Effects of Verapamil on Etoposide, Vincristine, and Adriamycin Activity in Normal Human Bone Marrow Granulocyte-Macrophage Progenitors and in Human K562 Leukemia Cells in Vitro

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ABSTRACT

We have examined the effects of verapamil on the cytotoxicity of etoposide, vincristine, and Adriamycin in human leukemia K562 cells as well as in normal human bone marrow granulocyte-macrophage progenitors (CFU-GM). Etoposide was 10-fold more potent against K562 cells than against normal human bone marrow CFU-GM. Similarly, vincristine cytotoxicity was about 10-fold greater against K562 cells than against human bone marrow CFU-GM. In contrast, Adriamycin exhibited little selectivity for K562 cells versus normal bone marrow CFU-GM during the 1-h incubation period of the experiments. In the presence of verapamil (2.5–10 μM), etoposide cytotoxicity was enhanced in both malignant and normal cells. Verapamil enhanced vincristine (0.1 μM) cytotoxicity in K562 cells but did not potentiate Vinca alkaloid toxicity in normal bone marrow CFU-GM. Adriamycin, on the other hand, did not display any calcium antagonist-mediated potentiation of cytotoxicity in either malignant or normal tissue. These results indicate that short-term (1 h) incubations with etoposide, vincristine, and Adriamycin yield different profiles of toxicities whether used alone or with chemosensitizing agents such as the calcium antagonists. These differences in activities are consistent with different mechanisms for intracellular disposition of these three classes of anticancer agents and are worthy of further investigation, especially with regard to combinations with calcium antagonists.

INTRODUCTION

Calcium antagonists such as verapamil have been shown to overcome resistance to anthracyclines and Vinca alkaloids in both murine and human tumor cells (8, 10–13). In addition, we have recently reported that calcium antagonists potentiate etoposide-induced cytotoxicity in L1210 cells in vitro (15, 16). Thus, chemosensitizers such as the calcium antagonists and calmodulin inhibitors have been suggested for clinical use in combination with anticancer agents to overcome clinically acquired resistance or to sensitize nonresponsive tumor types. Since bone marrow toxicity is the limiting factor for the clinical use of many chemotherapeutic agents, the potential clinical use of calcium antagonists with anticancer agents makes it desirable to examine the cytotoxic effects of these drug combinations in normal hematopoietic cells as well as in tumor cells. Such studies would determine whether calcium antagonist effects are selective for malignant cells. In this paper, we have examined verapamil-mediated enhancement of selective toxicity in normal human bone marrow CFU-GM versus human K562 myelogenous leukemia cells using 3 anticancer agents: vincristine; Adriamycin; and the epipodophyllotoxin, etoposide (VP-16).

MATERIALS AND METHODS

Chemicals. Etoposide was provided by Bristol Laboratories (Syracuse, NY). Verapamil was provided by Knoll Pharmaceuticals ( Whippany, NJ). All drugs were dissolved in either dimethyl sulfoxide or 50% ethanol. Solvent concentrations did not exceed 0.6% in the culture medium after drug treatment and were present in control flasks at equivalent levels. Cell culture medium and fetal calf serum were purchased from GIBCO (Grand Island, NY). Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO).

Cells, Media, and Incubation Techniques. Human leukemia K562 cells were grown in suspension culture in RPMI 1640 medium containing 10% fetal calf serum, penicillin, and streptomycin.

Colony-forming Assays. Human K562 cells were collected for experiments during the exponential growth phase at concentrations ranging from 5–8 x 10^5 cells/ml. These cells, which were maintained in RPMI 1640 medium containing 10% fetal calf serum, were exposed to the anticancer agents in the presence or absence of verapamil for 1 h at 37°C in 5% CO_2. Cells were washed twice in RPMI 1640 medium, counted, and subsequently plated at appropriate concentrations (10^2–10^5 cells/ml) in 0.3% agar medium containing Dubecco’s modified Eagle’s medium supplemented with 20% heat-inactivated fetal calf serum. Cultures were incubated for 7–10 days at 37°C in a humidified atmosphere of 5% CO_2 in air. Colonies containing greater than 50 cells were counted using an inverted microscope. Cytotoxicity was determined by comparing colony counts from drug-treated cells with counts from cells incubated only with solvent.

Assays for granulocyte/macrophage progenitors (CFU-GM) from normal human bone marrow were performed as described previously (9). Bone marrow was collected in heparin, diluted with Ca^2+- and Mg^2+-free HBSS, and separated on Ficoll/Hyphaque gradients (Ficoll/Paque; Pharmacia, Piscataway, NJ) at 250 g for 40 min. The interface cells were collected, washed in HBSS, resuspended in Medium 199 containing 25% human AB serum and 10% dimethyl sulfoxide and, unless otherwise noted, then frozen in vapor phase liquid nitrogen at a final concentration of 2 x 10^5 cells/ml. For experiments, cells were thawed at 40°C, diluted 10-fold in HBSS containing 6% fetal calf serum and heparin (5 units/ml), and then washed twice. Cells were then resuspended in α-MEM (Grand Island Biological Co., Grand Island, NY) containing 6% fetal calf serum, counted, and diluted to 1.4 x 10^5 cells/ml.

The abbreviations used are: CFU-GM, granulocyte-macrophage colony-forming units; VP-16, 4′-demethylepipodophyllotoxin-9-((4,5-O-ethylidene-3-O-glucopyranosyl)-(epotoposide, VP-16-213); IM, 50% growth-inhibitory concentration; HBSS, Hank’s balanced salt solution; α-MEM, α-minimal essential medium.

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These bone marrow cells were exposed to drugs for 1 h at 37°C in 5% CO₂, washed in α-MEM, and subsequently plated at 2 × 10⁶ cells/ml in 0.3% agar in α-MEM containing 5% fetal calf serum. Colony-stimulating activity was provided by the addition of giant cell tumor-conditioned medium (GIBCO). Aggregates of cells were scored as colonies (greater than 50) or clusters (less than 50) with an inverted microscope after 11 days of incubation at 37°C in 5% CO₂.

RESULTS

Verapamil-induced Potentiation of Etoposide Effects in K562 Cells and in Normal Human Bone Marrow CFU-GM.

Since bone marrow toxicity is the limiting factor in the clinical use of etoposide, it was of interest to compare the sensitivity of normal human bone marrow CFU-GM to K562 cells with respect to etoposide cytotoxicity. Chart 1 demonstrates that, as etoposide concentration was increased, there was a progressive decrease in granulocyte-macrophage colony formation in normal human bone marrow. Colony formation was decreased to only 48.5 ± 6.5% of control at 50 μM etoposide (not shown), a level which is achievable clinically using high-dose etoposide (4). Etoposide is at least 10-fold more potent against K562 cells than against normal human bone marrow CFU-GM as measured by the 50% growth-inhibitory concentration in both cell types. Chart 1 also demonstrates the effect of verapamil (10 μM) on etoposide-induced cytotoxicity in both normal bone marrow and K562 cells. In the presence of verapamil, there is a progressive enhancement of cytotoxicity in both normal and malignant cells as the etoposide concentration is increased.

In order to explore the dose-response relationship for verapamil further, CFU-GM and K562 cells were exposed to 25 μM etoposide in the presence of 2.5, 5.0, and 10 μM verapamil (Table 1). In these experiments, freshly obtained bone marrow was used to obviate any potential effects of prior freezing. As in the experiments in Chart 1, verapamil was nontoxic by itself but did potentiate the effects of etoposide at the lowest concentration used.

Vincristine Cytotoxicity in K562 Cells and in Normal Human Bone Marrow CFU-GM: Potentiation by Verapamil. Similar to results observed above for etoposide, Chart 2 indicates an order of magnitude difference in potency for vincristine when comparing 50% values in K562 cells and in normal bone marrow CFU-GM: 39 nM versus 310 nM, respectively. However, verapamil (10–40 μM) did not potentiate vincristine-induced cytotoxicity in normal bone marrow CFU-GM. In K562 cells, verapamil progressively enhanced vincristine cytotoxicity as the verapamil concentration increased.

Table 1

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of VP-16 with and without verapamil on colony formation by human CFU-GM and leukemic K562 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colony/10⁶ cells</td>
</tr>
<tr>
<td>Human CFU-GM</td>
<td></td>
</tr>
<tr>
<td>0 μM VP-16</td>
<td>85 ± 2</td>
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<tr>
<td>25 μM VP-16</td>
<td>68 ± 2</td>
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<tr>
<td>10 μM verapamil</td>
<td>102 ± 17</td>
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<td>25 μM VP-16 + 10 μM verapamil</td>
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<td>25 μM VP-16 + 5 μM verapamil</td>
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</tr>
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<td>K562 cells</td>
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</tr>
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<td>10 μM verapamil</td>
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<td>25 μM VP-16 + 10 μM verapamil</td>
<td>25 ± 6</td>
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<td>28 ± 8</td>
</tr>
<tr>
<td>25 μM VP-16 + 2.5 μM verapamil</td>
<td>40 ± 13</td>
</tr>
</tbody>
</table>

a From freshly obtained human bone marrow.

b Mean ± SE.

Chart 1. The effect of a 1-h incubation with various extracellular concentrations of etoposide alone (O, □) or in the presence of verapamil (10 μM; ●, ■) on the growth of normal human granulocyte/macrophage progenitors (CFU-GM) and tumor colony-forming units grown from K562 cells. Points, mean of 6–18 determinations made from 2–3 experiments run on separate days; bars, SE. Three to 6 determinations were made at each drug concentration in individual experiments.

Chart 2. The effect of a 1-h incubation with various concentrations of vincristine alone (O, □) or in the presence of verapamil (10–40 μM) on the growth of normal human granulocyte/macrophage progenitors (CFU-GM) and tumor colony-forming units grown from K562 cells. Points, mean of 6–18 determinations made from 2–3 experiments run on separate days; bars, SE. Arrows, 50% growth-inhibitory concentration.

The 50% growth-inhibitory concentration in both cell types. Chart 1 also demonstrates the effect of verapamil (10 μM) on etoposide-induced cytotoxicity in both normal bone marrow and K562 cells. In the presence of verapamil, there is a progressive enhancement of cytotoxicity in both normal and malignant cells as the etoposide concentration is increased.

In order to explore the dose-response relationship for verapamil further, CFU-GM and K562 cells were exposed to 25 μM etoposide in the presence of 2.5, 5.0, and 10 μM verapamil (Table 1). In these experiments, freshly obtained bone marrow was used to obviate any potential effects of prior freezing. As in the experiments in Chart 1, verapamil was nontoxic by itself but did potentiate the effects of etoposide at the lowest concentration used.

Vincristine Cytotoxicity in K562 Cells and in Normal Human Bone Marrow CFU-GM: Potentiation by Verapamil. Similar to results observed above for etoposide, Chart 2 indicates an order of magnitude difference in potency for vincristine when comparing 50% values in K562 cells and in normal bone marrow CFU-GM: 39 nM versus 310 nM, respectively. However, verapamil (10–40 μM) did not potentiate vincristine-induced cytotoxicity in normal bone marrow CFU-GM. In K562 cells, verapamil progressively enhanced vincristine cytotoxicity as the verapamil concentration increased.
was increased. Maximum verapamil effect was observed at 10 μM under the conditions used for these experiments. The results suggest that verapamil may increase the selectivity of vincristine at clinically relevant concentrations of the Vinca alkaloid (0.1 μM).

**Adriamycin Cytotoxicity in K562 and in Normal Human Bone Marrow CFU-GM.** Our experiments reveal a much different profile for Adriamycin cytotoxicity than for etoposide or vincristine (Chart 3). Adriamycin is not very selective against K562 cells compared to normal CFU-GM. The 50% inhibitory concentration of Adriamycin for the 2 cell types ranges between 0.2 and 0.4 μM. In addition, verapamil (10 μM) did not enhance cytotoxicity of Adriamycin for the 2 cell types ranges between 0.2 and 0.4 μM. In addition, verapamil (10 μM) did not enhance cytotoxicity in either tissue.

**DISCUSSION**

In previously untreated cancer patients, etoposide is generally less myelosuppressive than many other chemotherapeutic agents; yet the bone marrow is still the limiting factor. In order to assess more directly the effects of etoposide on hematopoietic tissue, we have studied normal cryopreserved bone marrow cells using a CFU-GM assay. The results suggest that hematopoietic cells, which ultimately form granulocytes and macrophages, are relatively insensitive to etoposide under the short drug exposure (1 h) of our experiments. The relative insensitivity of these cells to etoposide (Chart 1) as compared to K562 corresponds well with a previous report in which etoposide was used to selectively purge normal human bone marrow cells of “contaminating” K562 cells (6). The results in Chart 1 also indicate that, although normal hematopoietic tissue is relatively insensitive to etoposide, short periods of incubation with verapamil (10 μM) allow for a progressive enhancement of toxicity with increasing etoposide concentrations. Importantly, the verapamil concentration at which this effect is seen (2.5 μM; see Table 1) approaches that which may be achieved clinically. Although the clinical implications of these findings are unclear, the observed increased toxicity might serve as a caveat for the potential combined use of etoposide and calcium antagonists. Verapamil also potentiated etoposide effects against K562 cells (Chart 1), but it is unlikely that the magnitude of the difference in effect between the 2 cell types would result in therapeutic advantage.

We have recently reported that calcium antagonists potentiate etoposide-induced DNA damage and cytotoxicity in L1210 cells after short periods of incubation (15). We have extended these observations to an analysis of the effects of verapamil on etoposide membrane transport, demonstrating a direct correlation between etoposide levels, DNA damage, and cytotoxicity whether or not verapamil is present (16). On this basis, we have suggested that the main mechanism by which verapamil potentiates etoposide is by enhancing intracellular levels of the drug. We have observed a similar phenomenon in K562 cells (data not shown), and it is likely that this mechanism pertains to CFU-GM also, although we have not studied this. In cells which are resistant to etoposide by virtue of diminished accumulation of the drug, one might expect to observe an even greater degree of enhancement of cytotoxicity in the presence of verapamil.

Vincristine, like etoposide, is more selective in its lethal effects against the leukemia cell line than against normal CFU-GM (Chart 2). Based on the 50% inhibitory concentration for colony formation, vincristine is approximately 10-fold more potent in K562 cells. Verapamil decreased the IC50 value in K562 cells approximately 2-fold (Chart 2). This degree of potentiation in sensitive K562 cells corresponds well with the verapamil-mediated 3- to 5-fold increase in vincristine cytotoxicity observed by Tsuruo et al. (13). In contrast, verapamil (10–40 μM) did not potentiate cytotoxicity of vincristine used in concentrations up to 1 μM in normal hematopoietic CFU-GM (Chart 2). While this paper was in preparation, Fine et al. (2) similarly reported that vincristine cytotoxicity was not potentiated in normal bone marrow CFU-GM by 2 μM verapamil after a 24-h incubation.

Under the short period of incubation of our experiments (1 h), verapamil did not potentiate anthracycline cytotoxicity in either K562 cells or human bone marrow CFU-GM (Chart 3). These results are in agreement with those recently reported in which verapamil did not potentiate Adriamycin effects in human bone marrow CFU-GM after 1 or 24 h of incubation (2). In addition, after a 1-h incubation with verapamil (10 μM), we were unable to observe potentiation of Adriamycin-induced cytotoxicity or DNA damage in L1210 cells in vitro.5 Tsuruo et al. (13), using a 72-h continuous incubation with Adriamycin in K562 cells, have demonstrated a much lower IC50 value (0.024 μM) than we have observed (0.2 μM, Chart 3). In addition, these investigators demonstrated a greater than 2-fold enhancement of growth-
inhibitory action of Adriamycin in the presence of 10 μM verapamil. These data suggest that verapamil potentiation of Adriamycin cytotoxicity in some cells may require prolonged periods of drug exposure for expression.

Rogan et al. (7) recently demonstrated that resistance to Adriamycin could be reversed by verapamil (at concentrations up to 6 μM) in some human ovarian cancer cell lines after 24-h incubation in the presence of both drugs. They did not study shorter periods of incubation. Potentiation of Adriamycin effects was also noted in ovarian cancer cells which were sensitive to the anthracycline, but enhancement of activity was not as great as that seen with resistant cells. Interestingly, Adriamycin cytotoxicity was not potentiated by verapamil in a patient-derived fibroblast cell line, suggesting that Adriamycin effects can be selectively enhanced in tumors. Thus, our findings that verapamil does not potentiate Adriamycin effects in malignant cells must again be viewed within the context of short-term drug exposure and the types of cells studied.

In this paper, we have explored the possibility of using calcium antagonists to increase the selectivity of anticancer agents by analyzing verapamil-mediated enhancement of cytotoxicity in normal versus malignant tissues which are sensitive to the 3 classes of anticancer agent tested. The results have indicated that no easy predictions can be made as to the effects of chemosensitizing agents on anticancer drug activity. The heterogeneity of drug effects with regard to selectivity in the presence or absence of verapamil probably relates more to differences in the types of cells studied.

REFERENCES

Effects of Verapamil on Etoposide, Vincristine, and Adriamycin Activity in Normal Human Bone Marrow Granulocyte-Macrophage Progenitors and in Human K562 Leukemia Cells in Vitro


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