ABSTRACT

Verapamil, a calcium-influx blocker, enhanced the cytotoxicity of vincristine (VCR) in vitro 6- to 12-fold in eight human hematopoietic tumor cell lines established from acute lymphoblastic leukemia, acute myelogenous leukemia, and Burkitt's lymphoma. Great enhancement of VCR cytotoxicity was obtained in a VCR-resistant subline of K562 myelogenous leukemia. A maximum of approximately 100-fold increase in VCR cytotoxicity occurred in these human tumor cells. BALL and Daudi cells of B-cell type were more susceptible to VCR. At 6.6 or 20 μM of verapamil, the values for the concentration of drug required for 50% inhibition of cell growth for each cell line fell into a rather narrow range, and heterogeneity in VCR sensitivity among cell lines was circumvented in vitro. Verapamil also enhanced the cytotoxicity of Adriamycin, although the extent of enhancement was considerably small. Enhancement of VCR cytotoxicity also occurred with other calcium antagonists and calmodulin inhibitors. At maximum effective concentration of these reagents, a 3- to 5-fold increase in VCR cytotoxicity occurred in K562 cells. In VCR-resistant K562 cells, a more prominent enhancement (20- to 45-fold) was observed with these reagents. VCR resistance was circumvented in vitro. The mechanism of enhancement of VCR cytotoxicity was explained by the enhanced accumulation of VCR in K562, especially in resistant cells.

INTRODUCTION

The extent of penetration into and accumulation and retention within tumor cells of some antitumor agents is the most important determinant of the cytotoxicity of these drugs (26). We have reported that verapamil, a calcium influx blocker, greatly enhances the cellular level of VCR and vinblastine in P388 leukemia cells. This is especially apparent in P388/VCR where there is an inhibition of the VCR efflux function of the cells (33). The accumulation of VCR is directly related to a marked enhancement of VCR cytotoxicity. Verapamil enhances VCR cytotoxicity in P388/ADM and also enhances ADM cytotoxicity in P388 cells resistant to VCR and ADM (35). In a variety of experimental tumor cells, Vincar alkaloid-resistant cells are also resistant to anthracyclines (1, 5, 6, 12, 29, 37). Both classes of antitumor agents are actively transported outside the tumor cells, especially those that are drug resistant (6, 10, 21, 28, 29, 33). Verapamil seems to interfere with the drug efflux function of tumor cells common to VCR and ADM (35). In addition to verapamil, other calcium influx blockers and calmodulin inhibitors increase cellular accumulation of VCR and ADM in these drug-resistant cells through an inhibition of outward transport of these antitumor agents (34). This drug efflux mechanism seems to be controlled by the calcium-calmodulin complex (34).

Although all of the above results were obtained from animal and cellular experiments, they have potential importance for evaluation of methods to overcome drug resistance of tumor cells (33-35) and to circumvent the heterogeneity in drug sensitivity of tumor cells, both of which are crucial problems at present in cancer chemotherapy. To explore the possible clinical application, we first examined the effect of this approach on various human hematopoietic tumor cell lines and drug-resistant tumor lines. Prominent enhancement of VCR cytotoxicity occurred in these human cell lines, especially in VCR-resistant lines. The heterogeneity in VCR sensitivity among human hematopoietic cell lines used in the present study was circumvented by verapamil.

MATERIALS AND METHODS

Drugs. VCR and ADM, formulated for clinical use, were obtained from Shionogi and Co., Ltd., Osaka, Japan, and Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan, respectively. [G-3H]VCR sulfate (6.3 Ci/mmol) was purchased from Amersham Japan, Ltd., Tokyo, Japan. Verapamil (7) and caroverine (11) are calcium antagonists and were supplied by the Eisai Co., Ltd., Tokyo, and Mitsubishi Chemical Industries, Ltd., Tokyo, respectively. Prenylamine, trifluoperazine, clomipramine, and No. 233 are calcium channel blockers (3, 13, 16, 36) and were provided by Hoechst Japan, Ltd., Tokyo; Yoshitomi Pharmaceutical Industry, Ltd., Tokyo; Ciba-Geigy Japan, Ltd., Tokyo; and Mitsubishi Chemical Industries, Ltd., Tokyo, Japan, respectively. We refer to these drugs as “modifiers” throughout this paper.

Tumor Cell Lines. Eight tumor cell lines were used. A summary of the pertinent biological and clinical information on these lines is given in Table 1. Five lines are acute lymphoblastic leukemia of T-cell type except for BALL-1, which was derived from B-cells. K562/VCR was established from acute myelogenous leukemia K562 in this laboratory by an in vitro procedure. K562 was a gift of Dr. K. Ezaki of this center. Other cell lines were kindly supplied by Dr. M. Shimoyama, National Cancer Center Hospital, Tokyo, Japan.

Cell Culture and Drug Treatment. Tumor cells were maintained in suspension in plastic dishes (Coming Glass Works, Coming, N. Y.) in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.) and kanamycin (100 μg/ml) (growth medium) (33). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2. For the drug treatment experiment, tumor cells (4 × 10⁴) were cultured...
at 37° for 5 hr in Falcon No. 2054 culture tubes containing 2 ml of growth medium in a humidified atmosphere of 5% CO2. Then, they were treated with graded drug concentrations (0.1 to 100 nM VCR, 10 to 10^4 nM ADM) in the absence or presence of the modifier, reincubated for 72 hr in the presence of drugs, and counted with a Couter Counter (Model ZB1) (32). Three tubes were used for each drug concentration. In the control experiment, tumor cells grew exponentially during the incubation period, and the final cell numbers are described in the charts and tables. Modifiers were dissolved in dimethyl sulfoxide at a final concentration of 100 mM, and dilution was carried out with PBS. The final concentration of dimethyl sulfoxide in the culture was less than 0.1% (v/v), and no effect from dimethyl sulfoxide on cell growth was observed.

IC50 in the presence or absence of modifiers was determined by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells (32, 33).

**RESULTS**

Enhanced Cytotoxicity of VCR and ADM in Various Human Hemopoietic Tumor Cells by Verapamil. At verapamil concentrations up to 6.6 µM, no growth inhibition was observed for various human hemopoietic cells. The IC50 values of verapamil for K562, K562/VCR, P-12/ichikawa, CCRF-CEM, Molt-3, RPMI-8402, BALL-1, and Daudi cells were also highly susceptible to VCR. The sensitivity of other cell lines to VCR in the presence or absence of verapamil was determined by the same manner as described in Chart 1, and the IC50 values for these cell lines including K562 and K562/VCR were plotted against the concentration of verapamil (Chart 2). In the absence of verapamil, heterogeneity in VCR sensitivity was observed among tumor cell lines. BALL-1 was most sensitive to VCR (IC50 = 0.60 nM). Daudi and CCRF-CEM cells were also highly susceptible to VCR. BALL-1 and Daudi cells are of B-cell origin (15, 19). P-12/
Ichikawa was least susceptible (IC\textsubscript{50} = 3.3 nM). An approximately 82-fold difference in IC\textsubscript{50} values between BALL-1 and K562/VCR cells was observed. At 2.2 \(\mu\)M of verapamil, the range of IC\textsubscript{50} values is still significantly wide; however, at 6.6 \(\mu\)M, and especially at 20 \(\mu\)M, IC\textsubscript{50} values for each cell fell into a rather narrow range, indicating a circumvention of heterogeneity in VCR sensitivity among cell lines.

Verapamil at a nontoxic dose between 2.2 and 10 \(\mu\)M also enhanced the cytotoxicity of ADM for K562, CCRF-CEM, Molt-3, and BALL-1 cells (Table 2). However, the extent of enhancement was less then 2-fold as compared to the IC\textsubscript{50} values, and this enhancement was considerably lower than that observed for VCR where a 5- to 10-fold increase in VCR cytotoxicity occurred. A similar extent of enhancement of ADM cytotoxicity was also observed for P-12/Ichikawa, RPMI-8402, and Daudi cells (data not shown). K562/VCR cells were partly resistant to ADM (index of resistance, 3). Verapamil at 6.6 or 10 \(\mu\)M enhanced the cytotoxicity of ADM 4- to 5-fold in K562/VCR cells, and at 6.6 \(\mu\)M of verapamil, IC\textsubscript{50} of ADM shifted to 16.2 nM, indicating that the overcoming of ADM resistance in K562/VCR cells was accomplished as the IC\textsubscript{50} value of ADM for K562 cells was 24.3 nM.

Increased Cytotoxicity of VCR in Human Hemopoietic Tumor Cells by Calcium Antagonists and Calmodulin Inhibitors. In addition to verapamil, similar enhancement of VCR cytotoxicity was observed with other calcium antagonists and calmodulin inhibitors (Table 3). The concentrations of modifiers used did not induce cytotoxicity to the tumor cells during incubation for 3 days. At 6.6 \(\mu\)M of modifiers, an approximate 2- to 3-fold increase in VCR cytotoxicity occurred in K562 cells as compared to the IC\textsubscript{50} values. At 10 \(\mu\)M of modifiers, a maximum of about a 4.5-fold increase in VCR cytotoxicity occurred with clomipramine, and the effect was almost the same as that observed for verapamil (Chart 1). In K562/VCR cells, a more marked enhancement of drug cytotoxicity occurred. At 6.6 \(\mu\)M of modifiers, the enhancement of the VCR effect differed among the modifiers (4.6- to 45-fold, depending on the modifiers), and a prominent effect was observed for prenylamine. At 10 \(\mu\)M of modifiers, a 21- and 31-fold increase in VCR cytotoxicity occurred with caroverine and clomipramine, respectively. Circumvention of VCR resistance also was achieved with these modifiers, as the IC\textsubscript{50} of VCR for K562 cells was 2.9 nM. Enhancement of ADM cytotoxicity against K562/VCR cells also was observed for these modifiers (not shown), although the extent of maximum enhancement was approximately 2-fold.

Cellular Uptake of VCR and Effect of Calcium Antagonists and Calmodulin Inhibitors. The effect of modifiers on the cellular uptake of VCR at an identical concentration of 6.6 \(\mu\)M was examined. The cells excluded trypan blue after they were treated with 6.6 \(\mu\)M of modifiers for 3 hr. During the incubation for 2 to 3 hr at 37\(^\circ\), K562 cells accumulated approximately 1.6- to 1.8-fold the amount of VCR that was found in K562/VCR cells (Chart 3). Modifiers at 6.6 \(\mu\)M generally increased the cellular accumulation of VCR in both K562 and K562/VCR cells. In K562 cells, an approximate 1.5- to 2-fold increase in cellular VCR occurred by modifiers. In K562/VCR cells, the enhancement of VCR accumulation by modifiers was more prominent than that of K562 cells.

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Table 2

**Effects of verapamil upon growth-inhibitory actions of ADM on various human hemopoietic tumor cells**

<table>
<thead>
<tr>
<th>Verapamil ((\mu)M)</th>
<th>K562</th>
<th>K562/VCR</th>
<th>CCRF-CEM</th>
<th>Molt-3</th>
<th>BALL-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.3 ± 1.2(^a)</td>
<td>72.2 ± 1.4</td>
<td>24.7 ± 1.8</td>
<td>9.3 ± 1.5</td>
<td>18.7 ± 2.3</td>
</tr>
<tr>
<td>6.6</td>
<td>13.4 ± 0.5(^b)</td>
<td>16.2 ± 1.7</td>
<td>20.4 ± 1.8</td>
<td>6.3 ± 0.50</td>
<td>11.6 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>11.4 ± 0.7</td>
<td>15.4 ± 1.1</td>
<td>13.6 ± 3.1</td>
<td>5.9 ± 0.16</td>
<td>10.0 ± 0.7</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D. of 3 determinations.

\(^b\) The IC\textsubscript{50} values in the presence of verapamil for each cell line were statistically significant (\(p < 0.05\)) by \(t\)-test as compared to each IC\textsubscript{50} value without verapamil.
Effects of calcium antagonist and calmodulin inhibitors upon growth-inhibitory actions of VCR on K562 and K562/VCR cells

Tumor cells were treated with graded VCR concentrations with modifier at the indicated concentrations 5 hr after seeding the cells at 4 x 10⁴ per 2 ml of the medium. Cell number was counted 72 hr after the continuous drug exposure, and the IC₅₀ value was determined as is shown in Chart 1. In the absence of drugs, cells grew exponentially, and the final cell numbers were (3.3 ± 0.2) x 10⁵ and (3.2 ± 0.2) x 10⁵ per 2 ml of the medium for K562 and K562/VCR cells.

Table 3

<table>
<thead>
<tr>
<th>Modifier</th>
<th>K562 cells at modifier (µM)</th>
<th>K562/VCR cells at modifier (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6.6</td>
<td>10</td>
</tr>
<tr>
<td>Caroverine</td>
<td>1.5 ± 0.06 ± 0.09</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>0.94 ± 0.04</td>
<td>0.66 ± 0.21</td>
</tr>
<tr>
<td>Trifluoperazine</td>
<td>1.2 ± 0.07</td>
<td>2.0 ± 0.19</td>
</tr>
<tr>
<td>Prenylamine No. 233</td>
<td>1.1 ± 0.16</td>
<td>1.1 ± 0.17</td>
</tr>
</tbody>
</table>

* Mean ± S.D. of 3 determinations.

The level of intracellular VCR in K562/VCR cells in the presence of modifiers was higher than the level observed for K562 cells in the absence of a modifier, which may explain the circumvention of VCR resistance in K562/VCR cells by modifiers.

DISCUSSION

One of the major causes of failure in cancer chemotherapy is the selection and proliferation of specific drug-resistant tumor cells during treatment (23, 24, 27). In various experimental tumor systems, one of the most important mechanisms of drug resistance, especially to Vinca alkaloid and anthracycline classes of antitumor agents, has been attributed to the enhanced drug efflux function of resistant tumor cells (6, 10, 21, 28, 29, 33). These observations suggest that if we could control the drug efflux function of tumor cells appropriately, then we could expect anticancer agents to cause a therapeutic effect against resistant cells. We found that verapamil, a calcium influx blocker (calcium antagonist), efficiently inhibits the VCR and ADM efflux function of tumor cells, especially of resistant tumor cells (33–35). VCR and ADM accumulated greatly in resistant tumor cells by verapamil, and thus, the increased intracellular level of VCR and ADM was directly related to the marked enhancement of drug cytotoxicity. All of these data were obtained by animal tumor models.

In the present study, we found that verapamil also potentiated VCR cytotoxicity in human hemopoietic tumor cells and especially in VCR-resistant sublines. The enhanced cytotoxicity of VCR was also observed for other calcium antagonist and calmodulin inhibitors which are used as coronary vasodilator (3, 11), antipsychotic (16, 36), and thrombin inhibitors (13). The mechanism of enhancement of VCR cytotoxicity was attributed to the enhancement of cellular accumulation of VCR in tumor cells, especially in VCR-resistant tumor cells (Chart 3). The effects of modifiers were different among tumor cell lines. Trifluoperazine had a rather great effect for K562/VCR cells; however, the effects of clomipramine and especially of No. 233 for K562/VCR cells were rather weak when compared to the effects for K562 cells (Table 3). This suggests that there may be a different response of the modifier for different tumor cells. The enhancement of VCR accumulation was roughly related to the enhancement of which occurred in K562 cells. An approximate 2- to 4-fold increase in intracellular VCR occurred. The relationship between the extent of enhancements of cellular VCR accumulation and VCR cytotoxicity in these cell lines was not necessarily parallel.
VCR cytotoxicity by each modifier. For instance, No. 233 greatly enhanced the accumulation of VCR in K562 cells, whereas the accumulation was not efficient in K562/VCR cells, and in cytotoxicity experiments, No. 233 actually induced a rather weak enhancement of VCR cytotoxicity in K562/VCR cells. In some cases, however, the enhancement of VCR accumulation was not directly correlated with the enhancement of VCR cytotoxicity. Verapamil, clomipramine, and trifluoperazine induced efficient VCR accumulation in K562/VCR cells, while prenylamine induced a rather lower accumulation of VCR. Prenylamine, however, showed a rather stronger enhancement of VCR cytotoxicity than did other modifiers. One of the explanations for these discrepancies is that the drug accumulation was examined during a 3-hr incubation, while cytotoxicity was examined after a 72-hr incubation at 37°C. Prenylamine might show a lasting effect in cytotoxicity during long incubation. We need, of course, many basic investigations before we can examine a possible application of this approach clinically; however, these results with human hemopoietic tumor cells are a beginning basis for such studies.

Recent developments in analysis of the surface markers and membrane antigens of human leukemias and lymphomas have revealed the presence of different types of leukemias and lymphomas in human hemopoietic tumor cells (2, 4, 9, 22, 30, 31). Subclassification of human leukemia and lymphoma by immunological and cytochemical markers is useful not only for an understanding of pathophysiology and diagnosis but also for application of chemotherapy. T-cell acute lymphoblastic leukemia is known to respond poorly to current therapeutic regimens, but B-cell cancer responds fairly well to therapeutic agents (2, 4, 22, 31). We also found that B-cell tumors (BALL and Daudi) respond well to VCR in vitro, while T-cell tumors generally respond poorly to VCR (Chart 1), and heterogeneity in VCR sensitivity has been observed among tumor lines. The heterogeneous nature of human tumor cells in their response to chemotherapeutic agents is the crucial problem in the present cancer chemotherapy as has been observed in animal experiments (23, 24, 27). In most cases, although a complete remission could be reached, it is very difficult to attain a “cure” with regimens of only chemotherapeutic agents. In the present study, it was shown that the less sensitive tumor cells became more susceptible to VCR, and the heterogeneity in VCR sensitivity among established human hemopoietic tumor cells has been circumvented in vitro by a VCR efflux-inhibiting agent, verapamil. The human tumor lines used in this experiment were established from different patients and do not necessarily represent the heterogeneity of hemopoietic tumor cells of one patient. These results, however, might suggest a potential to render less sensitive populations of hemopoietic tumor cells sensitive to VCR. We are currently searching for calcium antagonists and calmodulin inhibitors which may prove to be effective clinically in cancer chemotherapy.

ACKNOWLEDGMENTS

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Potentiation of Vincristine and Adriamycin Effects in Human Hemopoietic Tumor Cell Lines by Calcium Antagonists and Calmodulin Inhibitors

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