Dipyridamole Enhancement of Etoposide Sensitivity

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ABSTRACT

Dipyridamole (DPM) enhanced the sensitivity of human ovarian carcinoma 2008 cells to etoposide (VP-16) producing a 5.5-fold reduction in 50% inhibitory concentration at a DPM concentration of 20 μM. This interaction was shown to be truly synergistic by isobologram and median effect analysis. DPM increased the steady-state VP-16 content of 2008 cells; a DPM concentration of 4 μM increased VP-16 content by 2-fold. DPM was 25 times less potent when cells were incubated in human plasma. In tissue culture medium 96% of the DPM was free, whereas in plasma only 15% was non-protein bound. DPM did not displace VP-16 from proteins under either condition. DPM did not increase the initial influx of VP-16 but did inhibit the initial efflux, reducing the efflux rate constant by 27%. DPM had no effect on the later stages of drug efflux, nor did it irreversibly bind VP-16 in the cell. The effect of DPM was evident within 1 min; once removed, the effect disappeared within 2 min. DPM is a potent nucleoside membrane transport inhibitor and can also inhibit cyclic AMP (cAMP) phosphodiesterase in platelets. Nitrobenzoylthioinosine, another nucleoside transport inhibitor which competes for binding with DPM, did not enhance sensitivity to VP-16 or increase VP-16 cellular accumulation and did not block the effect of DPM. In 2008 cells, DPM did not increase cAMP; when cAMP was increased by incubation with dibutyryl cyclic 3′,5′-AMP, there was no synergy with VP-16. The results indicate that enhanced sensitivity to VP-16 was not due to an effect of DPM on the protein binding of VP-16 or on cellular cAMP and suggest that it is not directly related to inhibition of nucleoside transport. This effect appears to be a newly identified mechanism of action for this agent.

INTRODUCTION

Etoposide is an important chemotherapeutic agent with activity against a wide variety of cancers including small cell lung cancer, testicular carcinoma, and lymphomas and leukemias (1). It appears to enter cells passively and to produce protein-associated strand breaks in DNA (2). VP-16 interacts with topoisomerase II and inhibits the rejoinder of the DNA strands following the strand-passing reaction mediated by this enzyme (3, 4). VP-16 leaves cells by a mechanism that appears to involve the gp150–180 product of the mdr1 gene, the amplification or overexpression of which is associated with the “multiple drug-resistant” phenotype (5). The efflux of VP-16 can be inhibited by verapamil and other calcium channel and calmodulin antagonists (6–8). Inhibition of efflux raises intracellular drug concentration, increases the VP-16-induced DNA strand breaks, and enhances the cytotoxicity of the drug (6–8). In most cases, agents capable of inhibiting VP-16 efflux enhance the sensitivity of highly drug-resistant cells to a greater extent than intrinsically drug-sensitive cells.

DPM has been used clinically for many years as a coronary vasodilator and as an inhibitor of platelet aggregation (9). Its major biochemical effects are inhibition of nucleoside membrane transport (10–13) and elevation of cAMP in platelets due to antagonism of phosphodiesterase (14). We report here that DPM can markedly enhance the sensitivity of human ovarian carcinoma cells to VP-16, that this interaction is truly synergistic, and that it is associated with enhanced VP-16 accumulation and inhibition of efflux.

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. Generally [3H]-VP-16 (900 mCi/mmol; Moravek Biochemicals, Brea, CA) was received as a stock solution of 0.5 mCi/ml in ethanol. For cell accumulation studies, [3H]-VP-16 was added to nonradioactive VP-16 to give a final specific activity of 2 mCi/ml. A stock solution of 15.44 mm DPM (Boehringer Ingelheim, Ltd., Ridgefield, CT) was prepared in water acidified to pH 2.74 with HCl. DbcAMP was purchased from Sigma Chemical Co., St. Louis, MO. NBTI, a generous gift from A. R. P. Paterson (University of Alberta), was dissolved at 24 μM directly in RPMI 1640 containing 10% fetal bovine serum.

Cell Line and Clonogenic Assay. The human ovarian carcinoma line 2008 was used in all experiments (15). 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 triplicate cultures, usually as 50-μl of stock solution, and the dishes 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 100–150 colonies.

Cellular VP-16 Accumulation. 2008 cells were seeded in 60-mm tissue culture dishes such that they approached confluence in 1 to 3 days. When the plates were almost confluent, the medium was aspirated and the designated concentrations of [3H]-VP-16 and DPM were added to the cells in 2 ml of culture medium at 37°C. At appropriate time points, the medium was aspirated and the cells were washed three times as rapidly as possible with 2 ml of phosphate-buffered saline (Oxoid, Columbia, MD) at 0°C. Time points were recorded from the addition of drug to the first wash. The cells were digested overnight with 1 ml of 1 N NaOH. An aliquot was removed for determination of protein content by the method of Bradford (16). Eight hundred μl of the remaining cell lysate were mixed with 8 volumes of Betaplate scintillation fluid (WestChem Products, San Diego, CA) and 3.5 m acetic acid and counted on a liquid scintillation counter. Each experimental point was performed in duplicate. The efflux of VP-16 from cells was determined after incubation of cells with drug for 1 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 were added back to the plates. At the designated time point, the fresh medium was aspirated and the cells were washed once with 2 ml PBS at 0°C. The actual time between addition of fresh medium and addition of cold PBS was recorded in each case. Cellular drug content in duplicate cultures was determined at 5 time points over the first 60 s.
and then at 1, 2, 4, 8, 16, 24, and 32 min. The efflux rate constants were determined by fitting the data by regression to a two compartment model.

**Protein Binding of VP-16.** The steady-state protein binding of \( ^{3}H \)-VP-16 and the effect of DPM on this binding were determined at a VP-16 concentration of 34 \( \mu \text{M} \) and a DPM concentration of 20 \( \mu \text{M} \). Free and bound drug was separated using a Centrifree YM-10 mem-

**cAMP Assay.** Cellular cAMP was measured by HPLC using the assay reported by Lin et al. (17). Cells were incubated either alone or with DPM or dbcAMP at 37°C in a water bath with constant agitation. At various time points, aliquots of 5 x 10^6 cells were removed, dispensed into 0.8 M perchloric acid, and left on ice for 10 min after vortexing. The precipitates were removed by centrifugation, and the supernatants were neutralized with 2.2 M potassium bicarbonate. The sample was centrifuged again for 10 min at 2000 x g, and the supernatant was injected onto the HPLC. The HPLC consisted of a Beckman model 110A pump, a model 160 variable wavelength detector set at 254 nm, and a Waters Z-module fitted with a C18 Bondapak cartridge and a guard column of the same material. The isocratic solvent used was 0.2 M NH4H2PO4 and 5% methanol (v/v), pH 3.0, pumped at a flow rate of 2.0 ml/min. The typical retention time for cAMP was 14 min, and peak identity was confirmed by coelution with purified standards purchased from Sigma and by comparing the 254/280 absorbance ratio. Values of cAMP were calculated from peak heights extrapolated from standard curves and normalized to nmol/million cells.

**RESULTS**

**Potentiation of VP-16 Cytotoxicity by DPM.** Fig. 1 shows the VP-16 dose-response curves for the killing of human ovarian carcinoma 2008 cells in the absence and presence of 20 \( \mu \text{M} \) DPM. Under both conditions the dose-response curves are nonlinear. DPM shifted the VP-16 dose response curve markedly to the left. The ratio of the VP-16 50% inhibitory concentration in the presence and absence of 20 \( \mu \text{M} \) DPM averaged 5.5 ± 0.9 (SD) (P < 0.001, t test). Under conditions where dose-response curves are nonlinear, the assessment of synergy is particularly difficult because of the problem of determining the expected effect of the two drugs in combination (18). In order to determine whether the interaction between VP-16 and DPM was truly synergistic, the interaction was examined by the construction of isobolograms (18). The concentration of the two drugs in combination required to produce 50% cell kill was determined at a VP-16:DPM molar ratio of 1:150, and this value is plotted in Fig. 2. The mean value for this data point lies well below the isobole (solid line), indicating a high degree of synergy. Using this kind of analysis, points lying on the isobole line indicate that the two drugs are simply additive in their effect and points lying above the isobole indicate antagonism.

The nature of the interaction between VP-16 and DPM was investigated further using “median effect analysis” (19). Ovarian 2008 cells were cultured in the presence of increasing concentrations of VP-16 and DPM alone and in a fixed molar ratio of 1:150. VP-16 and DPM, alone and in combination, produced linear median-effect plots with regression coefficients that averaged 0.93 ± 0.04 (n = 3) indicating that their dose-response relationships followed the basic mass-action principle. The slopes of the median effect plots for each drug alone and in combination did not differ significantly from each other, indicating that they were not acting by independent mechanisms (i.e., they were mutually exclusive). Fig. 3 shows a plot of the combination index as a function of the amount of cell kill for 3 separate experiments. The combination index provides a quantitative measure of the extent of drug interaction at each level of cell kill (19). A value of 1 indicates that the drugs are simply additive; a value of >1 indicates antagonism, and a value of <1 indicates synergy. As the curves in Fig. 3 demonstrate, the interaction between VP-16 and DPM at a molar ratio of 1:150 was synergistic over the whole range of cell killing.

All of the studies presented above were done using continuous exposure to both agents throughout the whole 10-day period of colony formation. Fig. 4 shows the effect of 20 \( \mu \text{M} \) DPM when

![Fig. 1. Dose-response curves for the killing of 2008 cells by VP-16 in the absence (■) and presence (▼) of 20 \( \mu \text{M} \) DPM. Data are expressed as percentage survival compared to control cultures containing either no drug or 20 \( \mu \text{M} \) DPM alone. Drugs were present in the culture for the full 10-day period of colony formation. Each point represents the mean of 3 separate experiments with each experiment performed using triplicate cultures; bars, SD.](image)

![Fig. 2. Isobologram analysis of the interaction between VP-16 and DPM at a molar ratio of 1:150. Each curve represents a separate experiment using triplicate cultures for each data point. Values of less than 1 indicate synergy.](image)

![Fig. 3. Median-effect plot of the combination index for the interaction between VP-16 and DPM at a molar concentration ratio of 1:150. Each curve represents a separate experiment using triplicate cultures for each data point. Values of less than 1 indicate synergy.](image)
compared to the 3.2-fold increase produced in medium contain
ing only 10% fetal bovine serum. At concentrations above 20 mM DPM (4 mM) produced a 2-fold increase in cellular VP-16 content and this effect occurred rapidly since it was evident within 2 min after addition of the drug.

Fig. 5. Cellular content of [3H]-VP-16 as a function of time when 2008 cells were incubated in tissue culture medium with 1 μM [3H]-VP-16, Fig. 5 shows that the cellular accumulation of VP-16 was rapid, reaching near steady state after approximately 4 min. When 10 μM DPM was added concurrently to the cultures, accumulation of [3H]-VP-16 was more extensive, but steady state had not yet been reached even by 10 min. Thus DPM increased the cellular content of VP-16, and this effect occurred rapidly since it was evident within 2 min after addition of the drug.

Fig. 6 (top) shows that when 2008 cells were incubated with 1 μM [3H]-VP-16 for 1 h in tissue culture medium, simultaneous exposure to DPM increased intracellular VP-16 content in a concentration-dependent manner. In tissue culture medium, the effect of DPM on VP-16 content was measurable at concentrations as low as 1 μM and appeared to plateau at DPM concentrations above 20 μM. DPM (4 μM) produced a 2-fold increase in cellular VP-16 content. Fig. 6 (bottom) shows that when 2008 cells were incubated in undiluted human plasma, approximately 100 μM DPM was required to double the VP-16 content and that the maximum enhancement was only 2.2-fold as compared to the 3.2-fold increase produced in medium containing only 10% fetal bovine serum.

In order to study the effect of DPM on the efflux of VP-16, 2008 cells were incubated with 1 μM [3H]-VP-16 with or without 10 μM DPM for 1 h until their content reached steady state. They were then rapidly washed and incubated in medium with or without DPM. DPM inhibited the efflux of VP-16, and visual inspection suggested that the effect was primarily on the initial phase of efflux. The data were fit by regression to a two compartment model. In the absence of DPM the initial efflux rate constant was $-0.637 \pm 0.071$ min$^{-1}$ ($n = 7$), whereas in the presence of DPM it was $-0.463 \pm 0.075$ min$^{-1}$ ($n = 4$). Thus DPM caused a 27% reduction in the initial efflux rate constant, and this was statistically significant ($P < 0.025$, both tails). Regression analysis yielded mean terminal efflux rate constants of $-0.0383 \pm 0.0032$ (n = 3) in the absence of DPM and $-0.0371 \pm 0.0377$ (n = 3) in the presence of DPM. Thus DPM had no discernible effect on the β phase of efflux. DPM also did not result in a reduction in the cellular content of VP-16.

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Effect of DPM did not persist for long after the removal of VP-16 but no DPM (El). Each point represents the mean of 3 separate experiments performed with duplicate cultures; bars, SD. Inset, representative experiment showing efflux of VP-16 over the first 60 s in the presence (●) or absence (□) of 10 μM DPM.

The rapidity with which DPM was able to inhibit the efflux of VP-16 was also examined by incubating cells in 1 μM [3H]-VP-16 alone or VP-16 plus 10 μM DPM, the former cells were then transferred to drug-free medium, and the latter were transferred to medium containing 10 μM DPM but no VP-16. Each point represents the mean of 3 separate experiments performed with duplicate cultures; bars, SD.

Fig. 8. Rapidity and duration of DPM effect on VP-16 cellular content. Top: Cells were incubated with 1 μM [3H]-VP-16 for 1 h, and then 10 μM DPM was added to one set of cultures (●) while incubation was continued for the other set without further drug addition (□). Bottom: Cells were incubated for 1 h with 1 μM [3H]-VP-16 and 10 μM DPM. One set of cultures continued incubation in the same medium (●), while the other was transferred to medium containing 1 μM VP-16 but no DPM (□). Each point represents the mean of 3 separate experiments each performed with duplicate cultures; bars, SD.

DISCUSSION

The intrinsic sensitivity of tumor cells and the development of resistance during treatment are important determinants of...
the effectiveness of chemotherapy. The observations that DPM can enhance the sensitivity of cancer cells to VP-16 and that it does so synergistically have important clinical implications. DPM is attractive as a modulator of drug sensitivity for a number of reasons. One reason is that DPM has a long history of safe clinical use as a coronary vasodilator and platelet anti-coagulant. Another is that we (26) and others (27) have shown that DPM-mediated enhancement of sensitivity to VP-16 was 25 times less potent as a modulator of sensitivity to VP-16 than tissue culture medium is unknown.

DPM may not be useful as a modulator of sensitivity to VP-16 when the DPM is administered p.o. Peak total plasma DPM concentrations in patients taking DPM in p.o. doses of 50–100 mg have been reported to be in the range of 2.8–4.6 μM (10, 21, 31). Our results suggest that these concentrations may be too low to effectively enhance cellular accumulation of VP-16 in vivo since free drug concentrations would be even lower. However, the fact that the interaction was truly synergistic and that both drugs are extensively protein bound makes this combination attractive for use in intracavitary therapy. We have previously shown that when DPM and VP-16 are administered i.p., the peritoneal exposure to non-protein-bound drug exceeds the plasma exposure by 53-fold for DPM and 65-fold for VP-16 (32, 33). The peak peritoneal free concentration was 84-fold greater than plasma concentration for DPM, and 188-fold for VP-16. When the VP-16 and DPM are given in saline via the i.p. route, the majority of both drugs are in the free form most favorable for synergistic interaction (32, 33). When they leak out of the peritoneal cavity, they “self-inactivate” by binding to plasma proteins, so that not only is the total drug concentration in the plasma much lower than that in the peritoneal cavity (32, 33), but so are the free forms required for synergy. In addition, our data demonstrated that DPM did not displace VP-16 from protein in plasma. Thus, one would not expect the low concentrations of DPM present in the plasma to increase VP-16 toxicity to normal tissues via this mechanism. Because of the concentration-dependent synergy between DPM and VP-16, one would expect a highly synergistic interaction in the peritoneal cavity but a much less synergistic interaction in the plasma where drug concentrations are much lower.

DPM was 25 times less potent as a modulator of VP-16 accumulation when suspended in human plasma than when suspended in tissue culture medium containing 10% fetal bovine serum, and while DPM increased VP-16 content by a maximum of 3.2-fold in tissue culture medium, the maximum effect in plasma was only 2.2-fold. This is explainable by differences in the extent of protein binding of both drugs. In tissue culture medium we found that approximately 96% of VP-16 was in a free form; the protein binding of DPM was likely to have been minimal as well, although this was not specifically tested. In contrast, in plasma we found that only 15% of the VP-16 was in a free form. In plasma DPM has been reported to be only 5% free, largely due to binding to α1-acidic glycoproteins (21, 34–36). Thus both drugs are less available in plasma. The reason why the maximal level of modulation was less in plasma than tissue culture medium is unknown.
consistent with the interpretation that DPM is inhibiting the initial efflux without affecting influx. Our results also establish that the ability of DPM to enhance sensitivity to VP-16 is not due to the displacement of VP-16 from protein either in tissue culture medium or in plasma. Our data also clearly showed that VP-16 efflux occurred in two phases, the initial phase being much more rapid than the terminal phase. Only the former was inhibited by DPM. The effect of DPM on the cellular pharmacology of VP-16 is very similar to that of verapamil, however, there are some reasons to believe that the drugs may not be working by the same mechanism. Verapamil, with some exceptions (7), is generally more effective as an inhibitor in cells that express the mdrl gene at a high level and manifest the multiple drug-resistant phenotype (37). However, the 2008 cells used in our experiments are very sensitive to VP-16 and do not manifest the multiple drug resistance phenotype. We speculate that the initial rapid phase is mediated by an active pump, whereas the terminal phase occurs by passive diffusion, but whether this pump is the mdrl gene product is unknown.

DPM is best known as an inhibitor of nucleoside membrane transport in many kinds of cells (11–13). NBPTI produces a qualitatively similar effect on nucleoside transport, and inhibition by both drugs is associated with binding to a specific site for which they compete (22, 24, 38). In Ehrlich ascites cells VP-16 can also interact with the nucleoside transporter; it inhibits the transport of the nucleoside cytarabine and alters the binding of NBPTI (38). Inhibition of nucleoside membrane transport by DPM is extremely rapid (39), as is its effect on VP-16 efflux. These associations suggest a possible mechanistic linkage between the ability of DPM to block VP-16 efflux and its effect on nucleoside membrane transport. However, the fact that NBPTI did not enhance sensitivity to VP-16 or increase its uptake and that it did not block the ability of DPM to do so argues in favor of some other mechanism. Thus, either the interaction between DPM and VP-16 does not involve the nucleoside transporter or DPM and NBPTI interact differently with the transporter such that DPM can inhibit both nucleoside and VP-16 efflux whereas NBPTI affects only the movement of the nucleoside. The former seems more likely based on the fact that DPM can block the efflux of methotrexate, indicating that it can inhibit yet another putatively unrelated transporter (40).

The other well recognized effect of DPM is inhibition of cAMP phosphodiesterase in platelets (9, 14). However, no relationship between cAMP levels and either the cellular pharmacology of VP-16 or the multiple drug resistant phenotype has been reported. DPM did not alter the cAMP content of 2008 cells at concentrations up to 20 μM, and elevation of cAMP content with dbcAMP did not produce synergy as was observed with DPM. These results indicate that elevation of cAMP is not a requirement for DPM enhancement of VP-16 sensitivity and argue strongly against a role for cAMP in the synergistic interaction between DPM and VP-16.

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