoretically, KB-GRC1 cells differ from the parental KB-3-1 cells only by the presence of a single protein, P-glycoprotein. We also compared modulation in KB-GRC1 cells with that in a more resistant variant, KBV1, which was derived from KB-3-1 cells by chronic exposure to high concentrations of VBL, and with modulation produced by VPL in the parental and KB-GRC1 cells. We report here that, although DPM and VPL were synergistic in some cases, they were antagonistic with the cytotoxic agent in others and that neither the level of resistance, the level of P-glycoprotein, nor the effect of the modulatory on C₅₀ predicted the presence of a truly synergistic interaction.

MATERIALS AND METHODS

Drugs. VP-16 was obtained in its clinical formulation as a lyophilized powder from the National Cancer Institute (Bethesda, MD). DPM was a gift of Boehringer Ingelheim Pharmaceutical, Inc. (Ridgefield, CT). VPL was purchased from American Regent Laboratories, Inc. (Shirley, NY). COL was obtained from Sigma Chemical Co. (St. Louis, MO), and VBL was obtained from Eli Lilly Co. (Indianapolis, IN). Stock solutions of the drugs were prepared in DMSO.
solutions were made by dissolving each drug in saline. Working solutions were prepared by further dilution in tissue culture medium. Solutions containing COL and VBL were protected from light to prevent light-sensitive chemical decomposition. \(^{1}H\)-labeled VP-16 (900 mCi/mmol) and \(^{1}H\)-labeled VBL (20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA), and \(^{1}H\)-labeled COL (20–40 Ci/mmol) was obtained from Du Pont New England Nuclear (Wilmington, DE). The final specific activities used for drug accumulation studies were: \(^{1}H\)-labeled VP-16, 2 μCi/μmol; \(^{1}H\)-labeled COL, 6.67 μCi/μmol; and \(^{1}H\)-labeled VBL, 6.67 mCi/μmol.

Tumor Cells and Culture Medium. Standard culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 units/ml), and 1% L-glutamine. The drug-sensitive KB-3-1 line, its COL-resistant subline (KB-GRC1), and a more highly resistant subline, KBV1, were obtained from Dr. Igor Roninson (University of Illinois, College of Medicine, Chicago, IL). The cells were grown at 37°C under 5% CO\(_2\) in air in T25 tissue culture flasks (Corning Glass Works, NY). The KB-GRC1 line was derived by transfection of the wild-type \(mdr1\) gene coupled to a Moloney murine leukemia virus long terminal repeat into KB-3-1 cells (17). After transfection, the KB-GRC1 cells were maintained in medium supplemented with 6 ng/ml COL. The more highly VBL-resistant clones, KBV1, were derived from KB-3-1 cells by gradual exposure to increasing concentrations of VBL (18) and were maintained in 0.25 μg/ml VBL.

Modulation of Cellular Pharmacology. Radiolabeled drugs and DPM or VPL were added to subconfluent cultures growing in 60-mm dishes in 2 ml of 37°C culture medium as previously described (13, 14). After 60 min, the medium was aspirated and the cells were washed 3 times with ice-cold PBS. The cells were digested overnight with 1 ml of 1 N NaOH. Aliquots were used for determination of protein content and of cell-associated radioactivity. Each experiment was performed in duplicate. The final concentrations used were 1 μM for VP-16 and 6 nM for COL and VBL.

Clonogenic Assay. Cytotoxicity was measured using a colony forming assay as previously reported (13, 14). Log-phase cells (48–72 h in culture) were harvested with 2 mM trypsin-0.05% EDTA, washed with PBS, and plated in triplicate onto 60-mm tissue culture dishes (Corning Glass Works, NY) at a density of 200 cells/dish in 5 ml of culture medium. Cells were allowed to attach for 2–4 h prior to addition of drug, usually as ≤50 μl of stock solution. Cultures were incubated at 37°C under 5% CO\(_2\) in air for 10 d with continuous exposure to drug. The resulting colonies were stained with Giemsa dye in methanol, and values greater than 1 indicate synergism; a value of 1 indicates additivity, percentage of survival compared to untreated control.

Median Effect Analysis. Median effect analysis permits a formal assessment of the nature of the interaction between drugs and yields the combination index, a measure of the extent of synergy, additivity, or antagonism at various levels of cell kill (19). Combination index values of less than 1 indicate synergy; a value of 1 indicates additivity, and values greater than 1 indicate antagonism. In clonogenic assays employing the median effect design, dose-response curves were determined for modulator and cytotoxic agent alone and for the combination of both agents at a fixed ratio equivalent to the ratio of the IC\(_{50}\) values. Actual concentrations used were on the order of 1/100 to 1/1 of the IC\(_{50}\) for each drug, as previously described (13, 14). The percentage of survival was converted to percentage of cell kill for calculation of the CI\(_{50}\) by computer analysis of the dose-response curves (20).

Surface Staining for P-glycoprotein. The \(mdr1\) P-glycoprotein gene product was quantified by using the monoclonal antibody HYB-241 (Hybritech, Inc., San Diego, CA) (21). After harvest by scraping with a rubber policeman, one million cells were suspended in 0.5 ml of PBS containing 5 μl of antibody. Forty-three μl of an anti-gardanilla antibody GDJ352 (Hybritech, Inc.) were added to another suspension as a control. The paired samples were processed simultaneously. The cells were stained at room temperature for 1 h and then washed in PBS (pH 7.4). Fifty μl of a 1:25 dilution of the fluorescein isothiocyanate-conjugated (F(ab)\(^{1}\)) fragment of goat anti-mouse IgG (Cappel, West Chester, PA) was added and incubated for 1 h at room temperature. The samples were washed 3 times with PBS and fixed in 2% paraformaldehyde in PBS, pH 7.4.

The fluorescence of the sample was measured by a FACSCAN flow cytometer (Becton-Dickinson, Mountain View, CA) using Consort 30 software. A computer-based statistical program calculated the difference in staining relative to the negative control antibody between each of the paired histograms, yielding the \(D\) value. Ten thousand cells were analyzed in each sample. The \(D\) value (range, 0–1.0), which is derived from the Kolmogorov-Smirnov statistical test (22), corresponds to the maximum difference between the cumulative frequency distributions of two samples expressed as a percentage of the total number of cells analyzed. A \(D\) value ≥ 0.15 indicates that there is a significant difference in antibody staining between two populations (23).

P-glycoprotein expression was quantified by determining the mean fluorescence of each cell line after staining with HYB-241 (corrected for background fluorescence by subtraction of fluorescence of the isotopic control). The fold increase in fluorescence relative to control was also determined by calculating the ratio of HYB-241 fluorescence relative to isotopic control fluorescence.

Statistical Analysis. Unless otherwise noted, the data are expressed as the group mean ± SD of triplicate determinations from each of \(n\) experiments. Student's \(t\) test for grouped data was used. In all cases, significance was at the level of \(P < 0.05\).

RESULTS

Surface Staining for P-glycoprotein. Table 1 shows the P-glycoprotein expression as quantified by fluorescence after staining of the KB-3-1 and subline KB-GRC1 and KBV1 cells with HYB-241. The KB-GRC1 and KBV1 cell lines both had high cell surface staining with statistical \(D\) values of 0.89 and 0.96, respectively. In comparison, the parental KB-3-1 line did not have significant antibody staining (\(D = 0.13\)). As determined from the data in Tables 1 and 2, the fold-increase in fluorescence after HYB-241 staining correlated with increasing drug resistance to VP-16 (\(r = 0.97\)), COL (\(r = 0.92\)), and VBL (\(r = 0.92\)) across the KB cell lines. These results indicate that HYB-241 detects the product of the \(mdr1\) gene and that, in agreement with a previous study (21), this gene is not expressed in the drug-sensitive parental cells.

Cytotoxicity Studies. Table 2 shows the IC\(_{50}\) values of the KB-3-1 and subline KB-GRC1 and KBV1 cells to 3 cytotoxic agents.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>p180 expression as quantified by staining with HYB-241</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>Mean fluorescence*</td>
</tr>
<tr>
<td>KB-3-1</td>
<td>3</td>
</tr>
<tr>
<td>GRC1</td>
<td>74</td>
</tr>
<tr>
<td>KBV1</td>
<td>135</td>
</tr>
</tbody>
</table>

* The mean fluorescence after HYB-241 staining was assessed in triplicate by subtraction of the background fluorescence (isotopic control).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Drug sensitivity of KB cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>DPM (μM)</td>
</tr>
<tr>
<td>KB-3-1</td>
<td>18.6 ± 3.1*</td>
</tr>
<tr>
<td>GRC1</td>
<td>31.3 ± 1.4</td>
</tr>
<tr>
<td>KBV1</td>
<td>24.2 ± 2.9</td>
</tr>
</tbody>
</table>

* Values are expressed as the mean IC\(_{50}\) ± SD, \(n = 20\) (except for KBV1 (\(n = 8\)) and studies using VPL (\(n = 3–6\)).

* Fold resistance relative to KB-3-1 cells, based on the ratio of the IC\(_{50}\) values. Results were obtained by using the colony forming assay with continuous drug exposure as described in "Materials and Methods."
agents (VP-16, COL, and VBL) and 2 modulators (DPM and VPL). The KB-GRC1 cells were minimally resistant to VP-16 but were 4.5 and 67 times resistant to COL and VBL, respectively, based on the ratio of the IC50 values obtained by colony forming assay. These results indicate that overexpression of the wild-type mdrl gene via a long terminal repeat promoter produced minimal resistance to VP-16, moderate resistance to COL, and substantial resistance to VBL. The KBV1 cells were more resistant to VP-16 (5.8-fold), COL (18.5-fold), and VBL (309-fold) than were the KB-GRC1 cells. We employed the technique of median effect analysis to determine the nature of the drug interaction between DPM and VPL and the cytotoxic agents. Fig. 1 compares the combination index plots for experiments involving DPM plus VP-16, COL, or VBL in the parental and KB sublines over the entire range of cell kill. The regression coefficients were ≥ 0.9 for each drug alone and in combination with modulator, indicating that the drugs followed basic mass action principles. High levels of synergy were observed only in the drug-resistant cells and only for COL or VBL. DPM produced high levels of synergy only with VBL in both the KB-GRC1 and KBV1 cell lines. Table 3 shows the combination index values at the 50% cell kill level for ease of comparison. As shown in Fig. 2, in KB-GRC1 cells there was good correlation (r = −0.92) between fold-resistance and extent of synergy with DPM as quantified by CI50. There was also a correlation (r = −0.79) between fold-resistance and CI50 in KBV1 cells. These data suggested that an increasing level of drug resistance was related to a decreasing combination index value in both cell lines irrespective of the absolute value of this parameter. However, despite the higher level of resistance to each drug in KBV1 cells, synergy was evident only with VBL and at a lower level than that observed in the KB-GRC1 cells. No synergy was evident between DPM and VP-16 in any cell line, and a significant antagonism was produced with these agents in KBV1 cells. In contrast, in parental KB-3-1 cells, DPM produced either an additive or an antagonistic interaction with each of the cytotoxic drugs. Thus, while synergy was observed in the drug-resistant cell lines, there was no consistent relationship between the level of resistance and the magnitude of the synergy produced by DPM. To compare the effects of DPM with a known modulator of the multidrug-resistant phenotype, the studies with the KB-3-1 and KB-GRC1 cells were repeated with VPL. Fig. 3 compares the combination index plots for experiments involving VPL plus VP-16, COL, or VBL in the KB-3-1 and KB-GRC1 cell lines over the entire range of cell kill, and Table 3 presents the CI50 values. In KB-3-1 cells, VPL was additive with VP-16 but antagonistic with COL and VBL. In KB-GRC1 cells, VPL was additive with VP-16 and COL but produced a high level of synergy with VBL. The mean CI50 value for the interaction between VPL and COL was not significantly different from that for the interaction between DPM and VBL. However, the magnitude of the mean CI50 values for the interaction in KB-GRC1 cells between DPM and COL was significantly different from that of the interaction between VPL and COL. Thus, DPM produced synergy in a situation where VPL did not, indicating additional components to the mechanism by which

![Combination Index Plots](image)

**Fig. 1.** Combination index plots for DPM and VP-16 (O), COL (A), or VBL (•) in KB-3-1 cells (A), GRC1 cells (B), and KBV1 cells (C) over the entire range of cell kill. A combination index of 1 (-----) indicates additivity; <1 shows synergy and >1 shows antagonism. In clonogenic assays employing the median effect design, dose-response curves were determined for modulator and cytotoxic agent alone and for the combination of both agents at a fixed ratio equivalent to the ratio of the IC50 values as described in "Materials and Methods." Each point is plotted as the mean combination index from 3-6 experiments, SD ± 22%.

**Table 3 Combination index values at 50% cell kill**

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Cell line</th>
<th>VP-16</th>
<th>COL</th>
<th>VBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPM</td>
<td>KB-3-1</td>
<td>1.08 ± 0.24*</td>
<td>1.11 ± 0.26</td>
<td>1.08 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>GRC1</td>
<td>1.27 ± 0.28</td>
<td>0.51 ± 0.39</td>
<td>0.07 ± 0.07d</td>
</tr>
<tr>
<td></td>
<td>KBV1</td>
<td>2.00 ± 0.12</td>
<td>1.01 ± 0.02</td>
<td>0.49 ± 0.16d</td>
</tr>
<tr>
<td>VPL</td>
<td>KB-3-1</td>
<td>1.07 ± 0.11</td>
<td>1.60 ± 0.26</td>
<td>1.76 ± 0.41f</td>
</tr>
<tr>
<td></td>
<td>GRC1</td>
<td>1.25 ± 0.25</td>
<td>1.18 ± 0.26</td>
<td>0.02 ± 0.01d</td>
</tr>
<tr>
<td></td>
<td>KBV1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* Values are mean CI50 ± SD.

* Experiments were performed in triplicate for each (n).

* P < 0.05.

* P < 0.01, different than 1.0.

n.d., not done.
DPM and VPL produced very similar increases in $C_{ss}$ for all 3 cytotoxic agents, given the ability of DPM and VPL to inhibit drug efflux, one might anticipate a correlation between the extent of synergy produced and the effect of the modulator on $C_{ss}$. Table 4 shows the effect of DPM and VPL on the $C_{ss}$ for VP-16, COL, and VBL in each of the cell lines. Cells were exposed to radiolabeled drug for 60 min until steady state content had been reached (24). A modulatory concentration of 80 $\mu$M was chosen because it induced a maximum increase in $C_{ss}$ for DPM and VPL (24). Under control conditions, $C_{ss}$ was higher in the KB-3-1 cells than in the KB-GRC1 or KBV1 cells; however, there was not a very close relationship between fold-resistance (Table 2) and $C_{ss}$. For example, the $C_{ss}$ for VP-16 in the KBV1 cells was 32% of that in the parental KB-3-1 cells, and this was associated with 5.8-fold resistance. In the case of COL, the $C_{ss}$ for DPM and VPL (24). Under control conditions, $C_{ss}$ was higher in the KB-3-1 cells than in the KB-GRC1 or KBV1 cells; however, there was not a very close relationship between fold-resistance (Table 2) and $C_{ss}$. For example, the $C_{ss}$ for VP-16 in the KBV1 cells was 32% of that in the parental KB-3-1 cells, and this was associated with 5.8-fold resistance. In the case of COL, the $C_{ss}$ for DPM and VPL was 36% of that in the KB-3-1 cells, but this decrease was associated with 18.5-fold resistance. DPM and VPL produced very similar increases in $C_{ss}$ for all 3 cytotoxic agents in all 3 KB cell sublines ($r = 0.97$). Surprisingly, the modulators produced a small increase in $C_{ss}$ in the KB-3-1 cells that was statistically significant for VP-16 (both modulators) and VBL (DPM only) ($P < 0.05$). This observation was unexpected since the KB-3-1 cells lack detectable levels of P-glycoprotein. The increase in $C_{ss}$ was not associated with a synergistic enhancement of cytotoxicity for either drug. VPL did not significantly augment the $C_{ss}$ of COL or VBL, but DPM modestly elevated the $C_{ss}$ of VBL in the KB-3-1 cells.

DPM and VPL enhanced the $C_{ss}$ of COL by an equivalent degree (2-fold) in the KB-GRC1 and KBV1 cells, yet synergy was evident only between DPM and COL and only in the KB-GRC1 cells (Table 3); VPL did not interact synergistically with COL in the same cell line. In contrast, DPM and VPL both markedly enhanced the accumulation of VBL in KB-GRC1 cells by ~9-fold and in KBV1 cells by ≥12.7-fold. The highest level of synergy (Table 3) was observed in the KB-GRC1 and not the KBV1 cells, contrary to what might have been predicted based on the ability of these modulators to enhance the $C_{ss}$ of VBL (Table 4). These results indicate a complex relationship between the effect of the modulators on $C_{ss}$ and their ability to interact synergistically. The fact that both modulators could increase $C_{ss}$ in cells not expressing the mdrl gene product, at least for VP-16, indicated a component to their mechanism that was independent of P-glycoprotein; this component was not associated with synergy. In contrast, in P-glycoprotein-expressing cells, slightly greater effects on $C_{ss}$ were associated with distinct levels of synergy, as for the interaction between DPM and COL in KB-GRC1 cells.

**DISCUSSION**

We employed parental KB-3-1 cells and their sublines KB-GRC1 and KBV1 to determine whether the ability of DPM to modulate drug sensitivity was linked with P-glycoprotein expression. First, there was a good correlation between the HYB-241 staining of these sublines and their level of drug resistance ($r \geq 0.92$). Second, transfection of the wild-type mdrl gene in KB-3-1 cells produced a high level of resistance to VBL, a moderate level of resistance to COL, and a low level of resistance to VP-16. Third, the modulators DPM and VPL increased the accumulation of drug at steady state to a similar extent. Fourth, DPM and VPL enhanced the cytotoxicity of VBL in a manner that was truly synergistic but only in the drug-resistant sublines. Neither the level of P-glycoprotein, the level of drug resistance, nor the ability of the modulator to alter
Cₚ accurately predicted whether the interaction between the cytotoxic agent and modulator would be truly synergistic.

Surface Staining for P-glycoprotein. The significant staining of parental KB-3-1 cells and their sublines KB-GRC1 and KBV1 by HYB-241 substantiated the reports (21, 25) that a M₁, 180,000 form of P-glycoprotein is found in multidrug-resistant cells. There was a good correlation between the amount of P-glycoprotein detected by the HYB-241 antibody and the level of drug resistance (r ≥ 0.92). While the amount of P-glycoprotein correlated well with drug resistance for a particular drug, the level of cross-resistance was unpredictable. Resistance to VBL increased to a large extent with increasing P-glycoprotein expression, resistance to COL increased less, and even high levels of P-glycoprotein expression were associated with only modest resistance to VP-16. The Cₚ in KB-GRC1 cells compared to KB-3-1 cells was reduced by equal fractions in the absence of modulators, but this resulted in 1.5-fold resistance for VP-16 and 4.5-fold resistance for COL, demonstrating that the level of cross-resistance cannot be determined simply by quantifying the level of P-glycoprotein in these drug-resistant cells. These results indicate either that there were differences in the ability of the P-glycoprotein pump to export these drugs or that P-glycoprotein accounted for a much larger fraction of total transmembrane flux for VBL and COL than for VP-16.

Resistance Levels. The levels of resistance to VBL in the drug-resistant cells were consistently greater than that of COL (15-fold) or VP-16 (45-fold). Although high levels of cross-resistance to VP-16 have been reported in many multidrug-resistant cells (1, 26-28), there are also numerous reports demonstrating a relatively low level of resistance to this agent (29-34), particularly when compared to that for the vinca alkaloids. Notably, VP-16 has been reported to antagonize the photoaffinity labeling of radioactive analogues of VPL to P-glycoprotein 100-500-fold less potently than does VBL (35). Along with our results, these reports demonstrate that mdr1 expression often confers a low level of cross-resistance to VP-16. We conclude that, if P-glycoprotein is functioning as an efflux pump in the KB-GRC1 and KBV1 cells, P-glycoprotein has a preference of VBL > COL > VP-16 for its export activity.

Using the Cₚ of drug reached over 60 min as an indirect measurement of P-glycoprotein activity, we examined the relationship between fold-resistance and level of drug accumulation and found that there was a significant correlation for VP-16 (r = 0.93). However, while there was a clear increase in resistance, the Cₚ did not correlate well for VBL (r = 0.70) or COL (r = 0.60). Thus, P-glycoprotein seemed responsible for reducing VBL and COL accumulation in the drug-resistant cells, but we could not find a clear relationship between this and the resulting resistance to these drugs.

Modulation of Cellular Pharmacology. We compared the ability of DPM and VPL to modulate the accumulation of VP-16, COL, and VBL at steady state in the KB sublines. Interestingly, in the KB-3-1 cells, we found that both modulators produced small increases in Cₚ that reached statistical significance for VP-16 and VBL (the latter with DPM only). This was surprising in view of the lack of mdr1 expression of these cells and may reflect nonspecific actions on cellular permeability (36) or a novel pharmacological activity of these modulators (13, 14). In any case, the enhancement was small (about 2-fold) and did not result in a synergistic augmentation of cytotoxicity. In the KB-GRC1 and KBV1 cells, we found that the DPM and VPL increased the Cₚ of VP-16 and COL to a similar extent (about 2-fold) but that the modulators markedly increased the Cₚ of VBL in the KB-GRC1 cells and increased it even more so in the KBV1 cells. Thus, DPM and VPL produced a much greater increase in the Cₚ of VBL in a similar fashion in the drug-resistant cell lines compared to the parental KB-3-1 cells. Since both DPM (15) and VPL (37) have been demonstrated to bind to P-glycoprotein, it is likely that they were acting to elevate the Cₚ of VBL at least in part by interfering with P-glycoprotein activity.

Cytotoxicity Studies. In KB-GRC1 cells, DPM produced synergy with both COL and VBL, whereas VPL produced synergy only with VBL. This difference could not be explained by the ability of the modulator to enhance accumulation because both enhanced the Cₚ of COL to the same extent. On the other hand, both modulators achieved the same high level of synergy with VBL and both were equally effective in elevating the Cₚ of VBL (Table 3). We conclude that synergy cannot be predicted exclusively by the effect of the modulator on Cₚ. Rather, we speculate that the intracellular compartmentalization of drug was changed by the action of DPM and VPL. In preliminary studies examining the efflux of VBL from KB-GRC1 cells, we found that DPM and VPL, at 20 μM concentrations, increased the non转运fraction of VBL by 6.6- and 1.9-fold over control (24), suggesting that the modulators enhanced the intracellular binding of VBL and that they did so to different degrees. Beck et al. (38) proposed that a prominent mechanism of resistance in drug-resistant cells involves reduced drug binding via an energy-dependent mechanism; further work is necessary to test the hypothesis that these modulators alter the intracellular binding of VBL.

In further studies, highly resistant KBV1 cells were compared with the KB-GRC1 cells to determine whether the synergy was related to the level of resistance. In KBV1 cells, DPM was less synergistic with VBL than in KB-GRC1 cells (CI₅₀ of 0.49 ± 0.16 versus 0.07 ± 0.07) and was actually antagonistic with VP-16 (Fig. 1). Thus, the good correlation between fold-resistance and extent of synergy in KB-GRC1 cells was absent in the KBV1 cells (Fig. 2). This result was unexpected because DPM increased the Cₚ of VBL to a much greater extent in the KBV1 than in the KB-GRC1 cells (Table 4). The level of synergy obtainable with DPM must involve a more complex mechanism(s) than can be explained by total accumulation of drug alone. As discussed, this mechanism may involve a change in the intracellular binding of VBL. The KB-GRC1 and not the KBV1 cells probably represent the best model line for the study of P-glycoprotein expression on DPM modulation of drug sensitivity because the KB-GRC1 cells were produced by transfection of the wild-type mdr1 gene whereas the KBV1 cells were selected with μg/ml concentrations of VBL. We conclude from the differences in the behavior of KB-GRC1 and KBV1 cells that selection of KBV1 cells with high levels of VBL may have markedly affected resistance-related processes such as the membrane transport or intracellular binding of VBL (38, 39). These alterations could be expected to significantly obfuscate the correlation between fold-resistance and extent of synergy that was evident in the KB-GRC1 cell line.

Analysis of Correlations. We attempted to determine the important relationships between the extent of synergy, the degree of drug resistance, and the ability of the modulators to enhance the Cₚ of drug. There was a good correlation between fold-resistance to VBL and the ability of DPM to enhance the Cₚ of VBL (r = 0.99), whereas no correlation was evident between the extent of synergy and the ability of DPM to enhance the Cₚ of VBL (r = 0.30). We also found a good
correlation between fold-resistance and the ability of DPM to enhance the $C_S$ of all 3 drugs across the KB-GRC1 and KBV1 sublines ($r = 0.98$ and 0.99, respectively). We did not find any other significant correlations. Notably, the extent of synergy did not correlate with the level of $C_S$ produced by DPM. Our inability to correlate the extent of synergy with the enhancement of $C_S$ by DPM demonstrated that the mechanisms involved are multifactorial and include other mechanisms in addition to the ability to elevate total cellular drug content.

In the KB cell system, synergy was observed only in cells expressing P-glycoprotein and the effects on $C_S$ were much larger in such cells. It is clear, however, that expression of P-glycoprotein is not an absolute prerequisite for a synergistic interaction with DPM or an effect of DPM on $C_S$. We previously reported that, in the human ovarian carcinoma cell line 2008, DPM enhanced the $C_S$ of VP-16 and VBL by 3.7-fold and 3.2-fold, respectively, and that this was associated with a strong synergistic interaction ($CI_{50} \pm 0.42 \pm 0.02$ and $0.30 \pm 0.05$) (14). Human ovarian 2008 cells do not contain detectable mdrl mRNA by Northern blot analysis and lack detectable levels of P-glycoprotein by either MRK-16 or C219 antibody staining. Thus, the small P-glycoprotein-independent effects of DPM on $C_S$ observed in the KB-3-1 cells were augmented and associated with synergy in the 2008 cells. This result establishes that DPM can produce synergy by both P-glycoprotein-independent and -dependent mechanisms and that the magnitude of the two effects varies between cell lines. The underlying mechanisms apparently are not related to ability to inhibit nucleoside transport or phosphodiesterase activity in either the drug-sensitive (14) or the drug-resistant (6, 15) cell lines. The novel mechanism(s) remain to be elucidated.

The data obtained in this study are indicative of a complex relationship between fold-resistance, effect on $C_S$, and synergy for which it is not easy to formulate a comprehensive model. The strongest conclusions emerge from the comparison of KB-GRC1 and KB-3-1 cells because of the fact that these cells differ in principle only by the expression of a single protein. The expression of P-glycoprotein in KB-GRC1 cells resulted in synergy with DPM that was not observed in the KB-3-1 cells. Therefore, there is a P-glycoprotein-dependent mechanism of synergy for DPM. This mechanism is most effective in promoting synergy with the cytotoxic agent for which expression of P-glycoprotein produces the greatest level of resistance and for which DPM has the greatest influence on $C_S$. However, there is also a P-glycoprotein-independent mechanism of synergy with DPM which is muted in the KB-3-1 cells but which is expressed in the 2008 cells. This mechanism also shows variation between cytotoxic agents, and synergy mediated by this mechanism appeared to be more closely correlated with effect on $C_S$ (14). Whether VPL also has P-glycoprotein-independent and -dependent mechanisms has not been fully elucidated, although the ability of VPL to enhance $C_S$ in cells that do not express P-glycoprotein (36) suggests that this will be the case. The determination of P-glycoprotein-independent and -dependent mechanisms is of particular interest since this may lead to a broader use of modulators such as DPM for enhancement of cytotoxicity against both drug-sensitive neoplasms and those to which drug resistance has developed.

**REFERENCES**

Modulation of Drug Sensitivity by Dipyridamole in Multidrug Resistant Tumor Cells *in Vitro*

David R. Shalinsky, Michael Andreeff and Stephen B. Howell


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/50/23/7537

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.