ABSTRACT

Dipyridamole (DPM) enhanced sensitivity to etoposide (VP-16), doxorubicin (DOX), and vinblastine (VBL) in a human ovarian carcinoma cell line that was already relatively sensitive to all three agents. This interaction was shown to be truly synergistic by median effect analysis over a 2 log cell kill. The combination index at 50% cell kill (CI50) was used to quantify the extent of synergy. The CI50% were 0.42, 0.66, and 0.30 for VP-16, DOX, and VBL, respectively. We compared the effect of DPM on the cellular pharmacology of each chemotherapeutic drug. DPM increased the steady state cellular content of VP-16 by a maximum of 3.2-fold, and that of DOX and VBL by 1.7- and 3.7-fold, respectively. There was a good correlation between the CI50% and the DPM-induced increase in cellular drug content (r = 0.94). DPM had no effect on the initial influx VP-16 or DOX but did increase the initial influx of VBL by 3.5-fold. DPM inhibited the initial efflux of all three compounds. However, there was no relationship between the extent of efflux inhibition and the magnitude of the DPM-induced increase in cellular drug content, indicating that DPM must have other effects as well. DPM has chemical characteristics similar to the MDR modulators of VP-16, DOX, and VBL sensitivity. When compared to verapamil, DPM was as efficacious but twice as potent in its synergistic enhancement of VP-16 sensitivity. These results demonstrate that DPM can markedly increase the cytotoxicity of VP-16, DOX, and VBL and suggest possible clinical applications.

INTRODUCTION

Many of the naturally occurring antibiotic antibiotic agents, including the anthracyclines, Vinca alkaloids, and epipodophyllotoxins, enter cells by passive diffusion (1, 2). In cells selected for high level resistance, these agents can also be transported out of the cell by an energy-dependent process that appears to involve the M, 170,000 glycoprotein coded for by the mdrl gene (3-5).Resistance in many (6), but not all (7) such cell lines exhibiting the multiple drug resistance phenotype is proportional to the amount of gp 170 present in the cell membrane. Resistance is usually associated with reduced accumulation of the chemotherapeutic agent, and more rapid efflux from the cell (6, 8, 9).

Treatment with a large variety of drugs, including the calcium channel antagonists and calmodulin inhibitors, can partially restore the drug sensitivity of many cell lines exhibiting the multiple drug-resistance phenotype, but generally these agents have little or no effect on the sensitivity of intrinsically drug-sensitive cells (10-17). In some cases, these agents have been shown to reduce efflux of the cytotoxic agent (10, 18, 19).

However, the concentrations of calcium antagonists and calmodulin inhibitors effective in tissue culture are thought to be well above the levels that can be maintained with safety in patients (20-21).

DPM is a coronary vasodilator and inhibitor of platelet aggregation with a long history of safe use in humans. Its major biochemical effects are inhibition of nucleoside membrane transport (22-25) and antagonism of phosphodiesterase in platelets (26). We have recently found that DPM can synergistically enhance the cytotoxicity of VP-16 to relatively drug-sensitive 2008 human ovarian carcinoma cells. We now report that DPM also interacts synergistically with DOX and VBL, and present a comparison of the effect of DPM on the cellular pharmacology of VP-16, DOX, and VBL. In addition, we compare the modulatory potency of DPM and VPL for VP-16 in these cells.

MATERIALS AND METHODS

Materials and Chemicals. Pure VP-16 was obtained as a lyophilized powder from the National Cancer Institute. A stock solution of 1 mg/ml was prepared by dissolving VP-16 in absolute ethanol. Working solutions were prepared by further dilution in ethanol. DOX and VBL were obtained in their clinical formulations from Adria Labs Inc. (Columbus, OH) and Bristol Labs Inc. (Wallington, CT), respectively.

[3H]VP-16 (900 mCi/mmol) and [3H]VBL (20 Ci/mmol) in methanol were obtained from Moravek Biochemicals (Brea, CA). [3H]DOX (56 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). The final specific activities used for drug accumulation studies were [3H]VP-16 2 mCi/µmol, VBL 6.67 mCi/µmol, and [3H]DOX 0.056 mCi/µmol. Pure DPM was obtained from Boehringer Ingelheim Ltd. (Ridgefield, CT); a stock solution of 15.44 mM was prepared in water acidified to pH 2.74 with HCl.

Cell Lines and Clonogenic Assay. Studies were performed using the human ovarian carcinoma line 2008 (25). Cells were maintained in logarithmic growth in RPMI 1640 containing 10% fetal bovine serum and 1% l-glutamine without antibiotics. Cells growing in log phase were harvested with trypsin, washed with medium and plated in triplicate onto 60-mm plastic tissue culture dishes (Corning Glass Works, NY) at a density of 300 cells/dish in 5 ml of culture medium. Varying amounts of drugs were added to the dishes, usually 50 µl of a 100X stock solution, and the cells were incubated under 5% CO2 at 37°C for 10 days. Clusters of more than 50 cells were counted as one colony; the control dishes generally contained 100 to 150 colonies.

Cellular Pharmacology. Radiolabeled drugs and DPM were added to subconfluent cultures growing in 60-mm dishes in 2 ml of 37°C culture medium. At appropriate time points, the medium was aspirated and the cells were washed three times as rapidly as possible with 2 ml of 0°C PBS (Oxoid, Columbia, MD). Time points were recorded from the addition of drug to the first PBS wash. The cells were digested overnight with 1 ml of 1 N NaOH. An aliquot was removed for the determination of protein content by the method of Bradford (26), and the remaining radioactivity determined by liquid scintillation counting. Each experimental point was performed in duplicate. The initial influx unidirectional rate was determined from the slope of the regression line fit to cellular contents determined at a minimum of five time points over the

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first 20 (VP-16), 40 (VBL), or 80 (DOX) s during which time uptake was linear. Cells were incubated in the presence or absence of 10 μM DPM for 10 min, and then exposed to 1 μM [3H]VP-16, 0.5 μM [3H]DOX, or 6 nM [3H]VBL for determination of initial influx rate.

Efflux was determined after incubation of cells with drug until steady state had been reached. Cells were loaded with 1 μM [3H]VP-16 for 1 h, 0.5 μM [3H]DOX for 3 h, and 6 nM [3H]VBL for 2 h. The medium was aspirated, and the cells washed three times with 0°C PBS. Five ml of fresh drug-free 37°C RPMI 1640 containing 10% fetal bovine serum was added back to the plates. At the designated time points, the fresh medium was aspirated and the cells washed once with 2 ml 0°C PBS. The actual time between addition of fresh medium and addition of cold PBS was recorded in each case. Duplicate cultures were counted for each data point.

**Regression Analysis.** Cellular content as a function of time after resuspension in fresh medium was fit to the equation:

\[ C(t) = A_i e^{-\lambda_i t} + M e^{-\lambda_0 t}, \]

where \( j = 0 \) (without DPM) or 1 (with DPM). This is a repeated-measures model allowing for free gross scale differences from one course to another. Values of the exponentials determined by least mean squares fitting were compared by Student’s t test.

**RESULTS**

**Potentiation of Cytotoxicity by DPM.** The human ovarian carcinoma cell line 2008 is intrinsically sensitive to VP-16, DOX, and VBL with IC50 values of 0.21 ± 0.020 μM, 21 ± 4 nM, and 5.4 ± 1.0 nM (SD, n = 3) μM, respectively. The interaction between DPM and these three agents was examined by the technique of median effect analysis because this approach allows a definitive determination of the nature of the drug interaction at multiple levels of tumor cell kill (27). The clonogenic survival of the 2008 cells was determined for increasing concentrations of each agent alone, and for DPM and each of the chemotherapeutic agents at increasing concentrations of a fixed molar ratio of the two drugs. In each case the molar ratio was that of the IC50 for DPM and the chemotherapeutic agent. The VP-16:DPM molar ratio was 1:150, while those for DPM:DOX and DPM:VBL were 1:1000 and 1:2000, respectively. Each agent alone and in combination with DPM produced a linear median-effect plot with regression coefficients greater than 0.99 indicating that the dose-response relationships followed the basic mass-action principle. The slopes of the median effect plots for VP-16, DOX, and VBL alone, DPM alone, and each of the three combinations did not differ significantly from each other. This indicates that in each combination, DPM was influencing the toxicity of the chemotherapeutic agent by some mechanism that was common to the two drugs (i.e., they were mutually exclusive).

**Effect of DPM on the efflux of VP-16, DOX, and VBL** was determined by sampling drug content after transfer to medium free of the chemotherapeutic agent. The data for VP-16 have been previously reported, and are included here for purposes of comparison. Figure 3 shows the long term efflux profiles. DPM slowed the efflux of all three chemotherapeutic drugs. The curves presented in Fig. 3 were subjected to regression analysis to determine whether the effect of DPM was on the initial or terminal phase of efflux. The efflux rate constants are presented in Table 4. In the case of VP-16 and DOX the frequency of sampling at short time periods was insufficient to permit the accurate estimation of a rate constant. However, this was possible for VBL, in which case DPM produced a 1.6-fold reduction in the rate constant. The terminal portion of the curve was well fit for all three drugs. DPM produced no significant effect on the terminal efflux of VP-16 or VBL, but did reduce the efflux of DOX by 1.9-fold.

**Comparison of DPM and VPL as Modulators of VP-16.** Figure 4 shows combination index plots of the ability of VPL to enhance the cytotoxicity of VP-16 for 2008 cells. It is apparent that in this cell line, which is intrinsically sensitive to VP-16, VPL does significantly increase cell kill in a synergistic manner. Similar to the situation for DPM, the slopes of the median effect plot for VPL and VP-16 alone and in combination were not significantly different, indicating that the drugs were not acting independently. The mean CI50 value was 0.32 ± 0.13 (SD, N = 3), which was not significantly different from the CI50 values for VP-16 and about 1.5-fold better modulator for VP-16, as it was for DOX.

**Effect of DPM on Cellular Pharmacology.** Figure 2 shows the effect of increasing concentrations of DPM on the steady-state 2008 content of VP-16, DOX, and VBL. A discernible effect of DPM was evident even at a concentration of only 1 μM for all three agents. In the case of VBL, maximum enhancement of steady state cellular content was produced by a DPM concentration of 10 μM, whereas in the case of VP-16 and DOX a plateau was approached only at DPM concentrations above 20 μM. Table 2 compares the fold-increase in steady state cellular content at 10 μM DPM for each of the three agents, and the maximum enhancement produced. There was a good negative correlation between the maximum-fold enhancement and the CI50 value (\( r = -0.94 \)).

The steady state content of VP-16 is a function of the relative influx and efflux rates. We have previously determined that DPM does not influence influx of VP-16, but does inhibit initial efflux. The initial influx rates in the absence and presence of DPM are presented in Table 3. Similar to the situation for VP-16, DPM did not alter the initial influx of DOX. In contrast, DPM did significantly increase the initial influx of VBL by 3.5-fold (\( P < 0.05 \), t test).

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of 0.42 ± 0.02 (SD) produced by DPM. Thus VPL was at least as effective as DPM at sensitizing 2008 cells to VP-16. Figure 5 compares the ability of DPM and VPL to increase the steady state cellular content of VP-16. DPM was more potent that VPL; it required only 4 μM DPM to double the VP-16 content whereas 9 μM VPL was needed to produce this same effect. DPM also produced a larger increase than VPL at the maximum concentration tested (50 μM), but this may be more apparent than real because VP-16 cellular content may continue to increase with higher concentrations of VPL. Of note is the fact that the correlation between CI50 and maximum increase in cellular VP-16 content remained good (r = −0.89) when the data for VPL and VP-16 were added to that for DPM and the three chemotherapeutic agents.

**DISCUSSION**

DPM synergistically enhances the cytotoxicity of VP-16 to 2008 human ovarian carcinoma cells, and this is associated with an increase in cellular VP-16 content and inhibition of VP-16 efflux. We have now established that DPM, a drug with a long history of safe use in humans, markedly enhances sensitivity to clinically important representatives of two other groups of chemotherapeutic agents which, like VP-16, can be pumped out of the cell by the gp 170 mdr1 gene product (6, 14). The major importance of this finding is that it identifies another class of clinically used compounds capable of modulating the activity of VP-16, DOX, and VBL.

The median effect plots indicated that, for all three chemotherapeutic agents, the DPM-induced enhancement of sensitivity resulted from a direct effect of DPM on the cellular pharmacology of VP-16, DOX, and VBL, and was not due to nonspecific toxicity of DPM acting alone. The CI plots indicated that the interaction was truly synergistic for all three chemotherapeutic agents over a 2 log range of cell kill. However, the extent of the synergy, as quantitated by the CI50, differed significantly for the three drugs. Synergy was strongest for VBL, intermediate for VP-16, and weakest for DOX. For those unfamiliar with the use of the CI50, the mean CI50 of 0.42 for the interaction between DPM and VP-16 corresponded to a 5.5-fold reduction in the concentration of VP-16 required to kill 50% of the cells when 20 μM DPM was added to the culture. Thus the CI50 values of 0.66 for DOX and 0.30 for VBL represent very substantial degrees of synergy. The ability of DPM to interact synergistically with these chemotherapeutic agents is a newly defined effect for DPM.

DPM increased the steady state cellular content of all three chemotherapeutic agents, and there was a very good correlation between the ability of DPM to increase steady state drug levels and the extent of synergy as quantitated by the CI50 (r = 0.94). In the case of VP-16, increasing the cellular content has been reported to increase both the damage done to DNA and the cytotoxicity of the drug (28–30). Our results indicate that this relationship between cellular content and cytotoxicity holds also for DOX and VBL. The data suggest that, at least in this cell line, one can use enhancement of steady state drug content as an accurate measure of synergy.

Whereas the extent of synergy was well explained by the increase in cellular drug content, the increase in drug content was not well explained by the effect of DPM on initial influx. DPM reduced the initial influx rate constants by 27% for VP-16, 80% for DOX, and 68% for VBL. The predicted increases in steady state drug content are 1.4-, 5-, and 3-fold respectively, assuming a dominant effect of the rapid phase of efflux. However, the observed maximum increases were 3.2-, 1.6-, and 3.7-fold respectively. Thus DPM must be altering steady state drug content in all three drug agents.

**Table 1** Combination indices at 50% cell kill for the interaction with DPM

<table>
<thead>
<tr>
<th>Drug</th>
<th>Molar ratio*</th>
<th>Mean (SD) combination index</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>150</td>
<td>0.42 ± 0.02b</td>
<td>3</td>
</tr>
<tr>
<td>DOX</td>
<td>1000</td>
<td>0.66 ± 0.07c</td>
<td>3</td>
</tr>
<tr>
<td>VBL</td>
<td>2000</td>
<td>0.30 ± 0.05</td>
<td>3</td>
</tr>
</tbody>
</table>

* Concentration ratio of DPM to chemotherapeutic agent.

b Significantly different from DOX and VBL, P < 0.05, t test.

c Significantly different from VBL, P < 0.05, t test.

**Fig. 1.** Combination index as a function of level of cell kill for the interaction between DPM and VP-16 (left), DOX (middle), and VBL (right). Each curve represents a separate experiment using triplicate cultures for each data point. Values of less than 1 indicate synergy.
Fig. 2. Steady state cellular drug content as a function of DPM concentration. Each point represents the mean of three experiments performed with duplicate cultures. Vertical lines, SD; (where vertical lines are missing the SD was less than the size of the symbol).

Table 2 Enhancement of steady state cellular content of VP-16, DOX, and VBL by DPM

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration of DPM required to cause a twofold increase (μM)</th>
<th>Maximum-fold increase caused by 50 μM DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>DOX</td>
<td>ND*</td>
<td>1.6</td>
</tr>
<tr>
<td>VBL</td>
<td>3.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*ND, not definable.

Table 3 Effect of 10 μM DPM on initial influx rate

<table>
<thead>
<tr>
<th>Drug</th>
<th>Initial Influx Rate ± SDf (fmol/sec/mg protein)</th>
<th>+DPMg Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>75 ± 8</td>
<td>59 ± 17</td>
</tr>
<tr>
<td>DOX</td>
<td>67 ± 13</td>
<td>56 ± 31</td>
</tr>
<tr>
<td>VBL</td>
<td>24 ± 1.0</td>
<td>8.2 ± 2.8</td>
</tr>
</tbody>
</table>

* Mean of three experiments using duplicate cultures.

cellular DOX content by approximately 1.5-fold after 24 h of incubation, which is in the same range as we found for the 2008 cells. They attributed the increase in cellular DOX content to an effect of DPM on influx rather than efflux, but did not determine initial rates for either process.

Fig. 3. Time course of the efflux of VP-16 (top), DOX (middle), and VBL (bottom) in the absence (C) or presence (•) of 10 μM DPM. Each point represents the mean of three experiments performed with duplicate cultures. Vertical lines, SD (where vertical lines are missing SD was less than the size of the symbol).
Table 4  Effect of 10 μM DPM on the efflux rate constants for VP-16, DOX, and VBL

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Initial</th>
<th>P value</th>
<th>Terminal</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>-DPM</td>
<td>ND*</td>
<td>ND</td>
<td>-0.0383 ± 0.0032</td>
</tr>
<tr>
<td>+DPM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-0.0371 ± 0.0032</td>
</tr>
<tr>
<td>DOX</td>
<td>-DPM</td>
<td>ND</td>
<td>ND</td>
<td>-0.00089 ± 0.00005</td>
</tr>
<tr>
<td>+DPM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-0.00048 ± 0.00006</td>
</tr>
<tr>
<td>VBL</td>
<td>-DPM</td>
<td>-0.0412 ± 0.0032</td>
<td>0.002</td>
<td>-0.00235 ± 0.00057</td>
</tr>
<tr>
<td>+DPM</td>
<td>-0.0257 ± 0.0032</td>
<td>-0.00132 ± 0.00057</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ND, not definable.  
† NS, not significant.

Fig. 4. Combination index as a function of level of cell kill for the interaction between VPL and VP-16. Each curve represents a separate experiment using triplicate cultures for each data point. Values of less than 1 indicate synergy.

SYNERGISTIC POTENTIATION OF VP-16, DOX, AND VBL BY DPM

Determined from sampling over initial 60-120 s

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Mean ± SD (min⁻¹)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16</td>
<td>-DPM</td>
<td>-0.637 ± 0.071</td>
</tr>
<tr>
<td>+DPM</td>
<td>-0.463 ± 0.075</td>
<td></td>
</tr>
<tr>
<td>DOX</td>
<td>-DPM</td>
<td>-0.00363 ± 0.00050</td>
</tr>
<tr>
<td>+DPM</td>
<td>-0.00074 ± 0.00001</td>
<td></td>
</tr>
<tr>
<td>VBL</td>
<td>-DPM</td>
<td>-1.146 ± 0.015</td>
</tr>
<tr>
<td>+DPM</td>
<td>-0.047 ± 0.031</td>
<td></td>
</tr>
</tbody>
</table>

in these cells, it is probably present only in relatively small amounts. Under these conditions one would expect that only a small fraction of drug efflux would be mediated by gp 170, and therefore that only a small fraction of efflux would be at risk for modulation by DPM. The large effect of DPM seems inconsistent with this model.

DPM (Figure 6) shares a number of common chemical features with VPL and other modulators of the multiple drug-resistance phenotype (32). It is lipid soluble at physiological pH, and contains two tertiary amines and two planar ring structures. When DPM was compared directly to VPL, it turned out that DPM was approximately twice as potent as VPL with regard to enhancing steady state drug content, but that both agents produced nearly the same degree of synergistic interaction. They are also similar in that they both produced a larger effect on sensitivity to the Vinca alkaloid than to DOX (12, 18, 33). The median effect analysis indicated that VPL, like DPM, was interacting with VP-16 in a mutually exclusive manner, suggesting that both were synergistic by virtue of a similar direct effect on the pharmacodynamics of VP-16 in the cell.

Our studies defined two distinct phases for the efflux of all three chemotherapeutic agents from 2008 cells, with the effect of DPM being entirely (VP-16 and VBL) or mostly (DOX) on the initial phase. The nature of these curves indicates that either there are at least two distinct efflux pathways (e.g., one mediated by gp 170 and the other by passive efflux), or several different compartments within the cell. In any case DPM has a much greater effect on the rapid efflux system, or rapidly emptying compartment, than it does on the slower efflux system, or more slowly emptying compartment.

These results have some important implications for the clinical use of DPM to modulate drug sensitivity. First, since DPM can enhance the sensitivity of 2008 cells, which whether or not they are expressing the mdr1 gene are quite sensitive to VP-16, DOX, and VBL, it offers the possibility of producing a much larger effect on initial tumor response than a modulator whose effect is confined to only those cells exhibiting high level resistance. Second, since VPL can modulate 2008 cell sensitivity, it may do so for other relatively sensitive tumor cells, and be more generally useful than heretofore appreciated. For both drugs there remains the problem that it is difficult to achieve high enough plasma concentrations to enhance sensitivity to VP-16, DOX, or VBL (20). When given by continuous i.v.
infusion at a maximum-tolerated dose, the plasma concentration of total dipyridamole was 11.9 \mu M (34). However, sufficiently high concentrations of DPM can be readily achieved by i.p. instillation (16-fold higher than plasma) (35), offering the possibility of using this approach for the treatment of ovarian carcinoma and mesothelioma.

REFERENCES


Comparison of the Synergistic Potentiation of Etoposide, Doxorubicin, and Vinblastine Cytotoxicity by Dipyridamole


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