Reversal of Vinca Alkaloid Resistance but not Multiple Drug Resistance in Human Leukemic Cells by Verapamil

William T. Beck, Margaret C. Cirtain, A. Thomas Look, and Richard A. Ashmun

Departments of Biochemical and Clinical Pharmacology [W. T. B., M. C. C.] and Hematology-Oncology [A. T. L., R. A. A.], St. Jude Children's Research Hospital, Memphis, Tennessee 38101

ABSTRACT

We examined the ability of verapamil, a Ca2+ channel blocker, to overcome Vinca alkaloid and multiple drug resistance in our CEM/VLB100 and CEM/DOX human leukemic lymphoblasts. Compared with the parent CCRF-CEM cells, CEM/VLB100 cells are ≈200- to 800-fold resistant to vinblastine and express cross-resistance to vincristine, doxorubicin, and other "natural product" drugs, as determined by comparing 50% inhibitory concentrations in a 48-h growth inhibition assay. Verapamil (10 μM) decreased the 50% inhibitory concentrations for Vinca alkaloids in CEM/VLB100 cells by ≈75- to 85-fold but caused only slight (≈2- to 5-fold) decreases in 50% inhibitory concentrations for anthracyclines, epipodophyllotoxins, and other tubulin-binding drugs (colchicine and podophyllotoxin). Qualitatively similar results were obtained with doxorubicin-resistant cells, termed CEM/DOX; verapamil caused a 19-fold increase in doxorubicin toxicity but 67- and 3500-fold increases in the toxicities of vinblastine and vincristine, respectively. These results indicate that the effect of verapamil is relatively greater for Vinca alkaloids, with less pronounced effects for the other natural product drugs against which these cells express multiple drug resistance. In flow cytometric studies, individually nontoxic or minimally toxic toxicities of tumor cells that have been selected for resistance to one of a few antitumor drugs, such as Vinca alkaloids, anthracyclines, epipodophyllotoxins, and other "natural products" or their semisynthetic derivatives. This pattern of resistance has been substantiated in experimental tumor systems both in vivo (1-4) and in vitro (5-11), and various pharmacological, biochemical, and molecular alterations have been linked with it (6). For example, a high molecular weight glycoprotein has been found on the surfaces of MDR cell lines of rodent (12, 13) and human (14) origin. However, despite clinical indications of MDR in patients with (usually) advanced neoplastic disease, such biochemical "markers" on the tumor cells from these individuals have yet to be consistently shown to be linked to this phenomenon.

Recent pharmacological studies in experimental systems have sought to circumvent MDR with the goal of applying this information in treatment strategies for unresponsive patients (15). Foremost among these efforts have been the studies of Tsuruo et al. (16-22) and others (23, 24), showing that Ca2+ channel blockers, such as verapamil, and calmodulin antagonists, such as trifluoperazine, can reverse alkaloid and anthracycline resistance in vitro and in vivo. This subject has been reviewed recently (25).

During the course of our investigations, we found that verapamil did not greatly reverse MDR but produced a rather specific and rapid enhancement of Vinca alkaloid cytotoxicity in our alkaloid- and anthracycline-resistant human leukemic cell lines; our results are the focus of this paper. A preliminary account of some of this work has been presented (26).

MATERIALS AND METHODS

Cells and Culture Conditions. CCRF-CEM human leukemic lymphoblasts (CEM) and their drug-resistant variants were grown as described earlier (14). Drug-resistant sublines were selected by growth in the continuous presence of sublethal concentrations of drug. After 1-2 months, cells were exposed to drug on an intermittent basis, usually only 3-4 days per week. The following cell lines were selected: CEM/VLB100, ≈200- to 800-fold resistant to VLB (14); CEM/DOX, ≈70-, to 190-fold resistant to DOX; CEM/ara-C, ≈3000-fold resistant to ara-C; and CEM/MTX, ≈133-fold resistant to MTX.

Quantitation of Drug Effects. The growth-inhibitory effects of drugs, either alone or in combination, were assessed by plating cells in multiwell dishes (Costar No. 3524) at a final density of ≈2.5 × 10^5/ml. Drug solutions were made in 0.9% NaCl solution and were ≤10% of the final volume of the cell suspension; epipodophyllotoxins were dissolved in dimethyl sulfoxide, the final concentration of which (≤0.69%) had no effect on cell growth (27).

Cell numbers were determined with a Coulter Counter (Model ZBI) using a Channelizer to distinguish cells from debris. The IC_{50} was defined as the concentration of drug that inhibits the 48-h cell growth by 50%, compared with untreated controls. The fold decrease in IC_{50} was determined by dividing the IC_{50} for the controls by that for verapamil-treated cells.
Flow Cytometry. After exposure to drugs for different times, cells were harvested for flow cytometry. DNA staining with propidium iodide, generation of DNA histograms, and analysis of the percentage of cells in G2-M, S, and G1 phases of the cell cycle were done as described earlier (28). The percentage of mitotic cells for each sample was determined in acetocarmine-stained preparations (28). Histochimical staining of cytospin preparations was done with an automated Ames Hema-Tek slide stainer.

Chemicals and Supplies. VLB and VCR were obtained from Eli Lilly and Co. (Indianapolis, IN), DOX and DNR were from Adria Laboratories (Wilmington, DE), VM-26 and VP-16-213 were from Bristol Laboratories (Syracuse, NY), and CLC and POD were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture media and fetal bovine serum were obtained from KC Biologicals (Lexena, KS) and Flow Laboratories (McLean, VA), respectively, and tissue culture plasticware was from standard sources.

RESULTS

Effect of Verapamil on MDR in CEM/VLB100 Cells. We showed previously that verapamil effectively enhanced the cytotoxicity of VLB in the VLB-resistant CEM/VLB100 cells (29): increasing concentrations of verapamil shifted the VLB dose-response curve to the left; at 10 μM verapamil, the IC50 for VLB against resistant cells was decreased to nearly that of the sensitive cells (29). Verapamil alone was somewhat toxic to the CEM/VLB100 cells: 10 μM often produced an approximate 20% decrease in cell number during a 48-h experiment (29). We nonetheless chose 10 μM as the standard concentration for the experiments to be reported here because it provided maximal inhibition of cell growth by other agents with only minimal verapamil-induced toxicity.

The CEM/VLB100 cells express the MDR phenotype, demonstrating cross-resistance to several natural product compounds (6, 29). As can be seen in Table 1, however, verapamil was mainly effective in decreasing the IC50 values of the Vinca alkaloids (VLB and VCR) against CEM/VLB100 cells, causing an 87- and 75-fold increase in alkaloid cytotoxicity, respectively. The Effect of verapamil on the cytotoxicity of the anthracyclines (DOX and DNR), the epipodophyllotoxins (VM-26 and VP-16-213), and the two other tubulin-binding drugs (CLC and POD) was much less pronounced, ranging between a 2- and 5-fold decrease in their IC50 values. Thus, in the presence of verapamil, CEM/VLB100 cells largely retain MDR despite increased susceptibility to Vinca alkaloid toxicity. It may be of importance that the ability of verapamil to enhance drug cytotoxicity is roughly proportional to the degree of resistance of the cells.

Effect of Verapamil on Drug Cytotoxicity of Other Drug-resistant CEM Cell Lines. It was important to determine if the verapamil effect was restricted to Vinca alkaloids in alkaloid-resistant cells or if it extended to cells selected for primary resistance to other drugs. Shown in Table 2 are results of experiments that test the effect of verapamil on our CEM/DOX cell line, which is =70- to =190-fold more resistant to DOX than is the parent line and =68- and =238-fold cross-resistant to VLB and VCR, respectively. Verapamil clearly enhanced the cytotoxicity of DOX against these cells in a progressive, concentration-dependent manner, but the effect was less striking than observed for Vinca alkaloids against CEM/VLB100 cells.

The MDR phenotype of CEM/DOX cells is characterized in part by cross-resistance to DNR, Vinca alkaloids, and epipodophyllotoxins (30). Addition of verapamil with Vinca alkaloids to cultures of CEM/DOX cells produced the rather unexpected results shown in Table 2. Not only did verapamil cause an apparent dose-related reduction in the IC50 values for both VLB and VCR but also the decreases were much greater than that observed for DOX. Moreover, effects on VCR cytotoxicity (>3500-fold enhancement) greatly exceeded those on VLB.

Finally, verapamil did not enhance MTX or ara-C cytotoxicity either in CEM cells selected, respectively, for resistance to these antimetabolites or in VLB- or DOX-resistant cells (data not shown). It did, however, appear to enhance the cytotoxicity of VLB in the cells selected for antimitabohite resistance (data not shown). Thus, the verapamil effect appears limited to certain natural product compounds. In this regard, this drug enhanced the cytotoxicity of VLB in the parent CEM cells but had no effect on MTX or ara-C cytotoxicity in these same drug-sensitive cells (data not shown).

Flow Cytometric Findings. Since 10 μM verapamil itself was somewhat cytotoxic to the cells (29), we wished to determine if the great enhancement of alkaloid cytotoxicity shown in Tables 1 and 2 was due to the alkaloid alone or to some unusual interaction of the two agents. We therefore used flow cytometric techniques to address this point. Cell cultures were analyzed at various times after the addition of drugs, singly or in combination; the results shown in Fig. 1 are DNA histograms generated at 24 h after beginning the treatments indicated. Fig. 1A shows results from untreated controls during this time period, and Fig. 1B shows identical results obtained with 0.1 μM VLB alone (a noncyotoxic concentration). Results with 10 μM verapamil alone were identical to these controls in Fig. 1, A and B (data not shown). When drugs that showed little or no toxicity as single agents were combined, however, the results were markedly different (Fig. 1D), the most notable effect being a striking increase in the G2-M peak and a decrease in the G1 peak. It is significant that the results shown in Fig. 1D are identical to those obtained after cells were treated with a toxic concentration of VLB (5 μM), as demonstrated in Fig. 1C. The black bars on the right side of each panel in Fig. 1 represent the mitotic indices. It is clear that the VLB-verapamil combination (Fig. 1D) had the same effect on mitotic index as a toxic concentration of VLB.

Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Degree of resistance</th>
<th>IC50 (μM)</th>
<th>Fold decrease in cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLB</td>
<td>Without verapamil</td>
<td>0.488 ± 0.121</td>
<td>0.006 ± 0.005</td>
</tr>
<tr>
<td>VCR</td>
<td>1163</td>
<td>4.85 ± 0.49</td>
<td>0.062 ± 0.042</td>
</tr>
<tr>
<td>DOX</td>
<td>109</td>
<td>4.05 ± 0.78</td>
<td>1.85 ± 0.95</td>
</tr>
<tr>
<td>DNR</td>
<td>102</td>
<td>2.65 ± 0.35</td>
<td>0.50 ± 0.27</td>
</tr>
<tr>
<td>VM-26</td>
<td>18</td>
<td>1.96 ± 1.68</td>
<td>0.50 ± 0.18</td>
</tr>
<tr>
<td>VP-16-213</td>
<td>59</td>
<td>12.0 ± 2.0</td>
<td>5.03 ± 3.36</td>
</tr>
<tr>
<td>CLC</td>
<td>44</td>
<td>0.66 ± 0.046</td>
<td>0.35</td>
</tr>
<tr>
<td>POD</td>
<td>19</td>
<td>0.048 ± 0.011</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* Cells, diluted to a density of ≈2 × 10^6/ml, were exposed to drugs for 48 h and then counted.
* Calculated by dividing the IC50 of the resistant CEM/VLB100 cells by that of the parent CEM cells.
* The fold decrease in cytotoxic concentration was determined by dividing the IC50 of controls by that of the verapamil-treated cells.
* Mean ± SD of two to four experiments. Where no SDs are given, only one experiment was done.
Table 2

<table>
<thead>
<tr>
<th>Verapamil concentration (µM)</th>
<th>IC₅₀ DOX (µM (range))</th>
<th>Fold decrease</th>
<th>IC₅₀ VLB (µM)</th>
<th>Fold decrease</th>
<th>IC₅₀ VCR (µM)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.25 (3.8–4.7)</td>
<td>1.07</td>
<td>131 ± 91</td>
<td>1.12</td>
<td>350 ± 14</td>
<td>1.12</td>
</tr>
<tr>
<td>1</td>
<td>0.93 (0.61–1.25)</td>
<td>4.6</td>
<td>53 ± 11</td>
<td>2.5</td>
<td>62 ± 17</td>
<td>5.7</td>
</tr>
<tr>
<td>10</td>
<td>0.22 (0.009–0.35)</td>
<td>19.5</td>
<td>&lt;1.95 ± 2.62</td>
<td>&gt;67.2</td>
<td>&lt;0.1</td>
<td>&gt;3500</td>
</tr>
</tbody>
</table>

* CEM/DOX cells were =70- to =190-fold less sensitive to the 48-h growth-inhibitory effects of DOX than were the sensitive CEM cells and were =68- and =238-fold cross-resistant to VLB and VCR, respectively, compared to the sensitive CEM cells.4

4 Mean ± SD.

DISCUSSION

We have shown here that the Ca²⁺ channel blocker, verapamil, greatly enhances the cytotoxicity of Vinca alkaloids against...
SELECTIVE ENHANCEMENT OF VINCA ALKALOID CYTOTOXICITY BY VERAPAMIL

When resistant cells were treated with other natural product compounds, a less striking enhancement of cytotoxic effects was seen for flow cytometric analysis (A), mitotic index (B), and cell number (C). Shown is a representative experiment.

Reversal of Vinca alkaloid resistance by verapamil appears to reflect an increase in VLB or VCR cytotoxicity, rather than a combined effect of verapamil and alkaloids, as was demonstrated clearly by our flow cytometric data. This confirms results of Tsuuro et al. (19-22) who showed that verapamil increases the cellular accumulation of [3H]VCR in alkaloid- and anthracycline-resistant leukemia cells. Further, our results demonstrate that, in the presence of verapamil, nontoxic concentrations of VLB rapidly become lytic to cells. This finding, not demonstrated before, mimics that seen with very high concentrations of VLB alone against either the CEM/VLB100 cells (data not shown) or the parent CCRF-CEM cells (31).

The mechanism by which verapamil enhances the cytotoxicity of certain antineoplastic drugs is not understood. Blockade of the slow calcium current, i.e., the inward passive diffusion of calcium across the cell membrane, is thought to be a major action of verapamil (32, 33). Other work has suggested that it acts on calmodulin, stimulates Ca\(^{2+}\)-ATPase activity and Ca\(^{2+}\) uptake, or inhibits Ca\(^{2+}\) release from cytosolic storage sites (reviewed in Ref. 34). It should be emphasized that current knowledge of the sites of action of Ca\(^{2+}\) channel inhibitors has been obtained through studies of excitable tissue. Whether or not leukemic lymphoblasts possess analogous verapamil-inhibitable Ca\(^{2+}\) channels remains to be determined. However, recent work of Ramu et al. (35) suggests that there is no correlation between the calcium channel-blocking ability of verapamil and similar types of compounds and their ability to "reverse" DOX resistance. Further, preliminary results of Fine et al. (36) suggest that DOX-resistant Chinese hamster ovary cells contain no nitrendipine-inhibitable Ca\(^{2+}\) channels. While this does not rule out a role for separate verapamil-inhibitable Ca\(^{2+}\) channels (37), the results tend to suggest that the verapamil enhancement of alkaloid cytotoxicity in MDR cells is probably due to a mechanism(s) other than inhibition of the slow Ca\(^{2+}\) current.

It can be asked if enhancement of alkaloid (or anthracycline) cytotoxicity in tumor cells, not only by "Ca\(^{2+}\) channel blockers" (16, 17, 19-22, 24) but also by the phenothiazines (18, 20, 21, 23), necessarily implies a role for Ca\(^{2+}\) or calmodulin at all. Tsuuro et al. (38) have recently reported that MDR cells have a higher Ca\(^{2+}\) content than their drug-sensitive counterparts. Moreover, Murray et al. (39) showed that increasing the extracellular concentration of Ca\(^{2+}\) produced a reciprocal decrease in the cytotoxicity of DNR in Ehrlich ascites cells. Both of these studies suggest that Ca\(^{2+}\) may be involved in the action of one or more classes of natural product compounds, but a precise role for this ion in drug action or resistance is unclear. By contrast, in the study cited above, Tsuuro et al. (38) could find no differences in calmodulin content between MDR cells and their drug-sensitive parents. We found the same to be true for our CEM/VLB100 and CEM cells, suggesting that calmodulin may not be directly involved in MDR or its circumvention by aryl alkyl amines, dihydropyridines, or phenothiazines, but this does not preclude a role for a calmodulin-dependent function, such as the stimulation of a Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase.

Finally, we should keep in mind the fact that these compounds are known to have other sites of action. Verapamil and phenothiazines are hydrophobic molecules that can have effects on cell membranes, verapamil exerting some of its actions through membrane binding (40, 41) and the phenothiazines by stabilizing cell membranes through nonspecific, hydrophobic interactions (42, 43). A recent study from Pastan's laboratory has apparently demonstrated another action of verapamil, inhibition of the lysosomal degradation of epidermal growth factor-Pseudomonas exotoxin conjugates in human KB cells in culture (44), although the mechanism for this effect is not clear. In this regard, it is of interest that the phenothiazines also affect lysosomes (45). Whether the effects of verapamil and/or VLB shown in the present paper (Fig. 3) are related to some action(s) on lysosomes is unknown at the present time.

Several groups of investigators have proposed that alkaloid and anthracycline resistance, manifested as decreased ability to retain drug, is mediated by the workings of an "active efflux pump" (46-49). Tsuuro et al. (16, 18, 19, 20) suggest that verapamil induces increased cellular accumulation of alkaloids by inhibiting this putative efflux pump. Moreover, recent studies of Kessel and Wilberding (50) indicate that verapamil itself may be handled by the same mechanism, suggesting that this drug competes in some way with the anthracyclines for release from the cells.

\( ^{5} \) W. T. Beck and E. A. Tallent, unpublished observation.

Fig. 2. Time course of verapamil action in CEM/VLB\(_{100}\) cells. Large flasks of cells were prepared, and at zero time verapamil (0.1 \( \mu \)M) and VLB (0.1 \( \mu \)M) were added in a small volume to one (C) and an equal volume of 0.9\% NaCl solution was added to another (O). Not shown are results with 0.1 \( \mu \)M VLB only or 10 \( \mu \)M verapamil only, which were identical to controls, and 5.0 \( \mu \)M VLB only, which was identical to the VLB-verapamil results. At the times indicated, aliquots were taken to determine DOX cytotoxicity to some extent in DOX-resistant CEM cells, epipodophyllotoxins, CLC, and POD. Although it enhanced DOX cytotoxicity to some extent in DOX-resistant CEM cells, verapamil had a considerably greater effect on Vinca alkaloid cytotoxicity in the same cells. Similarly, Tsuuro et al. (20) have reported that the effect of verapamil on VCR cytotoxicity in P388 cells was greater than its effect on DOX cytotoxicity. We conclude that verapamil does not effectively reverse the MDR phenotype of human leukemic blasts.

CANCER RESEARCH VOL. 46 FEBRUARY 1986

781
By contrast, we recently presented evidence favoring energy-dependent reduction of drug binding, rather than an efflux pump, as the basis for diminished drug retention in CEM/VLB_{100} cells (51). Based on this model, we propose that verapamil could perturb cell membranes, possibly affecting calcium metabolism or distribution in an (indirect) manner, and expose ordinarily "cryptic" targets to cytotoxic drug binding. This interpretation could explain the effect of verapamil and other calcium antagonists on etoposide action in drug-sensitive L1210 cells (52). Further, such perturbation by verapamil could likewise affect its own release and thereby explain the recent observation of Kessel and Wilberding (50). This mechanism could also account for the finding by these investigators that nitrendipine was apparently not a substrate for the proposed efflux pump.

The relative specificity of the verapamil effect for Vinca alkaloids and, to a lesser extent, for anthracyclines, but not other drugs listed in Table 1, is an enigma that has no easy explanation. Because the Vinca alkaloids bind to tubulin (53), verapamil might promote their binding to specific sites on tubulin or other cytotoxic targets that are ordinarily inaccessible or less accessible to these drugs. However, verapamil did not greatly enhance the cytotoxicities of CLC or POD, which share tubulin-binding sites that differ from those of Vinca alkaloids (54, 55), nor did it have the same magnitude of effect on the cytotoxicity of the anthracyclines, which are also large, heterocyclic compounds that interact with tubulin (56). These findings suggest that the modifier acts either relatively specifically on certain alkaloid (or anthracycline-) specific binding sites (possibly membrane-bound) or perhaps on cryptic, non-tubulin cytotoxic targets for Vinca alkaloids and, to a lesser extent, anthracyclines.

Another explanation for the specificity of the verapamil effect may be related to the degree of resistance of the cells. As seen in Table 1, the CEM/VLB_{100} cells are far less cross-resistant to the other drugs than they are to the Vinca alkaloids. However,
SELECTIVE ENHANCEMENT OF VINCA ALKALOID CYTOTOXICITY BY VERAPAMIL

the CEM/DOX cells are no more cross-resistant to VLB and CVR than they are resistant to DOX (Table 2), yet the effect of verapamil is greater on the former compounds. Our present and planned studies of cell membranes, organelles, and certain calcium-dependent enzyme activities should provide information about the mechanism of verapamil to only partially reverse the MDR phenotype while greatly enhancing the cytotoxicity of the Vinca alkaloids.

ACKNOWLEDGMENTS

We are grateful to Dr. Jack Yalowich for helpful discussion, to Graydon Nelson for technical assistance, and to Betsy Williford and the Audio-Visual Department for the skillful preparation of the artwork.

REFERENCES

SELECTIVE ENHANCEMENT OF VINCA ALKALOID CYTOTOXICITY BY VERAPAMIL


Reversal of *Vinca* Alkaloid Resistance but not Multiple Drug Resistance in Human Leukemic Cells by Verapamil

William T. Beck, Margaret C. Cirtain, A. Thomas Look, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/2/778

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, use this link http://cancerres.aacrjournals.org/content/46/2/778. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.