Schedule-Dependent Cytotoxicity of Etoposide (VP-16) and Cyclophosphamide in Leukemia Cell Line K-562

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In allogeneic bone marrow transplantation (allo-BMT) in patients with leukemia, the combination of VP-16 and cyclophosphamide (CY) is commonly used for the conditioning regimen. In the present study, we demonstrated schedule-dependent cytotoxicity of VP-16 and CY in K-562 cells. K-562 cells were pretreated with low concentrations (2.5 and 5 µg/mL) of 4-hydroperoxycyclophosphamide (40487S), which is a preactivated analog of CY. It was confirmed that these concentrations did not influence cell viability. Cells subsequently exposed to 0.5–100 µg/mL of VP-16 showed reduced the viability compared to that of control cells not treated with 40487S. In contrast, there was no change in the viability of K-562 cells pretreated with low concentrations (0.5 and 1 µg/mL) of VP-16. It was confirmed that these concentrations did not influence cell viability. Viability of subsequently exposed to 1–20 µg/mL was not different from that of control cells not treated with VP-16. VP-16 caused cell cycle arrest at G2/M phase. On the other hand, 40487S arrested the cell cycle at S phase. Thymidine-synchronized cells, VP-16 showed cell cycle specificity for cell killing from early-S to mid-S phase. On the other hand, 40487S showed cell cycle-independent cytotoxicity. Exposure of cells to VP-16 after 40487S induced a greater cytotoxic effect on K-562 cells. The findings may lead to improvements in clinical combination chemotherapy.

Key words etoposide; cyclophosphamide; schedule; cell cycle; allogeneic bone marrow transplantation

The combination of a topoisomerase II inhibitor (VP-16) and cyclophosphamide (CY) has been widely used in treatment of leukemias. Allogeneic bone marrow transplantation (allo-BMT) is an effective treatment for patients with acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and chronic myelogenous leukemia (CML). The standard conditioning regimen is CY and total body irradiation (TBI) (CY/TBI), but mortality rate due to relapse is high. Therefore, various conditioning regimens using chemotherapy agents such as busulphan,1) cytarabine2) and VP-163,4) combined with CY/TBI have been developed. However, some intense regimens led to a decrease in the relapse rate, but higher rates of toxicity and transplant related mortality (TRM) have been reported.5) Therefore, the best regimen has not yet been established. Recently, excellent outcomes of a conditioning regimen using medium-dose VP-16, CY, and TBI (medium-dose VP-16/CY/TBI) has been reported, and this regimen has been suggested to be effective and safe for adult patients with hematological malignancies, the 3-year overall survival rate, relapse rate, and TRM rate were 89.2%, 8.1%, and 5.4%, respectively.6) Medium-dose VP-16/CY/TBI for hematologic malignancies was associated with lower relapse rate and no increase in toxicity, resulting in better survival. However, the administration schedule of VP-16 and CY has not been based on the evidence and has differed among patients. Combination chemotherapy with multiple drugs is commonly used in treatment of cancer. Many studies has shown schedule-dependent effects between drugs such as melphalan and oxaliplatin,7) cisplatin and taxol,8) paclitaxel and gemcitabine,9) paclitaxel and carboplatin.10) However, the schedule for drug administration to achieve the best therapeutic efficacy is still uncertain. Therefore, the aim of the present study was to evaluate the schedule-dependent cytotoxicity of VP-16 and CY in K-562 cells, widely used as a human leukemia model.

MATERIALS AND METHODS

Chemicals VP-16 was purchased from LKT Laboratories, Inc. (St. Paul, MN, U.S.A.). It was dissolved in dimethyl sulfoxide (DMSO), and stored at −20°C. The final concentration of DMSO in the well was <0.5%, which was non-toxic in the assays. An activated analog of CY, 4-hydroperoxycyclophosphamide (40487S) was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Stock 40487S was stored at −80°C and diluted in cell culture medium immediately prior to use. WST-8 reagent was purchased from Wako Pure Chemicals (Osaka, Japan). Thymidine and RNase were purchased from WAKO. NP-40 was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). These agents were dissolved in phosphate buffered saline (PBS) buffer.

Cell Culture The human chronic myelogenetic leukemia cell line K-562 was purchased from RIKEN (Ibaraki, Japan). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 UI/mL), and streptomycin (100 µg/mL) at 37°C in a humidified atmosphere with 5% CO2 and 95% air.

Cytotoxicity Assay The cytotoxic effects of the drugs on K-562 cells was determined by the WST-8 assay. A suspension of exponentially growing cells (1×10⁴ cells/well) was added to 96-well microplates. Various concentrations of the drugs (0.5–100 µg/mL of VP-16 and 1–20 µg/mL of 40487S) were added and cultured for 24h or 48h. For the combination chemotherapy group, cells were pretreated with a low concentration of one of the drugs for 24h prior to treatment with the other drug. After exposure to the drugs, WST-8 reagent was added to each well, and the cells were incubated at 37°C for 3h. The two schedules used are shown in Fig. 1. The absorbance of each well was measured at 450nm with a microplate

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reader. Results were calculated as a percentage relative to unpretreated controls and plotted in logarithmic dose-effect curves, and EC_{50} was determined by using Origin® (version 8.1J). A curve was fitted using the following equation:

\[ Y = \frac{A_1 + A_2}{1 + (x + x_0)^P} + A_2 \]

Where \( A_1 \) is the initial value, \( A_2 \) is the final value, \( x \) is the concentration, \( x_0 \) is the EC_{50} value, and \( P \) is power. Both one word drugs were used at clinically relevant doses.11–13)

Cell Cycle Analysis The cell cycle was measured by flow cytometry. K-562 cells were incubated in culture medium containing 1 µg/mL VP-16 and 5 µg/mL 40487S for 24 and 48 h. The cells were washed with PBS, fixed in 90% cold EtOH, and then treated with staining buffer (final concentrations: 1 mg/mL RNase, 0.01% NP40, 20 µg/mL propidium iodide) for 20 min. Cell cycle distribution was determined by using a FACScan flow cytometer. All samples were compared to untreated control cells.

Cell Cycle Synchronization Cell cycle phase specificity of drugs was evaluated by a modification of the method of Kazuno et al.14) Cells were synchronized by a double thymidine block. Cells were incubated for 24 h in a medium containing 2 mM thymidine followed by incubation for 12 h in a thymidine-free medium. Synchronization of cells at the G1/S boundary was then achieved by further incubation for 14 h in a medium containing 2 mM thymidine. The thymidine block was released by washing the cells with PBS, followed by incubation in a fresh medium. At 0, 2, 4, 6, 8 and 10 h after release from the thymidine block, VP-16 and 40487S were added. Following 90-min exposure to the drugs, the cells were washed with PBS and suspended in culture medium. The cells were incubated in 96-well microplates for 3 d. Cell viability was evaluated by the WST-8 assay.

Statistical Analysis Data are presented as means±S.D. Student’s t-test was used to determine statistical differences between various experimental and control groups. Differences were considered statistically significant at a level of \( p<0.05 \).

RESULTS

Cytotoxic Effects of VP-16 and 40487S on K-562 Cells To clarify the cytotoxic effects of VP-16 and 40487S on K562 cells, we tested various concentrations of VP-16 and 40487S and the two exposure times of K-562 cells to the drugs. It was found that viability of cells exposed to VP-16 depended on concentration of the drug and exposure time (Fig. 2(A), Table 1). On the other hand, viability of cells exposed to 40487S depended only on concentration of the drug (Fig. 2(B), Table 1). The concentrations of VP-16 that had no influence on cell viability after 24 h and 48 h were 0.5 and 1 µg/mL, respectively, and those of 40487S were 2.5, 5 µg/mL, respectively.

Schedule-Depended Cytotoxicity of VP-16 and 40487S in K-562 Cells K-562 cells were pretreated with low concentrations (0.5 and 1 µg/mL) of VP-16 for 24 h and then treated continuously with 40487S (1–20 µg/mL) for 24 h. The logarithmic dose-effect curves and EC_{50} values showed no differences compared with those in the VP-16-untreated group (mean EC_{50} values: 12.39 and 12.22 µg/mL vs. 10.13 µg/mL). On the other hand, cells pretreated with low concentrations (2.5 and 5.0 µg/mL) of 40487S for 24 h and then treated continuously with VP-16 (0.5–100 µg/mL) for 24 h showed reduced viability compared with cells in the 40487S-untreated group (mean EC_{50} values: 3.83 and 2.74 µg/mL vs. 19.8 µg/mL) (Fig. 3, Table 2).

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>VP-16 (µg/mL)</th>
<th>40487S (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>25.2±8.12</td>
<td>9.72±0.93</td>
</tr>
<tr>
<td>48</td>
<td>4.17±0.78</td>
<td>9.25±1.27</td>
</tr>
</tbody>
</table>

Cell viability was assessed by WST-8 assay.
Cell Cycle Progression after Drug Exposure

K-562 cells were treated with 1 \( \mu \)g/mL VP-16 or 5 \( \mu \)g/mL 40487S for 24 and 48h. Following VP-16 treatment, cell cycle progression was blocked at the G2/M phase. Fractions of cells at G2/M phase after treatment with VP-16 for 24h and 48h were increased by 13.3% and 30.7%, respectively, compared with control cells. On the other hand, cell cycle progression of cells treated with 40487S was blocked at S phase. Fractions of cells at S phase after treatment with 40487S for 24h and 48h were increased by 27.6% and 32.8%, respectively, compared with control cells. Fractions of cells at G1 phase were decreased after treatment with VP-16 and 40487S (Fig. 4, Table 3).

Cell Cycle Phase Specificity of the Drugs

Firstly, to clarify cell cycle-specific cytotoxicity, cell cycle progression was checked every two hours after thymidine release. Cells were in S, G2/M and G1 phases at 0–6h, at 6–8h from 8h after thymidine release, respectively (Fig. 5). VP-16 showed the greatest cytotoxic effect in S phase and its effect decreased in G1 phase. On the other hand, Cytotoxicity of 40487S was cell cycle-independent (Fig. 6).

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Table 2. EC50 Values of Combination Chemotherapy

<table>
<thead>
<tr>
<th>Schedule A</th>
<th>EC50 value</th>
<th></th>
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<tbody>
<tr>
<td>Control (VP-16 free)</td>
<td>10.13±3.72</td>
<td></td>
</tr>
<tr>
<td>0.5 ( \mu )g/mL</td>
<td>12.39±3.11</td>
<td></td>
</tr>
<tr>
<td>1.0 ( \mu )g/mL</td>
<td>12.22±1.34</td>
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</table>

<table>
<thead>
<tr>
<th>Schedule B</th>
<th>EC50 value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (40487S free)</td>
<td>19.8±8.34</td>
<td></td>
</tr>
<tr>
<td>2.5 ( \mu )g/mL</td>
<td>3.83±0.54**</td>
<td></td>
</tr>
<tr>
<td>5.0 ( \mu )g/mL</td>
<td>2.74±0.41**</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Cell Cycle Distribution Change Relative to the Control at Each Cell Cycle after VP-16 or 40487S Treatment for 24 or 48h

<table>
<thead>
<tr>
<th>Variation (%)</th>
<th>( \text{G0/G1} )</th>
<th>( \text{S} )</th>
<th>( \text{G2/M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP-16 24 h treated</td>
<td>-21.2</td>
<td>+9.0</td>
<td>+13.3</td>
</tr>
<tr>
<td>VP-16 48 h treated</td>
<td>-43.6</td>
<td>+12.7</td>
<td>+30.7</td>
</tr>
<tr>
<td>40487S 24 h treated</td>
<td>-31.9</td>
<td>+27.6</td>
<td>+4.1</td>
</tr>
<tr>
<td>40487S 48 h treated</td>
<td>-43.1</td>
<td>+32.8</td>
<td>+10.2</td>
</tr>
</tbody>
</table>

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Fig. 3. Effects of Schedule Dependent Chemotherapy On Viability of K-562 Cells

Schedule A: viability of K-562 cells pretreated with 0.5 or 1.0\( \mu \)g/mL VP-16 for 24h and subsequently exposed to 1–20\( \mu \)g/mL 40487S compared to control cells not treated with VP-16. Schedule B: viability of K-562 cells pretreated with 2.5 or 5.0\( \mu \)g/mL 40487S for 24h and subsequently exposed to 0.5–100\( \mu \)g/mL VP-16 compared to control cells not treated with VP-16. Each point represents the mean±S.D. of 4–6 measurements.

Fig. 4. Cell Cycle Distribution of K-562 Cells

K-562 cells were treated with either 1.0\( \mu \)g/mL VP-16 or 5.0\( \mu \)g/mL 40487S for 24–48h. Cell cycle was analyzed by using a FACScan flow cytometer.

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Cell viability was assessed by WST-8 assay. Each point represents the mean±S.D. of 4–6 measurements. **: \( p<0.01 \)
Anticancer drugs are categorized as cell cycle-dependent and cell cycle-independent drugs. Cell cycle changes after treatment with anticancer drugs are well-known. We focused on the cell cycle to evaluate the mechanisms of schedule-treatment with anticancer drugs are well-known. We focused and cell cycle-independent drugs. Cell cycle changes after combination chemotherapy, we showed that pretreatment with 2.5 and 5 \( \mu \text{g/mL} \) of VP-16 did not influence the cytotoxicity of 40487S but that pretreatment with 2.5 and 5 \( \mu \text{g/mL} \) of 40487S increased the cytotoxicity of VP-16.

In the present study, we focused on only the schedule of VP-16 and 40487S. However, medium-dose VP-16/CY/TBI conditioning regimens include radiation. In addition, radiosensitivity is dependent on the cell cycle and is very effective in the G1 and G2 phases, while S phase has low radiosensitivity. It has been reported that docetaxel pretreatment increased cells in S phase and thus results in reduction of the cytotoxicity of radiation. Similarly, 40487S pretreatment led to an accumulation of cells at S phase, which may reduce the cytotoxicity of radiation. The blocking the cell cycle at S phase is important for combination with VP-16. These results suggested that 40487S pretreatment may enhance the cytotoxicity of other S phase specific anticancer drugs, and preadministration of CY may be useful for combination chemotherapy.

In conclusion, the results of the present study indicate that pretreatment of cells with 40487S enhances cytotoxicity of VP-16 blocking the cell cycle at S phase, which is a specific phase for VP-16. The schedule of chemotherapy may affect the therapeutic outcome. To achieve the best therapeutic efficacies, further studies will be needed to focus on the schedule-dependent cytotoxicity of VP-16 and CY in other cell line of ALL and AML, furthermore in vivo study is important data for the clinical setting.

**REFERENCES**


