Rhizoxin, a Macrocyclic Lactone Antibiotic, as a New Antitumor Agent against Human and Murine Tumor Cells and Their Vincristine-resistant Sublines

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ABSTRACT

Rhizoxin, isolated from a plant pathogenic fungus which causes rice seedling blight, inhibits the mitosis of the tumor cells in a manner similar to that of Vinca alkaloids as revealed by morphological study and flow cytometry analysis. This new 16-membered macrocyclic lactone showed similar chemotherapeutic effects to those of vincristine against L1210 and P388 leukemia-bearing mice. The drug is also effective against B16 melanoma inoculated i.p. or s.c. Rhizoxin, in contrast to the ansamacrolides, maytansine, was effective against human and murine tumor cells resistant to vincristine and Adriamycin in vitro and in vivo. A maximum 60% increase in life span was obtained in mice inoculated with P388 leukemia resistant to vincristine. Rhizoxin showed greater cytotoxicity in cultured tumor cells than did vincristine. Rhizoxin seems to bear consideration for further development as a new chemotherapeutic agent.

INTRODUCTION

Rhizoxin is a new 16-membered macrolide isolated from the plant pathogenic fungus Rhizopus chinensis Rh-2 (1). This fungus causes rice seedling blight, and the characteristic symptoms of this disease are abnormal swelling of seedling roots which is thought to be caused by the inhibition of cell division (2, 3). Our experiments indicated that rhizoxin inhibits the mitosis of tumor cells in a manner similar to that observed for Vinca alkaloids and ansamacrolides (4). Vinca alkaloids, such as VCR,2 vinblastine, and vindesine, are widely used clinically as antitumor agents. Among ansamacrolides, maytansine has been developed in the United States as a potential new antitumor agent. In spite of its activity against murine tumors (5), this compound has been disappointing in clinical trials (6). Rhizoxin is another macrocyclic lactone of interest as a potential new antitumor agent. Previously rhizoxin was found to be effective against MH134 mouse hepatoma.3 In this paper, we show that rhizoxin has considerable activity against L1210 or P388 leukemia and B16 melanoma. Rhizoxin was effective against vincristine- and Adriamycin-resistant tumor cells in vitro and in vivo, whereas maytansine has been shown to be inactive against the vincristine-resistant subline of P388 leukemia (7).

MATERIALS AND METHODS

Drugs. Rhizoxin was isolated from Rhizopus chinensis Rh-2 in the laboratory of one of the authors. The structure is shown in Chart 1 (M, 625). The isolation procedure and chemical nature have been reported previously (1). Vincristine, formulated for clinical use, was obtained from Shionogi & Co., Ltd., Osaka, Japan.

Animals and Tumor Cells. Adult female BALB/c × DBA/2CrF1 (hereafter called CD2F1) and female C57BL/6J × DBA/2CrF1 (hereafter called B6D2F1) mice were obtained from Charles River Japan, Inc., Tokyo, Japan. P388, P388/VCR, and P388/ADM cell lines were supplied by the National Cancer Institute, NIH, Bethesda, MD. The human myelogenous leukemia K562 cell line was provided by Dr. Ezaki, and the K562/VCR cell line was established in the laboratory of one of the authors (8).

Cell Culture and Drug Treatment. Tumor cells were maintained in suspension culture in plastic dishes (Corning Glass Works, Corning, NY) in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal bovine serum (Flow Laboratories, Stanmore, NSW, Australia) and kanamycin (100 µg/ml) (growth medium) (9). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2. For the drug treatment experiments, tumor cells (2 x 104 for L1210, P388, P388/VCR, and P388/ADM cells, and 4 x 104 for K562 and K562/VCR cells) were cultured at 37°C for 5 h in Falcon No. 2054 culture tubes containing 2 ml of growth medium in a humidified atmosphere of 5% CO2. Then the cells were treated with graded drug concentrations (0.1 to 100 nM for VCR and 0.01 to 30 nM for rhizoxin), reincubated for 72 h in the presence of drugs, and counted with a Model ZBI Coulter Counter (9). Three tubes were used for each drug concentration. In the control cultures, tumor cells grew exponentially during the incubation period. Rhizoxin was dissolved in ethanol to a stock concentration of 20 mM and diluted with phosphate-buffered saline (0.02 M sodium phosphate:0.15 M NaCl, pH 7.4).

IC50 was determined by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells (9).

Evaluation of Antitumor Activity. One-tenth ml of diluted ascites fluid, containing 105 L1210 cells or 106 P388 and P388/VCR cells, was transplanted i.p. into CD2F1 mice. Rhizoxin and VCR were dissolved with 0.9% NaCl solution and administered i.p. at 0.01 ml/g of body weight starting from the day after tumor inoculation. Treatment schedules and doses of the drugs are described in the legend of each chart and table. Five mice were used for each experimental group (9). Antitumor activity was evaluated by the mean survival time of a group of mice and also expressed by the ILS (percentage) value (9).

B16 melanoma (105) cells were inoculated i.p. into B6D2F1 mice, and rhizoxin was given i.p. on Days 1 to 4 and on Days 6 to 10 (9 injections). Antitumor activity was evaluated by the ILS (percentage) value as described above. Alternatively, fragments of B16 melanoma were inoculated s.c. into the flanks of B6D2F1 mice. Rhizoxin was given i.p. on Days 1 to 4 after tumor inoculation. Tumor diameters were measured in 2 directions with calipers on Day 10, and the tumor volume was calculated as follows: volume = ½ ab2, where a is long diameter and b is short diameter.

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2 The abbreviations used are: VCR, vincristine; ADM, Adriamycin; P388/VCR, P388 leukemia resistant to vincristine; P388/ADM, P388 leukemia resistant to Adriamycin; K562/VCR, human myelogenous leukemia K562 cells resistant to vincristine; IC50, concentration of drug required for 50% inhibition of cell growth; ILS, increase in life span.


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Morphological Changes and Cell Cycle Analysis of Tumor Cells. Light microscopic examination of L1210 cells, recovered from the peritoneal cavity of CD2F1 mice which were given rhizoxin at 2 mg/kg 6 h prior to the experiment, revealed the cells to be elongated, and many metaphase-arrested L1210 cells were present. Scanning electron microscopic examination of L1210 cells revealed spherical figures with an average diameter of approximately 7 μm. An abundance of microvilli approximately 0.1 μm in diameter was also observed on the surface of control L1210 cells (Fig. 1); however, at 6 h after rhizoxin administration, most of the cells were elongated, and the cell surface appeared smoother than controls and with less profuse microvilli (Fig. 1). Similar morphological changes have also been observed for VCR-treated cells (Fig. 1).

Cell cycle analysis by flow cytometry indicated that rhizoxin inhibits mitosis in tumor cells in a manner similar to that of VCR. L1210 cells recovered from mice 24 h after rhizoxin treatment were blocked on G2-M phase (Chart 2). More than 50% of the cells were distributed in G2-M phase as has been observed for VCR-treated L1210 cells (Table 1). These results clearly indicate that VCR and rhizoxin have the same mode of action, i.e., the inhibition of mitosis.

Chemotherapeutic Effects against L1210 and P388 Leukemias. For the comparison of in vivo effects of rhizoxin and vincristine, the doses of the drugs were expressed on a molar basis (μmol/kg). Rhizoxin given four successive days after the day of tumor inoculation increased the life span of L1210-bearing mice by 81%, 67%, and 43% at doses of 1.6, 0.8, and 0.4 μmol/kg (1.0, 0.5, and 0.25 mg/kg), respectively (Table 2), while VCR was slightly less effective with a 67% and 44% ILS obtained at 1.08 and 0.54 μmol/kg (1.0 and 0.5 mg/kg), respectively. The dose-dependent effect of rhizoxin is shown in Chart 3. When rhizoxin was given on Days 1 and 4 after the tumor inoculation, the maximum ILS of about 70% was obtained at doses of between 3.2 and 6.4 μmol/kg (2 and 4 mg/kg) of rhizoxin. At 0.8 μmol/kg (0.5 mg/kg), a small (25%) ILS was still observed, while above 9.8 μmol/kg (6 mg/kg), this regimen was toxic. The...
Chemotherapeutic effect of rhizoxin was compared to that of VCR against P388 leukemia (Chart 4). P388 leukemia was slightly more sensitive to VCR than to rhizoxin in this experiment. A maximum ILS of 108% was observed at 2.4 μmol/kg (1.5 mg/kg) of rhizoxin per kg, while a slightly higher maximum ILS (128%) occurred with VCR at 0.81 μmol/kg (0.75 mg/kg), a dose approximately 1/8 of that found most effective for rhizoxin on a μmol/kg basis.

Chemotherapeutic Effect of Rhizoxin against B16 Melanoma. Rhizoxin given i.p. at 0.8 μmol/kg showed efficacy (ILS, 91%) against B16 melanoma inoculated i.p. Even at a dose as low as 0.4 μmol/kg, a 24% ILS was observed. Against B16 melanoma inoculated s.c., rhizoxin given i.p. also induced tumor growth inhibition of 60 to 70% at 0.8 to 1.6 μmol/kg and of 40% at 0.4 μmol/kg.

Growth-Inhibitory Effect of Rhizoxin on Sensitive and Vincristine-resistant Tumor Cell Lines. Maytansine has been reported to be ineffective against tumor cells resistant to Vinca alkaloids (7). The cytotoxicity of rhizoxin against drug-resistant tumor cells would of course increase interest in the development of a new antitumor agent. P388/VCR, P388/ADM, and K562/VCR cells showed 15.4-, 25.7-, and 21-fold resistance to VCR, respectively, when the IC50 values of these tumor lines and the parent cells were compared (Table 3). These tumor cell lines, however, showed only 4.2-, 4.5-, and 2.1-fold resistance to rhizoxin, they were 8- to 43-fold more sensitive to rhizoxin than to VCR, while the sensitive cell lines (P388 and K562) were only 2- to 5-fold more sensitive to rhizoxin than to VCR. These results clearly indicate that rhizoxin is more effective to tumor cells, especially to the VCR- and ADM-resistant tumor cell lines, as compared to VCR. This information together with the observation that almost 3 times as much rhizoxin as VCR can be given to the mice (Chart 4 and below) suggested that rhizoxin should be effective in vivo in animals bearing VCR-resistant tumors.

Chemotherapeutic Effect of Rhizoxin against VCR-resistant Tumor-bearing Mice. VCR at doses ranging from 0.11 to 1.1 μmol/kg (0.1 to 1 mg/kg) given on Days 1, 5, and 9 after tumor inoculation showed no chemotherapeutic effect in mice bearing P388 leukemia resistant to VCR (Chart 5). VCR was toxic above 1.1 μmol/kg. Rhizoxin given at the same schedule resulted in a 30% ILS at 0.32 μmol/kg (0.2 mg/kg), with a maximum ILS of about 60% being achieved at doses of 2.4 to 3.2 μmol/kg (1.5 to 2.0 mg/kg). Although this survival advantage was less than that conferred by the drug at similar doses in the sensitive P388 leukemia-bearing mice, it is evident that rhizoxin is effective against VCR-resistant tumor cells in vivo.

General Toxicity of Rhizoxin in Mice. Acute toxicity of rhizoxin against C57BL/6N mice as expressed by the median lethal dose was about 1/8 of that of vincristine (median lethal dose, 8 to 16 μmol/kg (5 to 10 mg/kg) for rhizoxin and 2.7 to 5.4 μmol/kg (2.5 to 5 mg/kg) for VCR). In the case of rhizoxin, most of deaths occurred 24 h after injection, but in the case of VCR, it occurred 4 days after.

The main toxic signs observed in the higher dose of rhizoxin were loss of body weight gain, decrease of food consumption and water intake, diarrhea, hypothermia, mild leukopenia and thrombocytopenia, decrease of thymus weight, and hind limb paralysis. In comparison with VCR, rhizoxin at 4 μmol/kg (2.5 mg/kg) i.p. did not induce significant decrease of body weight; however, VCR at 2.7 μmol/kg (2.5 mg/kg) caused a 20% de-

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>P388</th>
<th>P388/VCR</th>
<th>P388/ADM</th>
<th>K562</th>
<th>K562/VCR</th>
</tr>
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<tr>
<td>Rhizoxin</td>
<td>0.91 ± 0.01*</td>
<td>3.84 ± 0.39 (4.2)</td>
<td>4.13 ± 0.49 (4.5)</td>
<td>0.51 ± 0.04</td>
<td>1.28 ± 0.05 (2.5)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>2.10 ± 0.10</td>
<td>32.4 ± 2.3 (15.4)</td>
<td>54.0 ± 2.3 (25.7)</td>
<td>2.68 ± 0.25</td>
<td>56.3 ± 1.3 (21.0)</td>
</tr>
</tbody>
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* Mean ± SD of three determinations.
Numbers in parentheses, degree (x-fold) of resistance as compared to parent cells.
crease of body weight after 5 days. Rhizoxin at 8 \( \mu \text{mol/kg} \) caused a 10% decrease of body weight on Day 2, but body weight was recovered by Day 5. Rhizoxin at 4 to 16 \( \mu \text{mol/kg} \) caused no abnormal values in the erythrocyte counts or hemoglobin/hematocrit values. It caused only a marginal decrease in the numbers of leukocytes and platelets, and these toxicities were lower than VCR. Rhizoxin at 8 \( \mu \text{mol/kg} \) caused a slight decrease in thymus weight, while a significant decrease of thymus weight occurred with VCR at 1.35 and 2.71 \( \mu \text{mol/kg} \). Rhizoxin at 4 and 8 \( \mu \text{mol/kg} \) did not induce a decrease in spleen weight, while VCR at 2.7 \( \mu \text{mol/kg} \) caused an evident decrease in spleen weight. Both drugs showed similar toxicity against testis as estimated by the decrease in weight; however, recovery was faster in the case of rhizoxin.

The presence of neurotoxicity in rhizoxin is not clear from the results of this experiment, but clinical signs such as hind limb paralysis and ataxic gait were observed 24 h after injection of rhizoxin at 16 \( \mu \text{mol/kg} \) (10 mg/kg). The same signs were also seen 2 or 3 days after injection of VCR at 5.4 \( \mu \text{mol/kg} \) (5 mg/kg). Neurotoxicity could be one of the major toxicities of rhizoxin; however, toxicology studies including the cause of death remain to be solved in the future.

DISCUSSION

Rhizoxin showed mitosis-inhibiting properties similar to those of Vinca alkaloids. Maytansine, which is a naturally occurring ansamacrolide originating from the East African shrub *Maytenus ovatus*, also has been reported to possess similar properties (4). It belongs to a new class of compounds characterized by the presence of a macrocyclic lactam ring, with a bridged aromatic moiety. Maytansine has been of substantial interest because of its novel structure (5). However, it has shown essentially no therapeutic effects in clinical studies (6). This compound was also inactive against the vincristine-resistant subline of P388 leukemia (7). The structure of rhizoxin is somewhat similar to that of maytansine, although the former is characterized by a macrocyclic lactone and the latter by a lactam ring. Both compounds possess mitosis-inhibiting properties. Rhizoxin, in contrast to maytansine, was effective against the sublines of P388 and K562 human myelogenous leukemias resistant to vincristine and Adriamycin *in vivo* and *in vitro* as reported in this paper. The chemotherapeutic effects of rhizoxin are similar to those of VCR against L1210 and P388 leukemia-bearing mice. Approximately 3 times as much rhizoxin as VCR were needed to obtain the maximum therapeutic effect. Rhizoxin showed a somewhat greater cytotoxicity than did VCR against cultured tumor cells; however, its toxicity in animal experiments appears lower than that of VCR. Approximately 2 to 3 times as much rhizoxin as VCR induced similar toxicity in mice. Thus the therapeutic index was similar between rhizoxin and VCR. Rhizoxin seems to be a worthwhile candidate for further evaluation as a new antitumor agent with possible efficacy in VCR- and ADM-resistant tumors also.

REFERENCES

Fig. 1. Rhizoxin- or VCR-treated cells examined by scanning electron microscopy. CD2F mice were inoculated with $10^6$ L1210 cells. After 6 days, rhizoxin or VCR was given at 2 mg/kg. L1210 cells were recovered from the peritoneal cavity 6 h after drug administration and examined by scanning electron microscope (x 8000). Left, control L1210 cells; center, rhizoxin-treated L1210 cells; and right, VCR-treated L1210 cells.
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