Promotion by Verapamil of Vincristine Responsiveness in Tumor Cell Lines Inherently Resistant to the Drug

Takashi Tsuruo, Harumi Iida, Kuniko Naganuma, Shigeru Tsukagoshi, and Yoshio Sakurai

ABSTRACT

Cultured cell lines LL, B16, C26, and C38 established from mouse solid tumors of Lewis lung carcinoma, B16 melanoma, and colon adenocarcinomas 26 and 38, respectively, showed inherently different resistance to vincristine (VCR) in vitro. The inherent resistance to VCR of these cell lines was related to the ability of the cells to accumulate VCR. Verapamil, a calcium antagonist with coronary vasodilator activity, enhanced the cytotoxicity of VCR against these cell lines depending upon their susceptibility to VCR. C26 cells, the most resistant, became the most susceptible to VCR with a nontoxic dose of verapamil. A 12-fold increase in VCR cytotoxicity occurred. Only a 2.5-fold increase in VCR cytotoxicity was observed for B16 cells, the most sensitive cells. VCR cytotoxicity against each cell line reached almost the same level by verapamil (2.2 to 6.6 μM). Thus, the inherent resistance to VCR among the tumor lines was circumvented. Verapamil enhanced the cellular accumulation of VCR. A 3- to 4-fold increase in cellular VCR occurred in C26 cells, while approximately a 2-fold increase was observed for B16 and LL cells. A similar rate of enhancement was observed for both bound and free VCR, indicating that verapamil does not enhance the affinity of VCR to tubulin. Verapamil inhibited the outward transport of VCR from the cells. The most prominent inhibition was observed for C26 cells. Circumvention of inherent resistance of tumor cells to VCR by verapamil could be attained through an enhanced cellular accumulation of VCR in each of the tumor cells. The enhancement of VCR cytotoxicity and circumvention of inherent VCR resistance by verapamil could be explained by the cellular concentration of VCR, and also it might be related to the extent of VCR binding to tubulin in the cell. The chemotherapeutic effect of VCR is significantly enhanced by verapamil in colon adenocarcinoma 26-bearing mice.

INTRODUCTION

A previous report from this laboratory described that verapamil, a coronary vasodilator, enhanced the cytotoxicity of VCR against P388 and P388/VCR cells through an effective accumulation of VCR in tumor cells (22, 24). Enhanced accumulation of VCR was attained by the inhibition of the VCR efflux function of the cells, especially those of P388/VCR cells. Overcoming of VCR resistance has been attained in vitro and in vivo by verapamil (22) and also by other calcium antagonists and calmodulin inhibitors (23).

MATERIALS AND METHODS

Drugs. VCR sulfate formulated for clinical use was obtained from Shionogi and Co., Ltd., Osaka, Japan. [3H]VCR sulfate (7.2 Ci/mmol) and [3H]CLC (5.7 Ci/mmol) were purchased from Amersham Japan, Ltd., Tokyo, Japan. Verapamil was kindly supplied by the Eisai Co., Ltd., Tokyo, Japan.

Tumors and Animals. Lewis lung carcinoma, B16 melanoma, and colon adenocarcinomas 26 and 38 were obtained from the National Cancer Institute, NIH, Bethesda, Md. BALB/c x DBA/2 F1 (hereafter called CD2F1) mice were purchased from Charles River Japan, Inc., Tokyo, Japan.

Preparation of Tumor Cell Cultures. Solid tumor tissue was removed surgically from the mouse, trimmed from the necrotic portion, and finely minced using a sterile technique. Tissue (1 g) was mixed in a flask with 10 ml of 0.05% trypsin:EDTA (Grand Island Biological Co., Grand Island, N. Y.). The mixture was gently stirred with a stirring bar at 37° for 10 min. Two sequential enzyme treatments of the same tissue were performed, each time decanting the cell suspension through sterile gauze into a glass beaker containing 10 ml of culture medium in an ice bath. The pooled cell suspension was centrifuged at 150 x g for 5 min at 4°. The cell pellet was suspended in culture medium, and the viable cells were quantitated. Viable cells were cultivated at an initial cell density of 5 x 10⁵ to 10⁸ cells/ml of RPMI Medium 1640 (Grand Island Biological Co.) containing 10% fetal bovine serum (growth medium).

Drug Treatment of the Cultured Cells. Cells established from solid tumors were subcultured 5 times before use in experiments. Cells were grown in plastic dishes (Corning Glass Works, Corning, N. Y.) at 37° in a humidified atmosphere of 5% CO₂. The initial cell density of each cell line was 6 x 10⁶ cells per dish (60 mm diameter) which contained 3 ml of RPMI Medium 1640 with 10% fetal bovine serum. The cells grew exponentially for at least 4 days. Twenty-four hr after the cells...
were seeded, verapamil and VCR dissolved in PBS were added successively to the culture, and the cells were cultivated further for another 72 hr. The cell layer was then washed with PBS and trypsinized with 0.5 ml of 0.05% trypsin:EDTA. PBS (2 ml) containing 2% fetal bovine serum was added to neutralize the trypsin. The cells were suspended by pipetting and counted with a Model ZBI Coulter Counter as described previously (21). The cytoxic activity of VCR in the presence or absence of verapamil was measured by determining the IC_{50} which was obtained by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells (21). The initial cell number was subtracted in the calculation.

Cellular Uptake and Retention of [3H]VCR. Tumor cells (6 x 10^{6}) were seeded in plastic dishes (60 mm diameter; Corning Glass). Twenty-four hr after seeding, [3H]VCR (33 nM; specific activity, 7.2 Ci/mmol) was added with or without verapamil at 6.6 mM. At various time intervals, the medium was removed, and the cell layer was washed 3 times with cold PBS (5 ml), trypsinized (0.05% trypsin:EDTA, 0.5 ml), and suspended after the addition of 2.5 ml of PBS serum as described above. An aliquot of 2 ml of cell suspension was removed and added to 0.2 ml of 4 N NaOH. The mixture was heated at 60° for 30 min to ensure the lysis of the cells, transferred to scintillation vials, and counted in 20 ml of acidified Aquasol 2 (New England Nuclear) (21). The radioactivity was counted in a Beckmann LS 7500 liquid scintillation system equipped with automatic quench compensation. Cells were enumerated with a Coulter Counter on a 0.5-ml aliquot.

For the drug retention experiment, LL, B16, C26, and C38 cells were treated for 3 hr with [3H]VCR at 33, 14, 49, and 49 nM, respectively, so that a similar concentration of VCR in each of the cell lines was achieved. The average concentration of VCR in repeated experiments in these cells was 0.89, 1.0, 0.95, and 1.1 mM, respectively. The medium was removed, and the cell layer was washed 2 times with cold PBS (5 ml). The cells were resuspended at a density of 10^{6} cells/ml with or without verapamil at 6.6 mM. At various time intervals, the amount of [3H]VCR retained in the cells was determined as described above.

Determination of the Levels of Tubulin in Tumor Cells. In order to determine the levels of tubulin in each line, CLC binding to extracts of tumor cells was carried out by a slightly modified method as described previously (6, 17). Briefly, tumor cells in log phase growth were collected from culture dishes by trypsinization, washed with PBS, and centrifuged, and the pelleted cells (4 to 6 x 10^{6}) were suspended in 1 ml of 0.24 M sucrose:10 mM potassium phosphate buffer (pH 6.5):1 mM MgCl_{2}. The cells were disrupted by brief sonication and centrifuged at 10,000 x g for 60 min at 4 °C. A 0.7-ml aliquot of the supernatant solution was incubated at 37°C for 90 min with 0.02 ml of [3H]CLC (3.5 μCi) at a final concentration of 3.5 mM (6). The mixture was subjected to gel filtration on a Sephadex G-100 column (0.8 x 22 cm) and eluted with 10 mM potassium phosphate (pH 6.5):10 mM MgCl_{2} as described previously (6, 17). Fractions of 0.80 ml were collected, and the radioactivity in an 0.5-ml aliquot from each fraction was counted in 10 ml Aquasol 2 as described above. Bound CLC emerged at Fractions 6 to 10, while free CLC appeared at the bed volume of the column (Fractions 13 to 19). The total amount of bound CLC was calculated from the elution pattern. Under our experimental conditions, binding reached saturation as observed previously (6), and the amount of CLC which can bind to the precipitate was less than 5% of that bound to a supernatant macromolecule as has been reported previously (6). We assumed that the amount of CLC bound to the extract from 10^{6} tumor cells represents the amount of CLC binding in 10^{6} tumor cells. The amount of bound CLC represents the amount of tubulin (dimer) in the cells as 1 mol of CLC binds to 1 mol of tubulin dimer (1, 16).

Determination of Bound and Free VCR in VCR-treated Cells and the Effect of Verapamil. Tumor cells were treated with [3H]VCR for 3 hr at 37°C as described in the VCR uptake experiment. The cell layer was washed, trypsinized, and suspended in PBS as above. The cells were pelleted by centrifugation at 300 x g for 5 min, and suspended in 1 ml of 0.24 M sucrose:10 mM potassium phosphate (pH 6.5):1 mM MgCl_{2}. The cell extract was prepared and applied to a column of Sephadex G-100 as described in the CLC binding experiment. Bound and free VCR were quantitated from the elution pattern. Radioactivity in the precipitate of the disrupted tumor cells was less than 10% of the amount that was found in the supernatant. We assumed that the amount of bound VCR in the extract from 10^{6} cells represents the total amount of bound VCR in 10^{6} tumor cells.

Evaluation of Antitumor Activity. Cell suspension in Hanks balanced salt solution (20%, w/v) of colon adenocarcinoma 26 was prepared from surgically removed tumor by disaggregating the tumor pieces by gentle homogenization in a loosely fitted glass homogenizer. After the cell suspension was passed through a 40 mesh sieve, a volume (0.2 ml) of 5 x 10^{6} viable cells, as determined by trypsin dye exclusion, was implanted i.p. into CD2F1 mice. Verapamil and VCR were dissolved in 0.9% NaCl solution. Both drugs were mixed, and the mixture was administered at a constant rate of 0.02 ml/g body weight i.p. daily for 10 days starting from the day after the tumor inoculation. Doses of verapamil and VCR were in the range of 30 to 100 mg/kg and 30 or 100 μg/kg, respectively. Antitumor activity was expressed by the mean survival time of a treated group of mice divided by the mean survival time of a control group. Ten mice were used for each experimental group.

RESULTS

Characteristics of Cultured Mouse Solid Tumor Cells. Some characteristics of cultured mouse solid tumor cells are described in Table 1. The cells were cultured in RPMI Medium 1640 with 10% fetal bovine serum. B16 and C26 cells can also grow in the medium supplemented with calf serum, while LL and C38 cells absolutely required fetal bovine serum. LL, B16,
and C26 cells grew rapidly, while C38 cells grew rather slowly. LL, B16, and C26 cells were fibroblastic in morphology, while the cellular morphology of C38 was epithelioid without any characteristics of fibroblastic cells. The tumor of C38 rarely metastasized to other organs (7). LL and C38 cells were rather large. The B16 and C26 cells were about 70 and 80% of the size of LL and C38 cells. The cell sizes were well correlated with the amounts of protein in the cells and also well correlated with the amount of tubulin (as determined by CLC binding) in the cells. This might suggest an identical nature of the cellular components among the 4 cell lines used in this experiment. The data for the amount of tubulin in the cells was used later for the evaluation of VCR cytotoxicity.

Enhanced Cytotoxicity of VCR in LL, B16, C26, and C38 Cells by Verapamil. At verapamil concentrations up to 6.6 μM, no growth inhibition was observed for these cells. At 10 μM verapamil, a slight growth inhibition (less than 5%) occurred for LL, B16, and C38 cells. At 20 μM verapamil the growth of LL, B16, C26, and C38 cells was inhibited by 44, 62, 31, and 63%, respectively. The IC50 values of verapamil for these cells were 24, 19, 31, and 18 μM, respectively. We used verapamil at nontoxic doses of 0.7, 2.2, or 6.6 μM in this experiment. The sensitivities of LL, B16, C26, and C38 cells to VCR and the effect of verapamil on these sensitivities was examined. The IC50 values calculated and the increase in VCR cytotoxicity by verapamil are summarized in Table 2. The order of sensitivity of VCR against cultured tumor cells was B16 (most sensitive), LL, C38, and C26 (least sensitive). Verapamil at a nontoxic dose (0.7 to 6.6 μM) generally enhanced the cytotoxicity of VCR for these cells. However, the extent of enhancement is different among the cells. At 6.6 μM verapamil, the cytotoxicity of VCR increased 12-, 6.4-, 2.6-, and 2.5-fold in C26, C38, LL, and B16 cells, respectively. At other verapamil concentrations, the highest enhancement was also observed to be for C26 cells and then for C38 and LL cells, and the lowest enhancement was observed to be for B16 cells. It is of interest to note that the less susceptible cell line to VCR became more sensitive to VCR by verapamil. The IC50 value of VCR for each cell line reached almost the same level by 2.2 or 6.6 μM verapamil, indicating that a circumvention of the inherent resistance to VCR has been attained in vitro.

Intracellular Uptake of \[3H\]VCR and Effect of Verapamil. The amount of VCR accumulated in \(10^6\) cells was estimated. The intracellular VCR increased with time in LL, B16, C26, and C38 (Chart 1). The amount of VCR at 3 hr in C26, C38, LL, and B16 cells was 0.61, 0.86, 0.94, and 1.15 pmol/\(10^6\) cells, respectively. We used verapamil at nontoxic doses of 0.7, 2.2, or 6.6 μM in this experiment. The intracellular VCR increased with time in LL, B16, C26, and C38 cells (Chart 1). The amount of VCR at 3 hr in C26, C38, LL, and B16 cells was 0.61, 0.86, 0.94, and 1.15 pmol/\(10^6\) cells, respectively. We used verapamil at nontoxic doses of 0.7, 2.2, or 6.6 μM in this experiment.

Table 2

<table>
<thead>
<tr>
<th>Verapamil (μM)</th>
<th>IC50 (μM) of VCR against</th>
<th>LL (IC50 μM)</th>
<th>B16 (IC50 μM)</th>
<th>C26 (IC50 μM)</th>
<th>C38 (IC50 μM)</th>
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<tr>
<td>0</td>
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<td>1.1</td>
<td>14</td>
<td>5.0</td>
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<tr>
<td>0.7</td>
<td>2.5 (1.6)a</td>
<td>1.0 (1.1)</td>
<td>4.5 (3.1)</td>
<td>4.3 (1.2)</td>
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</tr>
<tr>
<td>2.2</td>
<td>1.8 (2.2)</td>
<td>1.0 (1.1)</td>
<td>1.8 (7.8)</td>
<td>1.5 (3.3)</td>
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</tr>
<tr>
<td>6.6</td>
<td>1.5 (2.6)</td>
<td>0.4 (2.5)</td>
<td>1.2 (12)</td>
<td>0.8 (6.4)</td>
<td></td>
</tr>
</tbody>
</table>

a Numbers in parentheses, increase (-fold) in cytotoxicity as compared to the value without verapamil.

Verapamil added to the culture at 6.6 μM increased the amount of cellular VCR more prominently in the less VCR-sensitive C26 cells. At 3 hr after incubation with verapamil, an average of 1.9-, 2.2-, and 2.7-fold amounts of VCR was found in LL, B16, and C38 cells, respectively, while a 3.5-fold increase in VCR concentration occurred in verapamil-treated C26 cells. These results might suggest that inherently resistant cells to VCR usually accumulate a lesser amount of VCR in the cells; however, the cells efficiently accumulate VCR after treatment by verapamil and become as equally susceptible to VCR as the inherently less resistant cells.

VCR Binding in the Cells and Effect of Verapamil. The levels of bound and free VCR in the VCR-treated cells in the absence or presence of verapamil were measured (Table 3). The amount of bound VCR increased in the presence of verapamil in each cell line. However, verapamil also increased the amount of free VCR in the cells, and the extent of increase was more prominent or equal when compared to that of bound VCR. These results might suggest that verapamil does not stimulate the binding of VCR to tubulin. The increase in bound VCR in the cells might be simply mediated by the increased free VCR by verapamil.

Release of VCR from the Cells and the Effect of Verapamil.
Circumvention of Inherent Drug Resistance

Each cell line was treated for 3 hr at 37° with different concentrations of [\(^3\)H]VCR so that almost equal intracellular VCR concentrations were achieved. Release of VCR from LL, B16, and C38 cells proceeded with similar kinetics (Chart 2). Release of VCR from C26 cells is faster than from other cell lines. A lower content of VCR in the C26 cells might partly be explained by this mechanism. Release of VCR from these cells was well inhibited by 6.6 \(\mu\)M verapamil, and VCR-release in the presence of verapamil follows similar kinetics among all the cells. Insofar as we examined, verapamil did not enhance significantly the inward transport of VCR (18), nor did the drug enhance the binding of VCR to tubulin as described above. The enhanced accumulation of VCR by verapamil might be explained by the inhibition of outward transport of VCR. An average increase of 1.7- to 2.5-fold occurred in the amount of VCR by verapamil for LL, B16, and C38 cells, and a 3.6-fold increase occurred for the C26 cells as described previously (Chart 1). The extent of enhanced accumulation of VCR in each cell line by verapamil might be related to the extent of inhibition of VCR release by verapamil.

Combined Effect of VCR and Verapamil on C26-bearing Mice. The C26 tumor has been reported not to be sensitive to VCR (5). In this experiment, VCR administered daily for 10 days starting from Day 1 did not significantly increase the life span of C26-bearing mice (Table 4). Verapamil given 10 times with VCR did significantly increase the life span of the C26 bearer. In repeated experiments, an approximately 30 to 35% increase in life span was obtained when verapamil at 50 to 63 and 75 to 88 mg/kg was administered along with VCR at 100 or 30 \(\mu\)g/kg, respectively. A single administration of verapamil at 125 mg/kg was toxic. Verapamil at 75 and 100 mg/kg with VCR at 100 and 30 \(\mu\)g/kg, respectively, also manifested toxicity.

DISCUSSION

Mouse Lewis lung carcinoma, B16 melanoma, and colon adenocarcinomas 26 and 38 are widely used as signal solid tumors in cancer chemotherapy (10). VCR showed antitumor activity against B16 melanoma (10). Against LL, C26, and C38, the activity of VCR is in the range of slightly effective to not effective (7, 10). In an in vitro system, B16 cells are most sensitive to VCR, while LL, C38, and especially C26 cells are less susceptible to VCR (Table 2).

To order to rationalize the different VCR cytotoxicities among the cell lines with or without verapamil, we have summarized all the in vitro findings in Table 5. Although the VCR uptake did not reach equilibrium after 3 hr of incubation, we did observe some relationship between the cytotoxicity and the cellular uptake of VCR. The cytotoxicity of VCR in each cell line in the absence or presence of verapamil might be explained by the intracellular VCR concentration, since the concentration of VCR in a more susceptible cell line (B16 cells) is usually higher than that in a more resistant cell line. After treatment of the cells with verapamil, the most prominent increase in intracellular concentration of VCR was observed in C26 cells. Also,

<table>
<thead>
<tr>
<th>Drug and dosage</th>
<th>Survival time (days)</th>
<th>T/C (%)</th>
<th>Survival time (days)</th>
<th>T/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.2 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td>16.8 ± 1.0</td>
<td>100</td>
</tr>
<tr>
<td>Verapamil (75 mg/kg)</td>
<td>16.3 ± 1.4</td>
<td>97</td>
<td></td>
<td></td>
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<tr>
<td>VCR (100 (\mu)g/kg)</td>
<td>23.9 ± 3.9</td>
<td>99</td>
<td>19.4 ± 2.1</td>
<td>115</td>
</tr>
<tr>
<td>+ Verapamil (30 mg/kg)</td>
<td>25.2 ± 4.0</td>
<td>104</td>
<td>19.2 ± 1.0</td>
<td>114</td>
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<tr>
<td>+ Verapamil (60 mg/kg)</td>
<td>32.4 ± 9.8</td>
<td>134&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.1 ± 1.5</td>
<td>126</td>
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<tr>
<td>+ Verapamil (75 mg/kg)</td>
<td>22.1 ± 15.9</td>
<td>91</td>
<td>21.3 ± 0.8</td>
<td>127&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>VCR (30 (\mu)g/kg)</td>
<td>24.7 ± 4.0</td>
<td>102</td>
<td>17.3 ± 1.9</td>
<td>103</td>
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<tr>
<td>+ Verapamil (30 mg/kg)</td>
<td>26.0 ± 4.4</td>
<td>107</td>
<td>19.0 ± 1.4</td>
<td>113</td>
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<tr>
<td>+ Verapamil (60 mg/kg)</td>
<td>26.3 ± 3.7</td>
<td>109</td>
<td>19.4 ± 1.9</td>
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<tr>
<td>+ Verapamil (75 mg/kg)</td>
<td>31.9 ± 5.8</td>
<td>132&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22.4 ± 6.9</td>
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<tr>
<td>+ Verapamil (100 mg/kg)</td>
<td>26.1 ± 13.4</td>
<td>108</td>
<td></td>
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</tr>
</tbody>
</table>

<sup>a</sup> T/C, mean survival time of a treated group divided by the mean survival time of a control group.  
<sup>b</sup> Mean ± S.D.  
<sup>c</sup> Statistically significant (\(p < 0.05\)) by \(u\) test as compared with that of mice treated with VCR alone at each dosage of VCR.
the strongest enhancement of VCR cytotoxicity occurred in C26 cells. The cytotoxicity of VCR in each cell line with or without verapamil could also be explained by the percentage of VCR-bound tubulin in the cells. B16 cells are the most susceptible to VCR in the absence or presence of verapamil, and the percentage of VCR-bound tubulin in B16 cells is the highest among the lines. A rough correlation was observed between the cytotoxicity of VCR and the percentage of VCR-bound tubulin in each cell line. From these observations, we can speculate that the VCR susceptibility of an individual cell line could be related to the ability of the cells to accumulate VCR (intracellular VCR concentration of the cells), and also it might be related to the extent of VCR binding to tubulin (the percentage of VCR-bound tubulin) in the cells, if the inhibition of tubulin polymerization with subsequent arrest of dividing cells in metaphase is the main action of VCR in the cells. At the present time, however, the cellular action of VCR is not fully elucidated (3).

In C26 cells, the outward transport of VCR is faster than that of other cells, and the concentration of VCR in C26 cell is the lowest among the cells. The outward transport of VCR in LL, B16, and C38 cells followed similar kinetics. These results indicate that the intracellular concentration of VCR is partly, but not fully, explained by the outward transport of VCR. Verapamil inhibited the enhanced drug efflux function of P388/VCR cells, leading to an impressive enhancement of cellular accumulation and cytotoxicity of VCR (22-24). Here, in solid tumor cells in culture, verapamil also inhibited the drug efflux function of the cells. A stronger effect of verapamil was obtained in the less sensitive C26 cells to VCR, where the efflux of VCR is faster and the intracellular VCR was kept at a relatively low level. Generally, the effect of verapamil on the cytotoxicity of VCR depends upon the insensitivity of each cell line to VCR. The cell line less susceptible to VCR became sensitive to VCR, and the IC50 value of VCR for the less susceptible cell lines reached almost the same level as that of the sensitive cell line. This indicates that inherent resistance to VCR was circumvented by verapamil in vitro.

In an in vivo experiment, verapamil enhanced the chemotherapeutic effect of VCR in C26-bearing mice. We speculate that the present approach could render less sensitive tumor susceptible to VCR in vivo. The less sensitive tumor cells might possess a stronger VCR efflux function, as observed for C26 cells. Verapamil could efficiently inhibit the enhanced drug efflux function of the cells (22). The implication of this finding seems to be interesting for cancer chemotherapy.

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REFERENCES


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