Schedule-Dependent Cytotoxicity of Etoposide and Cyclophosphamide in P-Glycoprotein-Expressing Human Leukemic K-562 Cells

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Combination chemotherapy is often used to treat cancer. Many studies have shown schedule-dependent effects between anticancer drugs. Our previous studies showed that K-562 cells pretreated with non-cytotoxic concentrations of 4-hydroperoxycyclophosphamide (4-HPC), which is a preactivated analog of cyclophosphamide (CY), enhanced the cytotoxicity of etoposide (VP-16). The appearance of cellular resistance to anticancer drugs is a major problem in cancer chemotherapy. P-Glycoprotein (P-gp) plays an important role in drug resistance, and VP-16 is a substrate for this efflux pump. In the present study, we demonstrated schedule-dependent cytotoxicity of VP-16 and CY in P-gp-overexpressed K-562/P-gp cells. Cytotoxicity of VP-16 was enhanced in K-562/P-gp cells that were pretreated with a non-cytotoxic concentration of 4-HPC compared to that of cells not treated with 4-HPC. 4-HPC arrested the cell cycle at S phase. Cells in S phase are most sensitive to VP-16. The results suggest that cell cycle arrest by 4-HPC pretreatment may be responsible for the enhanced cytotoxicity of VP-16. The findings in this study should lead to improvements in clinical combination chemotherapy.

Key words etoposide; P-glycoprotein; schedule; cyclophosphamide; cell cycle

Etoposide (VP-16) is an anticancer drug that is derived from podophyllotoxin. The cytotoxic mechanism of VP-16 inhibits DNA synthesis by forming a complex with topoisomerase II and DNA.[1)] Cells in S phase are most sensitive to VP-16, and the cytotoxicity is decreased rapidly as cells progressed to G_1 through G_2 and M.[2,3)] VP-16 has been used in a number of combination chemotherapies with multiple drugs. Many studies have shown schedule-dependent effects between anticancer drugs. Chresta *et al.* reported that pretreatment with arabinosyl cytosine (Ara-C) enhances cytotoxicity of VP-16 in a human lymphoblastoid cell line (CCRF-CEM),[4)] and Lorico *et al.* reported that pretreatment with methotrexate (MTX) increased cytotoxicity of VP-16 in a human histiocytic lymphoma cell line (U937).[5)]

The combination of VP-16 and cyclophosphamide (CY) has been widely used for treatment of leukemias.[6–9)] We previously showed that pretreatment of K-562 cells with 4-hydroperoxycyclophosphamide (4-HPC), which is a preactivated analog of CY, enhances the cytotoxicity of VP-16 *via* blocking by 4-HPC of the cell cycle at S phase, which is a specific phase for VP-16.[10)]

In clinical treatment, multidrug resistance (MDR) is one of the major obstacles for successful chemotherapy in patients with cancer. MDR has been associated with overexpression of P-glycoprotein (P-gp) in cancer cells.[11,12)] P-gp is a 170-kDa protein encoded by the MDR-1 gene.[13,14)] P-gp belongs to the superfamily of ATP-binding cassette (ABC) transporters and actively effluxes various anticancer drugs. The substrates of P-gp include anthracyclins and epipodphyllotoxins (*e.g.*, VP-16), which have been used in the treatment of a variety of malignancies, especially leukemia and lymphoma.[15)] A number of studies have shown that a lower complete remission (CR) rate in leukemia patients is associated with an increase in P-gp expression.[16,17)]

There has been an accumulation of evidence showing that schedules of anticancer drugs affect the cytotoxicity not only VP-16 but also other anticancer drugs.[4,5,18)] However, the schedule for the optimal use of anticancer drugs in clinical treatment has still not been established. Moreover, the effect in P-gp-positive cells has not been investigated. In the present study, we investigated that schedule-dependent cytotoxicity of VP-16 and CY in P-gp-overexpressed K-562 cells. An understanding of the schedule-dependent cytotoxicity of VP-16 and CY may be useful for improving chemotherapy regimens.

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) (stock concentration: 20 mg/mL) and stored at −20°C. An activated analog of CY, 4-hydroperoxycyclophosphamide (4-HPC), was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Stock 4-HPC was stored at −80°C and diluted in cell culture medium immediately prior to use. Quinidine sulfate was purchased from AstraZenace Inc. (Osaka, Japan). WST-8 reagent, thymidine and RNase were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NP-40 was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). These agents were dissolved in phosphate buffered saline (PBS). All other chemicals were of the highest grade available.

Cell Culture The human chronic myelogenetic leukemia cell line K-562 was purchased from RIKEN (Ibaraki, Japan). K-562 and K-562/P-gp 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% CO_2 and 95% air.

Cytotoxicity Assay For combination chemotherapy, a

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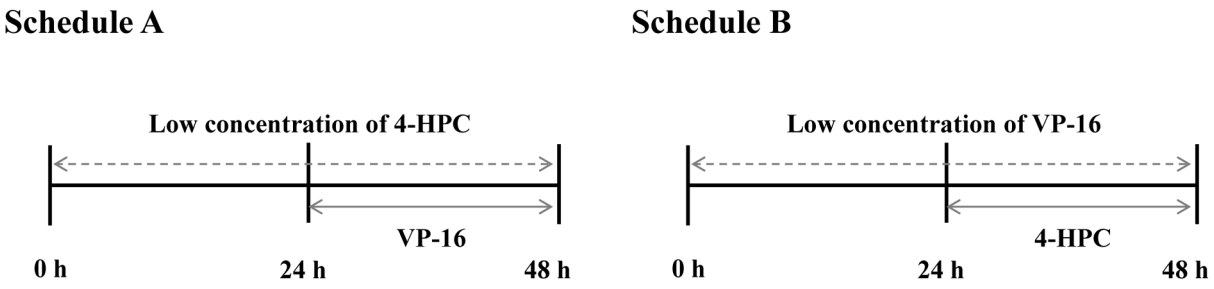


Fig. 1. Schedules of the Two Protocols for Combination of VP-16 and 4-HPC

suspension of exponentially growing cells (1×10^4 cells/well) was added to 96-well microplates. Cells were pretreated with a non-cytotoxic concentration of VP-16 or 4-HPC for 24 h prior to treatment with the other drug. The concentrations of VP-16 and 4-HPC for pretreatment were 1.0 μg/mL and 5 μg/mL respectively, and it was confirmed that these concentrations did not influence cell viability by the WST-8 assay as described previously.[10] After exposure to the drugs, the cytotoxic effects of the drugs on K-562 and K-562/P-gp cells were determined by the WST-8 assay. The two schedules used are shown in Fig. 1. The absorbance of each well was measured at 450 nm with a microplate 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 9.0J). 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.

Cell Cycle Analysis The cell cycle was measured by flow cytometry. K-562 cells were incubated in culture medium containing 5 μg/mL 4-HPC for 24 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% NP-40, 20 μg/mL propidium iodide) for 20 min. Cell cycle distribution was determined by using a FACScan flow cytometer (Becton Dickinson).

Cell Cycle Synchronization Cells were synchronized by a double thymidine block as described previously.[10] Briefly, 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 G_1/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.

Uptake Studies K-562 and K-562/P-gp cells were suspended at 5×10^7 cells/mL in a transport buffer (25 mM Tris-*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose), and 200 μL of the cell suspension was added to 1.5 mL microcentrifuge tubes. The cells were preincubated for 15 min at 37°C. After preincubation, 200 μL of transport buffer containing 25 μg/mL VP-16 was added and incubated for 60 min at 37°C. Uptake experiments were terminated after 60 min by adding 1 mL ice-cold transport buffer and centrifuged at 210×***g*** for 5 min at 4°C. The cells were washed twice with 1 mL ice-cold transport buffer and centrifuging at 210×***g*** for 5 min at 4°C. The cells pellets were resuspended in 200 μg phosphate buffer (10 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.0) and disrupted by freezing at −80°C for 10 min. The disrupted cell suspension was divided for protein analysis and HPLC analysis. The HPLC system consisted of an L-7110 pump, L-7300 column oven, L-7420 UV-VIS detector, and D-2500 integrator (HITACHI, Tokyo, Japan). The column was an Inersil ODS-4 (100 mm×2.1 mm i.d., 3 μm) (YOKOHAMARIKA CO., Yokohama, Japan). Fifty microliter of the sample and 50 μL of acetonitrile were added. After shaking the mixture vigorously, the sample was centrifuged at 21500×***g*** for 10 min at 4°C. The injection volume of the sample was 15 μL. A mobile phase containing methanol–distilled water–acetonitrile (42.7 : 55 : 2.3, v/v/v) was used at a flow rate of 0.2 mL/min. The detector was monitored at 229 nm. The protein content was measured by the method of Lowry.[19]

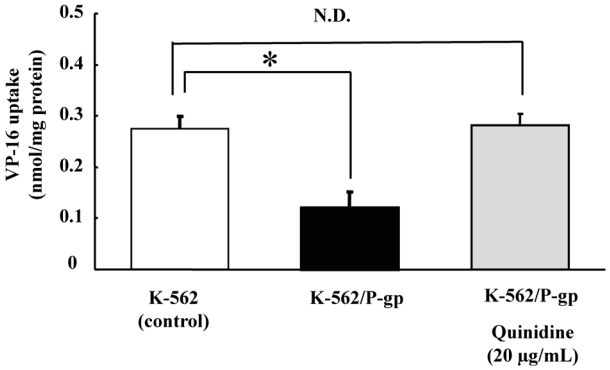


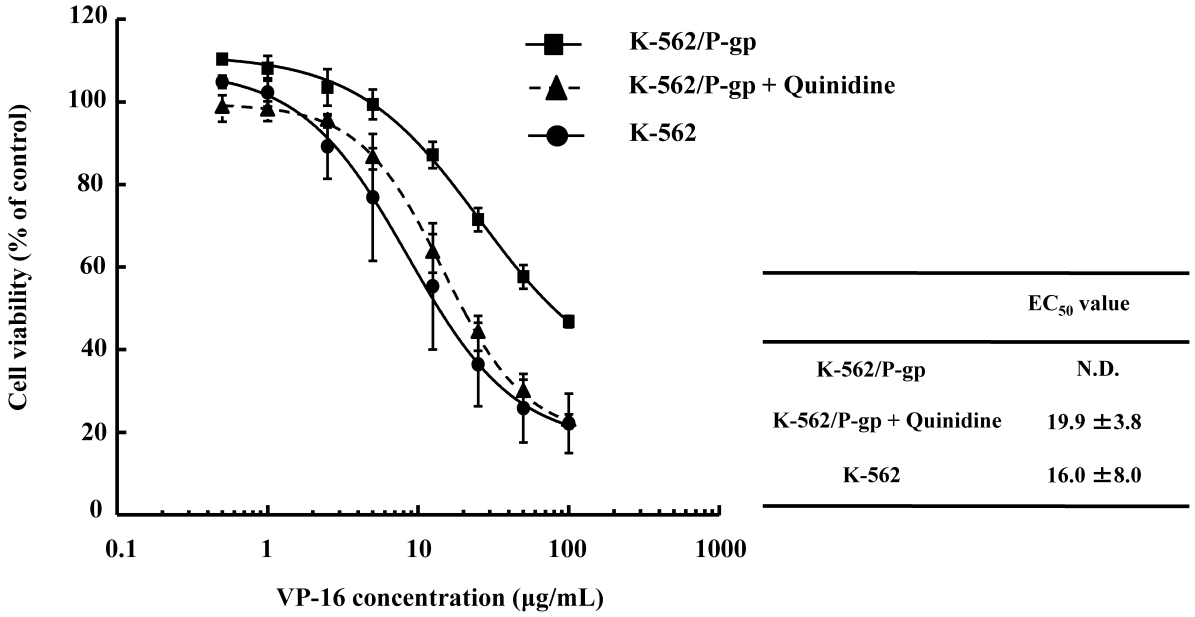
Fig. 2. Uptake of VP-16 by K-562 and K-562/P-gp Cells

Cells were incubated for 60 min at 37°C at pH 7.5 with 25 μM VP-16 in transport buffer. For inhibitory assays, K-562/P-gp cells were preincubated with 20 μg/mL of the P-gp inhibitor quinidine for 24 h. The concentration of VP-16 was determined using an HPLC system. Each column represents the mean+S.D. of 3 measurements. *Significantly different from control at $p<0.05$.

Statistical Analysis Data are presented as means with S.D. Student's *t*-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was evaluated using ANOVA followed by the Tukey–Krammer test. Statistical significance was defined as $p<0.05$.

RESULTS

Function of P-gp in K-562/P-gp Cells Firstly, we investigated VP-16 uptake by K-562 and K-562/P-gp cells for 60 min (Fig. 2). The uptake of VP-16 for 60 min by K-562/P-gp cells was significantly decreased compared with that by K-562



	EC_{50} value
K-562/P-gp	N.D.
K-562/P-gp + Quinidine	19.9 ±3.8
K-562	16.0 ±8.0

Fig. 3. Effects of VP-16 on Viability of K-562 and K-562/P-gp Cells

K-562 and K-562/P-gp cells were exposed to 0.5–100 μg/mL VP-16 for 24 h. For inhibitory assays, K-562/P-gp cells were preincubated with 20 μg/mL of the P-gp inhibitor quinidine for 30 min. Cell viability was assessed by the WST-8 assay. Each column represents the mean±S.D. of 4–7 measurements.

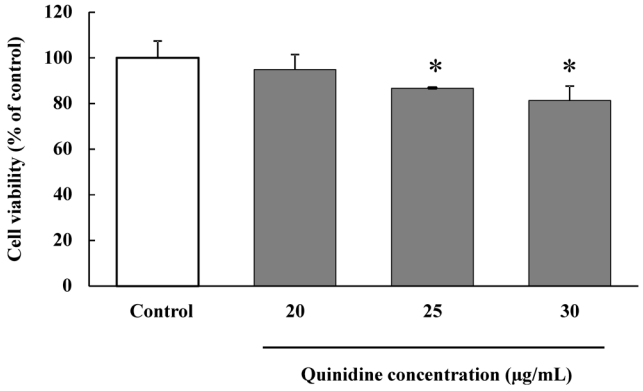


Fig. 4. Effects of Quinidine on Viability of K-562/P-gp Cells

K-562/P-gp cells were exposed to 20, 25 and 30 μg/mL quinidine for 24.5 h. Cell viability was assessed by the WST-8 assay. Each column represents the mean±S.D. of 3 measurements. *Significantly different from control at $p<0.05$.

cells (control). On the other hand, the decrease in uptake was recovered to the control level by preincubation with the P-gp inhibitor quinidine.

Effect of P-gp on Cytotoxicity of VP-16 The cytotoxicity of VP-16 in K-562/P-gp cells was decreased compared with that in K-562 cells (control), and the decrease in cytotoxicity was recovered to the control level by preincubation with quinidine (Fig. 3). We confirmed that 20 μg/mL quinidine did not influence cell viability (Fig. 4).

Schedule-Depended Cytotoxicity of VP-16 and 4-HPC in K-562/P-gp Cells K-562/P-gp cells were pretreated with a low concentration (1 μg/mL) of VP-16 for 24 h and then treated continuously with 4-HPC (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: 11.1 μg/mL *vs.* 10.1 μg/mL) (Fig. 5 schedule B, Table 1B). On the other hand, cells pretreated with a low concentration (5 μg/mL) of 4-HPC for 24 h and then treated continuously with VP-16 (0.5–100 μg/mL) for 24 h showed

Table 1. EC_{50} Values of Combination Chemotherapy on K-562/P-gp Cells

	EC_{50} value
A: Low-concentration 4-HPC→VP-16	
Low-concentration 4-HPC free	N.D
5.0 μg/mL	N.D
B: Low-concentration VP-16→4-HPC	
Low-concentration VP-16 free	10.1±0.52
1.0 μg/mL	11.1±0.30

Cell viability was assessed by WST-8 assay. Each value is the mean±S.D. of 3 measurements.

reduced viability compared with that of cells in the 4-HPC-untreated group. EC_{50} values could not be determined because EC_{50} was not less than 50% (Fig. 5 schedule A, Table 1A).

Cell Cycle Progression after Drug Exposure K-562/P-gp cells were treated with 5 μg/mL 4-HPC for 24 h. Following 4-HPC treatment, cell cycle progression was blocked at S phase (Fig. 6).

Effect of Cell Cycle Progression on VP-16 Uptake by K-562/P-gp Cells To clarify cell cycle-specific VP-16 uptake, we investigated VP-16 uptake by K-562/P-gp cells that were synchronized by a double thymidine block. After release of the block, cells were allowed to progress through the cell cycle and collected at every one or two hours. The degree of synchronization was confirmed by flow cytometry analysis. Cells were in G_1, S and G_2/M phases at 1 h, at 6 h and 10 h after removinig thymidine, respectively (Fig. 7). We previously reported that K-562 cells were in S, G_2/M and G_1 phases at 0–6 h, at 6–8 h from 8 h after thymidine release, respectively.[10] Based on these results, to investigate VP-16 uptake in different phases of the cell cycle. There was no significant difference between each fraction and non-synchronized cells (control) in the rate of VP-16 uptake (Fig. 8).

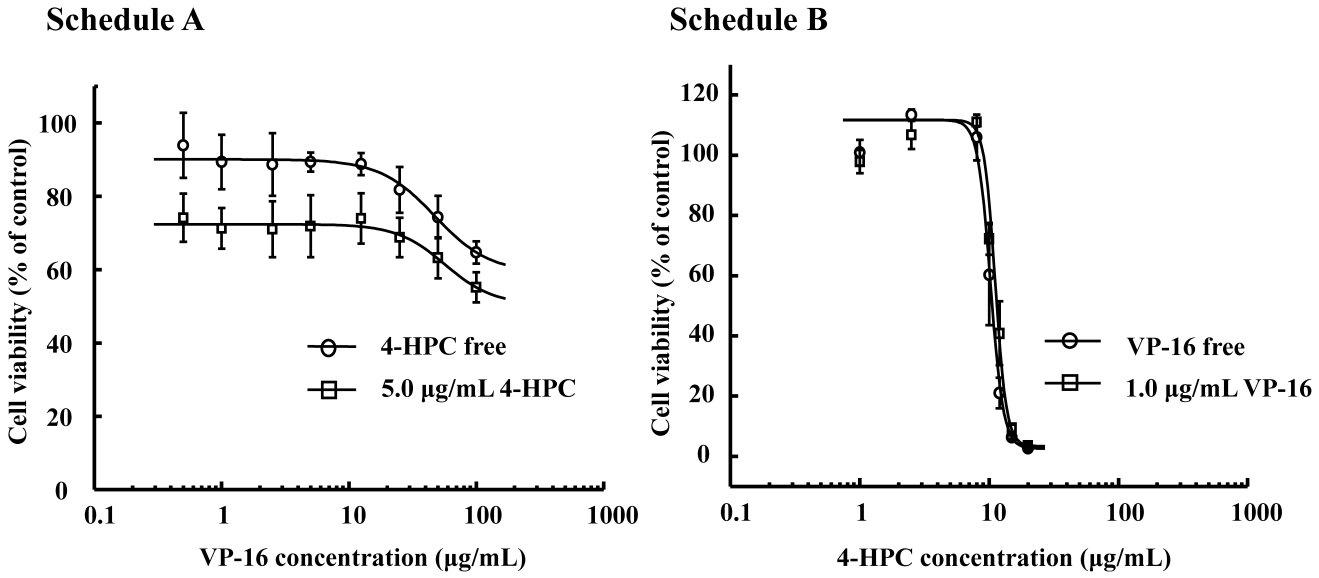


Fig. 5. Effects of Schedule-Dependent Chemotherapy on Viability of K-562/P-gp Cells

Schedule A: Viability of K-562/P-gp cells pretreated with 5.0 μg/mL 4-HPC for 24 h and subsequently exposed to 0.5–100 μg/mL VP-16 compared to that of cells not treated with VP-16. Schedule B: Viability of K-562/P-gp cells pretreated with 1.0 μg/mL VP-16 for 24 h and subsequently exposed to 1–20 μg/mL 4-HPC compared to that of cells not treated with VP-16. Cell viability was assessed by the WST-8 assay. Each point represents the mean±S.D. of 3 measurements.

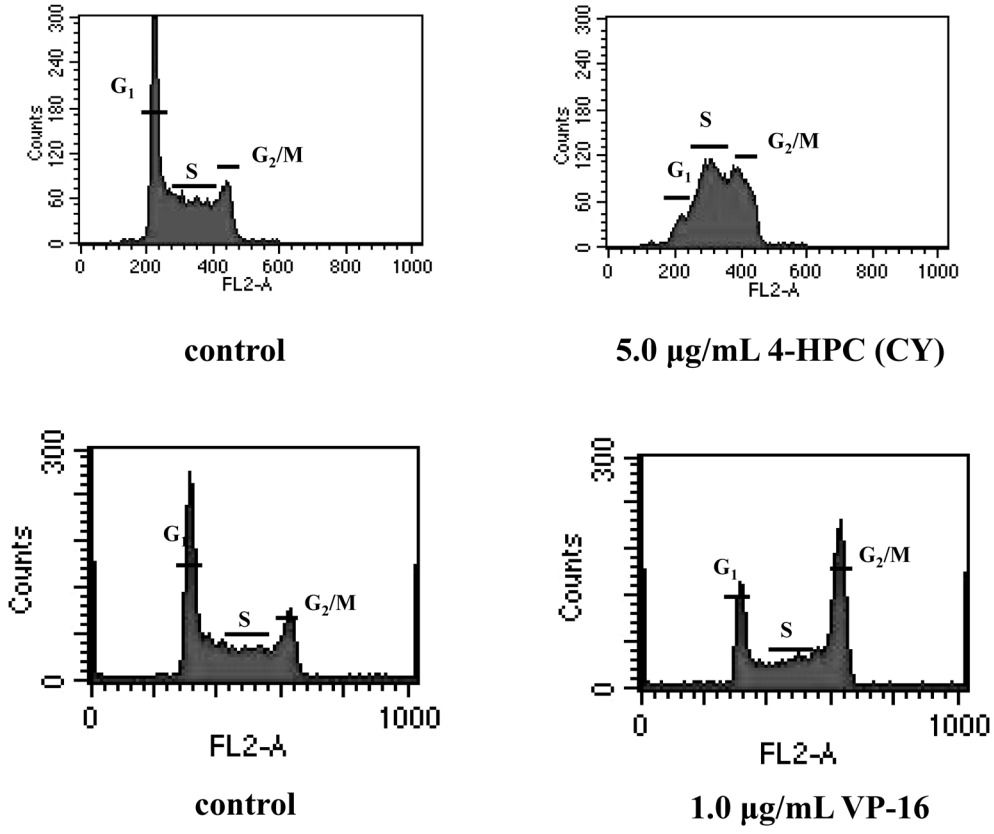


Fig. 6. Cell Cycle Distribution of K-562/P-gp Cells

K-562/P-gp cells were treated with either 1.0 μg/mL VP-16 or 5.0 μg/mL 4-HPC for 24 h. Cell cycle was analyzed by using a FACScan flow cytometer.

DISCUSSION

Some previous clinical studies have suggested that the combination chemotherapy of VP-16 and CY is effective and safe for adult patients with hematological malignancies.[8,9] However, the optimal schedule for drug administration to achieve the best therapeutic efficacy is still uncertain. In clinical chemotherapy, overexpression of P-gp is often an obstacle of treatment. Therefore, we investigated the schedule-dependent cytotoxicity of VP-16 and CY in K-562/P-gp cells.

First, we confirmed that MDR-1 mRNA was expressed in K-562/P-gp cells but not in K-562 cells (data not shown). Up-

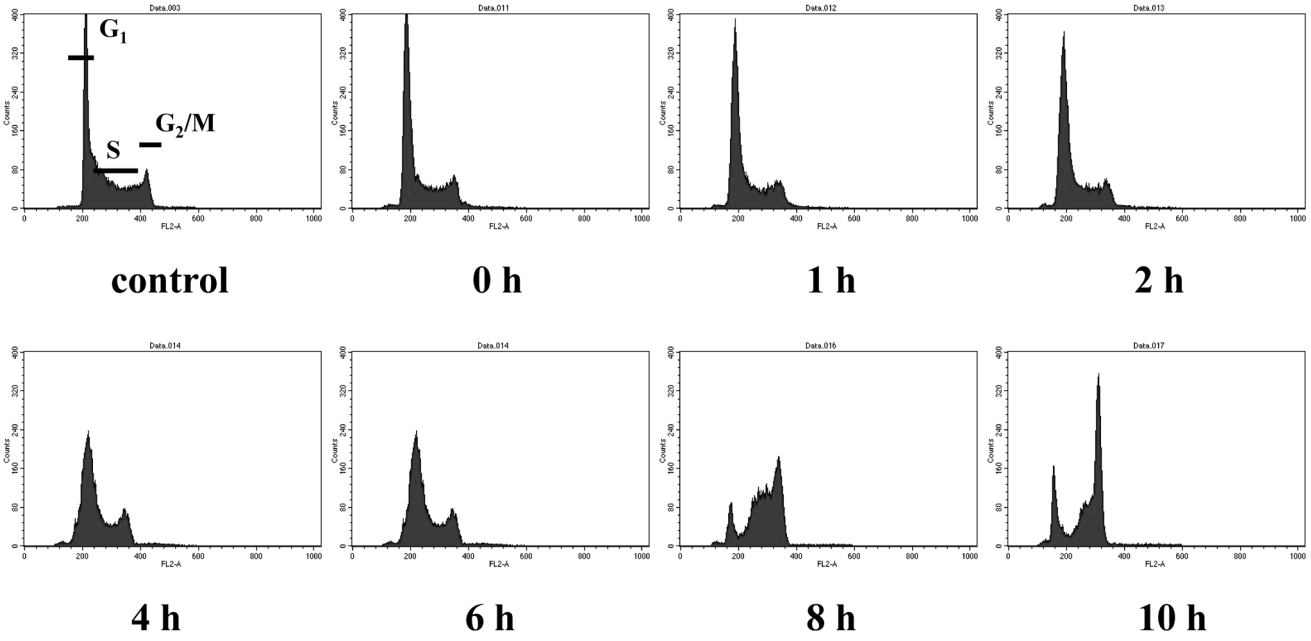


Fig. 7. Cell Cycle at 0, 1, 2, 4, 6, 8 and 10 h after Release from Thymidine Block

The thymidine bock was released by washing K-562/P-gp cells with PBS, followed by incubation in a fresh medium.

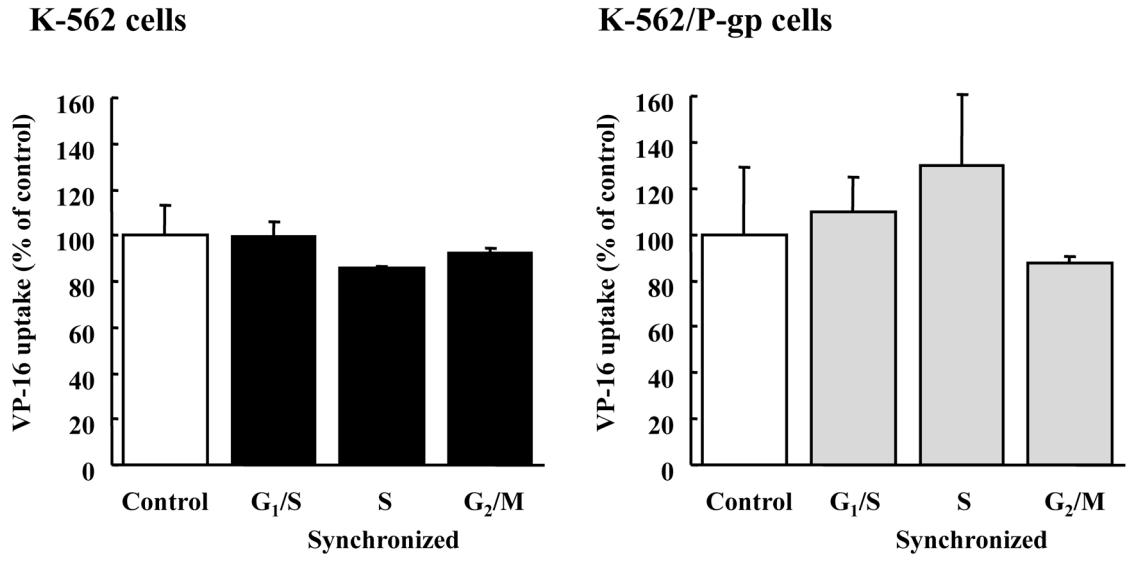


Fig. 8. Cell Cycle Phase Specificity of Uptake

K-562 cells and K-562/P-gp cells were incubated at 37°C for 60 min at pH 7.5 with 25 μM VP-16 in transport buffer. The concentration of VP-16 was determined using an HPLC system. Each column represents the mean±S.D. of 3 measurements.

take of VP-16 by K-562/P-gp cells was significantly decreased compared with that by K-562 cells, and the decrease in uptake was recovered to the control level by the P-gp inhibitor quinidine (Fig. 2). We also investigated the effect of P-gp expression on cytotoxicity of VP-16. The cytotoxicity of VP-16 in K-562/P-gp cells was decreased compared with that in K-562 cells, and the decrease in cytotoxicity was recovered to the control level by quinidine (Fig. 3). These results indicate that K-562/P-gp cells actively transport VP-16 out of cells.

P-gp is highly expressed in many clinically resistant cancer cells. Previous clinical trials have shown that several drugs including verapamil and cyclosporine inhibit P-gp and modulate MDR.[20,21] However, few of these MDR modulators achieve clinical success.[22] Therefore, strategies for overcoming resistance to anticancer drugs are important.

We focused on the schedule of anticancer drugs and investigated the effect of cell cycle arrest on cytotoxicity of VP-16. For combination chemotherapy, we showed that pretreatment with a non-cytotoxic concentration of 4-HPC enhanced the cytotoxicity of VP-16. On the other hand, pretreatment with a non-cytotoxic concentration of VP-16 did not influence the cytotoxicity of 4-HPC (Fig. 5, Table 1). The cell cycle progression of cells treated with 4-HPC was blocked at S phase, which is a specific phase for VP-16 (Fig. 6). Differences in cellular topoisomerase II levels have been reported in relation to different phases of the cell cycle.[23] Cells that are actively synthesizing DNA are more sensitive to topoisomerase II inhibitors.[23] Lorico *et al.* reported that pretreatment of U937

cells with a low concentration of MTX increased the proportion of cells in S phase and the levels of topoisomerase II in cells and subsequently increased DNA breakage and cytotoxicity of VP-16.[5] We also investigated whether the function of P-gp is cell cycle-dependent. K-562 and K-562/P-gp cells were synchronized in G_1, S and G_2/M phases by a double thymidine block (Fig. 7). For each cell cycle, VP-16 uptake was not affected by the cell cycle (Fig. 8). These results suggest that 4-HPC enhances the cytotoxicity of VP-16 due to cell cycle arrest at phase specificites of VP-16. In our study, we demonstrated that pretreatment of K-562/P-gp cells with a non-toxicity concentration of 4-HPC increased the cytotoxicity of VP-16. The enhanced cytotoxicity of VP-16 occurs at a clinically relevant concentration.[24–26]

In conclusion, we demonstrated schedule-dependent cytotoxicity between VP-16 and 4-HPC *in vivo.* The results indicate that pretreatment of cells with 4-HPC enhances cytotoxicity of VP-16 by blocking the cell cycle at S phase, which is a specific phase for VP-16. This schedule may affect the therapeutic outcome. Moreover, cell cycle progression after drug exposure may influence the cytotoxicity of cell cycle dependent anticancer drugs. However, these data are cell line-specific and limited *in vitro.* Further clinical studies are needed to establish the schedule of VP-16 and CY administration.

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