Effects of Vitamin K_3 and K_5 on Daunorubicin-resistant Human T Lymphoblastoid Leukemia Cells

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Abstract. *Background/Aim: Anticancer efficacy of vitamin K derivatives on multidrug-resistant cancer cells has been scarcely investigated. Materials and Methods: The effects of vitamins K_3 and K_5 on proliferation of human leukemia MOLT-4 cells and on daunorubicin-resistant MOLT-4/DNR cells were estimated by a WST assay. Apoptotic cells were detected by Annexin V and propidium iodide staining, followed by flow cytometry. Results: Vitamins K_3 and K_5 significantly inhibited proliferation of leukemic cells at 10 and 100 μM ($p<0.05$), and these effects were almost equally observed in both MOLT-4 and MOLT/DNR drug-resistant cells. Vitamin K_3 induced cell apoptosis at 10 and 100 μM in both MOLT-4 and MOLT-4/DNR cells ($p<0.05$). Vitamin K_5 also increased apoptotic cells, while rather inducing necrotic cell death. Conclusion: Vitamins K_3 and K_5 suppress MOLT-4 and MOLT-4/DNR cell-proliferation partially through induction of apoptosis, and these vitamin derivatives can overcome drug resistance due to P-glycoprotein expression.*

There have been a number of reports concerning anticancer properties of lipophilic vitamins, which include, differentiation-inducing efficacy of tretinoin against clinical promyelocytic leukemia (1), the anti-proliferative and apoptosis-inducing efficacy of vitamin D3 in breast cancer and colorectal cancer (2), and the apoptosis-inducing and chemosensitivity-enhancing effects of vitamin E succinate in bladder cancer cells (3). We also found apoptosis-inducing effects of vitamin K derivatives on human melanoma cells (4). In addition, the antitumor effects of vitamin K_2 (menaquinone), K_3 (menadione), and K_5 (4-amino-2-methyl-1-naphtol) against colorectal cancer (5) and hepatocellular carcinoma (6) models have also been demonstrated. Vitamin K_2 and K_3 were shown to posses apoptosis-inducing effects in human tumorigenic cells (5, 7). Vitamin K_2, K_3, and K_5 exerted antitumor effects on established colorectal cancer in mice by inducing apoptotic death of tumor cells (5).

Thus, while vitamin K derivatives have been known to induce apoptotic cell death in several types of cancer cells, their effects on drug-resistant cancer cells have not been clarified. The success of chemotherapy in cancer treatment is frequently limited by intrinsic or acquired multidrug resistance due to increased expression of a plasma membrane P-glycoprotein (8). This protein is an ATP-dependent transporter that effluxes a number of structurally-unrelated anticancer agents out of cells, thereby reducing intracellular drug concentration, permitting cancer cells to survive against high concentrations of anticancer drugs. We developed multidrug-resistant cell line MOLT-4/DNR from a human T lymphoblastoid leukemia MOLT-4 cell line by exposing parent cells to increasing concentrations of daunorubicin over 3 months (9). This resistant sub-line, MOLT-4/DNR has been revealed to overexpress functional P-glycoprotein and *MDR1* mRNA (9). Moreover, drug resistance in MOLT-4/DNR has been shown to be closely related to the expression of P-glycoprotein and *MDR1* mRNA (9), and therefore, this sub-line will be a suitable model to investigate the agents which overcome drug resistance due to functional P-glycoprotein expression.

The present study was undertaken to evaluate anti-proliferative effects of vitamin K derivatives, K_3 and K_5, on a P-glycoprotein-expressing multidrug-resistant cell line MOLT-4/DNR, and to provide an insight into their mechanisms of action by examining apoptotic cells treated with the vitamin K derivatives.

Materials and Methods

Reagents. RPMI-1640 medium and fetal bovine serum were purchased from Gibco BRL Co. (Grand Island, NY, USA). Daunorubicin, vitamin K_3 and K_5 were obtained from Sigma Chemical Co. (St.Louis, MO, USA). Daunorubicin stock solutions were prepared at a concentration of 10 mmol/L with ethanol and

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diluted to working concentrations before use. Test-compound solutions were made at a concentration of 5 mmol/L with ethanol and diluted to working concentrations before use. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). FITC Annexin V Apoptosis Detection Kit I was obtained from BD Biosciences (Pharmingen, CA, USA). All other reagents were of the best available grade.

Cell culture and cell proliferation assay. MOLT-4 cells were purchased from DS PharmaBiomedical Co., Ltd. (Osaka, Japan). Human breast cancer cell line MCF-7 was obtained from Riken BRC Co. (Ibaraki, Japan). MOLT-4/DNR cells have been developed from MOLT-4 cell line by exposing the parent cells to increasing concentrations stepwise of daunorubicin over 3 months (9). MOLT-4 and MOLT-4/DNR cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/ml streptomycin (9, 10).

The leukemia cells were washed and re-suspended with the above medium to 5×10^5 cells/mL, and 196 μL of this cell suspension were placed in each well of a 96 well flat-bottom plate. Four micro litters of ethanol solution containing each vitamin K derivative or 4 μl of ethanol solution containing daunorubicin were added to yield final concentrations of 0.001, 0.01, 0.1, 1, 10 and 100 μM, respectively. Four micro litters of ethanol were added to the control wells. The cell suspensions were mixed and incubated for 72 h in 5% CO_2 /air at 37˚C.

MCF-7 cells were maintained in MEMα medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (11). The cells at densities of $1\text{-}5\times10^5$ cells/ml suspended with each medium were incubated in 5% CO_2/air at 37˚C for 3-4 days in a humidified chamber. The cells were washed and re-suspended with the medium to 5×10^5 cells/ml, and 196 μL of this cell suspension were placed in each well of a 96 well flat-bottom plate. Four micro litters of ethanol solution containing each vitamin K derivative were added to yield final concentrations of 0.1, 1, 10 and 100 μM, respectively. Four micro litters of ethanol were added to the control wells. The cell suspensions were mixed and incubated for 72 h in 5% CO2 /air at 37o C.

Cell proliferation was analyzed with a WST assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacture's instructions. After culturing, 20 μL of Cell Counting Kit-8 reagent solution was added to each well, and the plate was incubated for another 2 h. Then, the proliferated cells were analyzed by measuring the optical density at 450 nm absorbance (reference, 650 nm).

Apoptosis assays. After 5×10^5 cells/mL of MOLT-4 and MOLT-4/DNR cell suspensions were incubated in the presence of indicated concentrations of vitamin K_3 or K_5 for 72 h in 5%CO_2/air at 37˚C. One ml of this cell suspension was placed in a 1.5-mL tube, and the cells were washed twice in cold phosphate-buffered saline (pH 7.2). Cells were re-suspended with 500 μL of phosphate-buffered saline containing 1% fetal bovine serum, and 5 μL of Annexin V-FITC solution and 2.5 μl of propidium iodide solution (50 μg/mL) were added to the tube. Then, the cells were incubated for 15 min at room temperature in the dark. After the incubation, 400 μl of binding buffer were added to each tube, and the cells were analyzed by flow cytometry (BD FACSCaliburTM, BD Biosciences) within one hour after staining (12). A total of 30,000 non-gated cells were analyzed. Apoptotic cells were detected as Annexin V-positive and propidium iodide-negative cells, while necrotic cells were detected as Annexin V and propidium iodide double-positive cells.

Statistics. Comparison of the data between the two groups was carried out by Student's *t*-test. Comparison of the data in multiple groups was carried-out by the Dunnett test. In each case, *p*-values less than 0.05 were considered significant.

Results

Effects of vitamin K_3 and K_5 on proliferation of MOLT-4 and drug-resistant MOLT-4/DNR cells. Daunorubicin dose-dependently suppressed proliferation of MOLT-4 cells, while the drug was less effective on MOLT-4/DNR cell proliferation (Figure 1A). The difference in the daunorubicin effect on cell proliferation between MOLT-4 cells and MOLT-4/DNR cells was statistically significant at a drug concentration of 1 μM ($p<0.05$). The IC_{50} value of daunorubicin on proliferation of MOLT-4/DNR cells was 15.5-times higher than that of the drug on the proliferation of MOLT-4 cells. Daunorubicin treatment at 0.1 and 10 μM significantly increased the number of apoptotic cells in MOLT-4 cells, compared to the number of apoptotic cells in the drug-resistant MOLT-4/DNR cells ($p<0.05$) (Figure 1B).

Then, cells of the parental MOLT-4 and the drug-resistant MOLT-4/DNR cell lines were continuously treated with 0.1 to 100 μM of vitamin K_3 and K_5 for 72 h, and cell proliferation was assessed by WST assay procedures (Figure 2A and B). Vitamins K_3 and K_5 inhibited the proliferation of these cells at a concentration range of 10-100 μM. In contrast to the effects of daunorubicin, proliferation of MOLT-4 and MOLT-4/DNR cells were suppressed almost equally by the vitamin K derivatives, K_3 and K_5, in a dose-dependent manner. The effects of these vitamins on MOLT-4 and MOLT-4/DNR cell proliferation were not significantly different at any concentrations used in this study. Anti-proliferative effects of vitamins K_3 and K_5 were also examined using human breast cancer cell line MCF-7 (Figure 2C). Both vitamin K derivatives inhibited MCF-7 cell proliferation dose-dependently, whereas the effects on this cell line were somewhat weaker compared to the effects on the leukemia cell lines.

Then, the additive effects of vitamin K_3 combined with daunorubicin were examined using MOLT-4 and MOLT-4/DNR cells (Figure 3). The daunorubicin dose-response curves to suppress proliferation of these cells appeared to be unchanged in the absence or presence of 0.1 and 1 μM vitamin K_3. As described above, MOLT-4/DNR cells, compared to parental MOLT-4 cells, showed resistance to the suppressive effects of daunorubicin (Figure 3B). The daunorubicin IC_{50} values on MOLT-4/DNR cell proliferation were more than ten-times higher than those on the parent MOLT-4 cell proliferation, even in the presence of 0.1 and 1 μM vitamin K_3. However, the dose-response curves of daunorubicin on the proliferation of MOLT-4 (Figure 3A) and MOLT-4/DNR cells (Figure 3B) were almost the same in the presence of 10 μM vitamin K_3. The IC_{50} values of daunorubicin in the presence

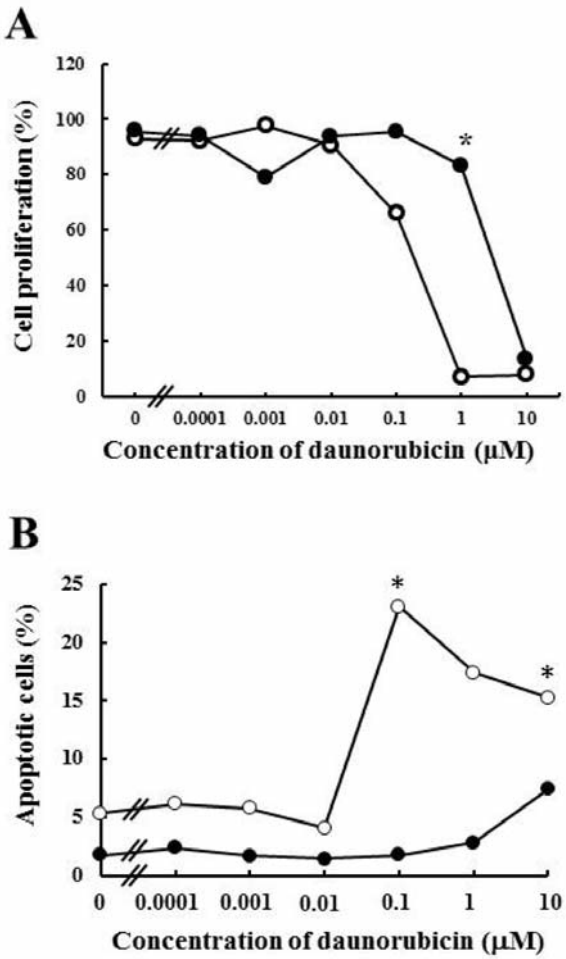

Figure 1. *Different daunorubicin responses in cells of the parent MOLT-4 and the drug-resistant MOLT-4/DNR cell lines. A: Cells of the parent MOLT-4 (open circle) and the drug-resistant MOLT-4/DNR (closed circle) cell lines were cultured in presence of serial concentrations of daunorubicin for 72 h, and cell proliferation was analyzed by WST assay procedures. B: MOLT-4 (open circle) and MOLT-4/DNR (closed circle) cells were treated with serial concentrations of daunorubicin, stained with Annexin V and propidium iodide, and analyzed with flow cytometry. Apoptotic cells were detected as Annexin V-positive and propidium iodide-negative cells (see also Figure 4A-D). Experiments were carried-out three times, and the differences in the percentages of apoptotic cells between the two cells lines were analyzed by unpaired t-tests. *p<0.05 between the two cell lines.*

of 10 μM vitamin K_3 on the proliferation of MOLT-4 and MOLT-4/DNR cells were less than 10 μM.

Apoptosis induction in MOLT-4 and drug-resistant MOLT-4/DNR cells by vitamin K_3 and K_5 treatment. Parental MOLT-4 and the drug-resistant MOLT-4/DNR cell lines were cultured in presence of vitamin K_3 or K_5 at concentrations of 0.1, 1.0, 10, and 100 μM for 4 8h, stained with Annexin V and

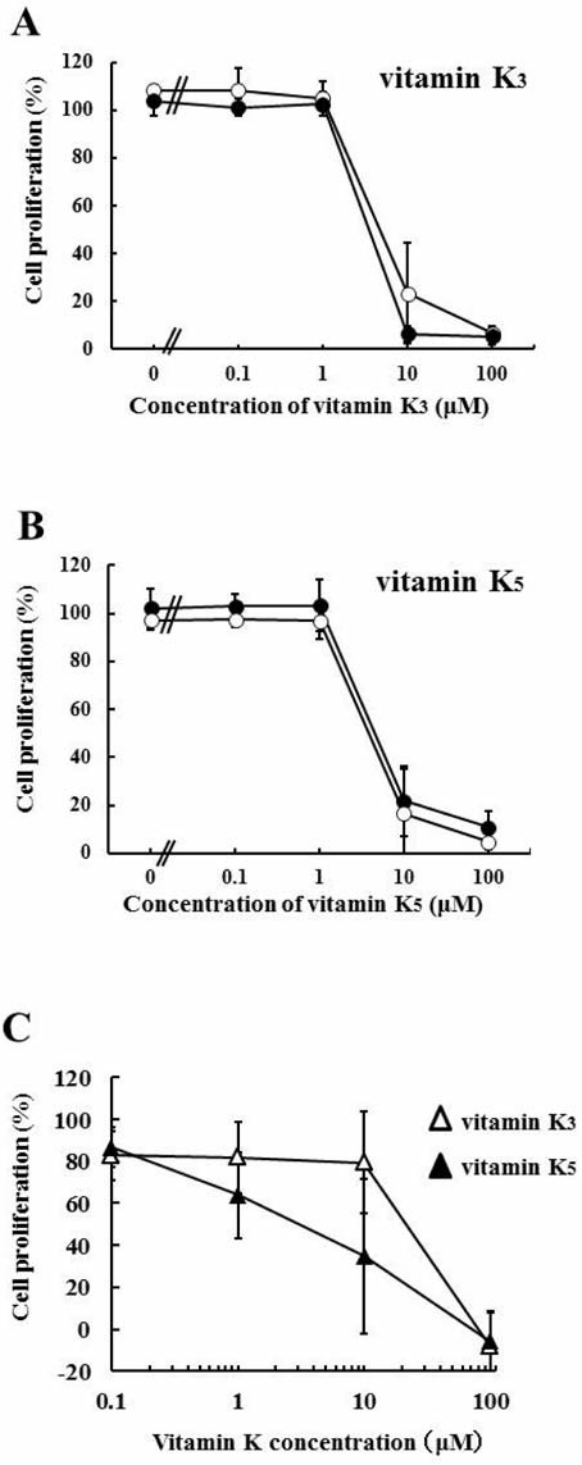

Figure 2. *Effects of vitamins K_3 and K_5 on in vitro proliferation of MOLT-4 cells, MOLT-4/DNR cells, and MCF-7 cells. Cells were treated with serial concentrations of vitamin K derivatives for 72 h, and cell proliferation was estimated by WST assay. A, B: Effects of vitamin K_3 (A) and K_5 (B) on MOLT-4 (open circle) and MOLT-4/DNR (closed circle) cells. No statistically significant differences were observed in the vitamin K effects between MOLT-4 and MOLT-4/DNR cells. C: Effects of vitamin K_3 (open triangle) and K_5 (closed triangle) on MCF-7 cells.*

propidium iodide, and the percentages of apoptotic cells in an Annexin V-positive and PI-negative area were estimated (Figure 4; lower right area in each right figure). Typical dot plot data of apoptotic cells treated by vitamin K derivatives and analyzed with flow cytometry are shown in Figure 4A-D. Number of apoptotic cells increased in both MOLT-4 and MOLT-4/DNR cells after the treatment by vitamin K_3, especially in cells treated by 10 and 100 μM of vitamin K_3. The mean percentages of apoptotic cells in MOLT-4 and MOLT-4/DNR cells treated with 0.1-100 μM vitamin K_3 or K_5 are also shown in Figures 4E and F. These vitamin K derivatives increased the number of apoptotic cells in both MOLT-4 and MOLT-4/DNR cells, and the effects of vitamin K_3 at 10 and 100 μM were statistically significant, compared to control ($p<0.001$) (Figure 4E). The effect of vitamin K_3 at 1 μM on MOLT-4 cells was also statistically significant ($p<0.05$) (Figure 4E). When compared to the effects of vitamin K_3, vitamin K_5 by 10 and 100 μM tended to cause necrotic cells in both MOLT-4 and MOLT-4/DNR cells, as can be seen in the right upper area (Annexin V and propidium iodide double-positive cells) of each right-side Figure. Differences in inducing apoptotic cells and necrotic cells between vitamin K_3 or K_5 can be also seen in Figure 4E and F.

Discussion

The data described above show that vitamins K_3 and K_5 inhibit growth of both T lymphoblastoid leukemia MOLT-4 cells and P-glycoprotein-expressing daunorubicin-resistant MOLT-4/DNR cells almost equally. Vitamins K_3 and K_5 also inhibited proliferation of cells of a breast cancer cell line MCF-7. However, the vitamin K effects on this cell line were somewhat weaker, when compared to the effects of vitamin K derivatives on T lymphoblastoid leukemia cells. The anti-proliferative effects of vitamin K_3 and K_5 on leukemic cells were apparent at a concentration range of 10-100 μM, that was almost the same as the concentration range of these vitamin K derivatives exhibiting apoptosis induction. These observations suggest that the anti-proliferative effects of the vitamin K derivatives are partially due to apoptotic-cell induction in these cell lines. The apoptosis-inducing ability of vitamin K_3 was relatively stronger than that of vitamin K_5. Whereas, vitamin K_5 suggested to cause necrotic dell death, as can be seen in Figure 4E and F.

In the present study, we revealed that the IC_{50} value of daunorubicin on the proliferation of MOLT-4/DNR cells was approximately 15.5-times higher than that of the drug on the proliferation of MOLT-4 cells. MOLT-4/DNR cell line was shown to be persistently resistant to the anti-proliferative effect of daunorubicin by expressing functional P-glycoprotein (9). Multidrug resistance is recognized as one of the most common causes for failure of chemotherapy in treating cancer patients (8). P-glycoprotein is an ABC transporter, which hydrolyses ATP and extrudes cytotoxic drugs from mammalian cells. Herein, we found that both parental MOLT-4 and daunorubicin-resistant MOLT-4/DNR cells were sensitive to the suppressive effects of the vitamin K derivatives, and the effects were suggested to be additive in combination with daunorubicin. Morerover, the vitamin K derivatives especially vitamin K_3 induced apoptosis in both MOLT-4 and MOLT-4/DNR cells at concentrations higher than 1 μM. Thus, current data suggest that vitamin K_3 or K_5 are not excluded from the drug-resistant leukemia cells by P-glycoprotein, and are possible candidates for overcoming multidrug resistance in leukemia cells expressing functional P-glycoprotein.

We have reported in our previous study that vitamin K_3 and K_5 suppress *in vitro* growth of human melanoma A375 cells at concentrations lower than 10 μmol/L, which are suggested to results from apoptosis-induction in the melanoma cells (4). We also found that vitamins K_3 and K_5 diminished T cell immunity by inhibiting the proliferative response and inducing apoptosis in mitogen-activated human peripheral-blood lymphocytes (13). The antitumor effects of vitamin K_2 (menatetrenone), K_3, and K_5 against colorectal cancer (2) and hepatocellular carcinoma (6) models have been reported. Moreover, vitamin K_2 and K_3 have been shown to have apoptosis-inducing effects in human tumorigenic cells (5, 7). Vitamin K_2 was reported to induce apoptosis in a cell line established from a patient with myelodysplastic syndrome in blastic transformation (14), while vitamins K_2, K_3, and K_5 exerted anti-tumor effects on established colorectal cancer in mice by inducing apoptotic death of tumor cells (5). Shah *et al*. reported that ubiquitin ligase inhibition by vitamin K_2 attenuates hypoxia and Ras/mitogen-activated protein kinase (MAPK) signaling, which results in blockade of melanoma tumorigenesis (15). On the other hand, Chowdhury *et al*. suggested that vitamin K_2 enhanced arsenic-induced apoptosis in melanoma cells through generation of reactive oxygen species, p38 signaling, and activation of p53 (16). Thus, blockade of these signaling cascades in combination is suggested to be part of the underlying mechanisms of anti-tumor effects of vitamin K derivatives.

The present study, in conclusion, showed that vitamin K_3 and K_5 suppress cell proliferation of both MOLT-4 and MOLT-4/DNR cells by inducing apoptosis, and that these vitamin derivatives can overcome drug resistance due to functional P-glycoprotein expression.

Conflicts of Interest

The Authors declare that there exist no conflicts of interest with regard to this study.

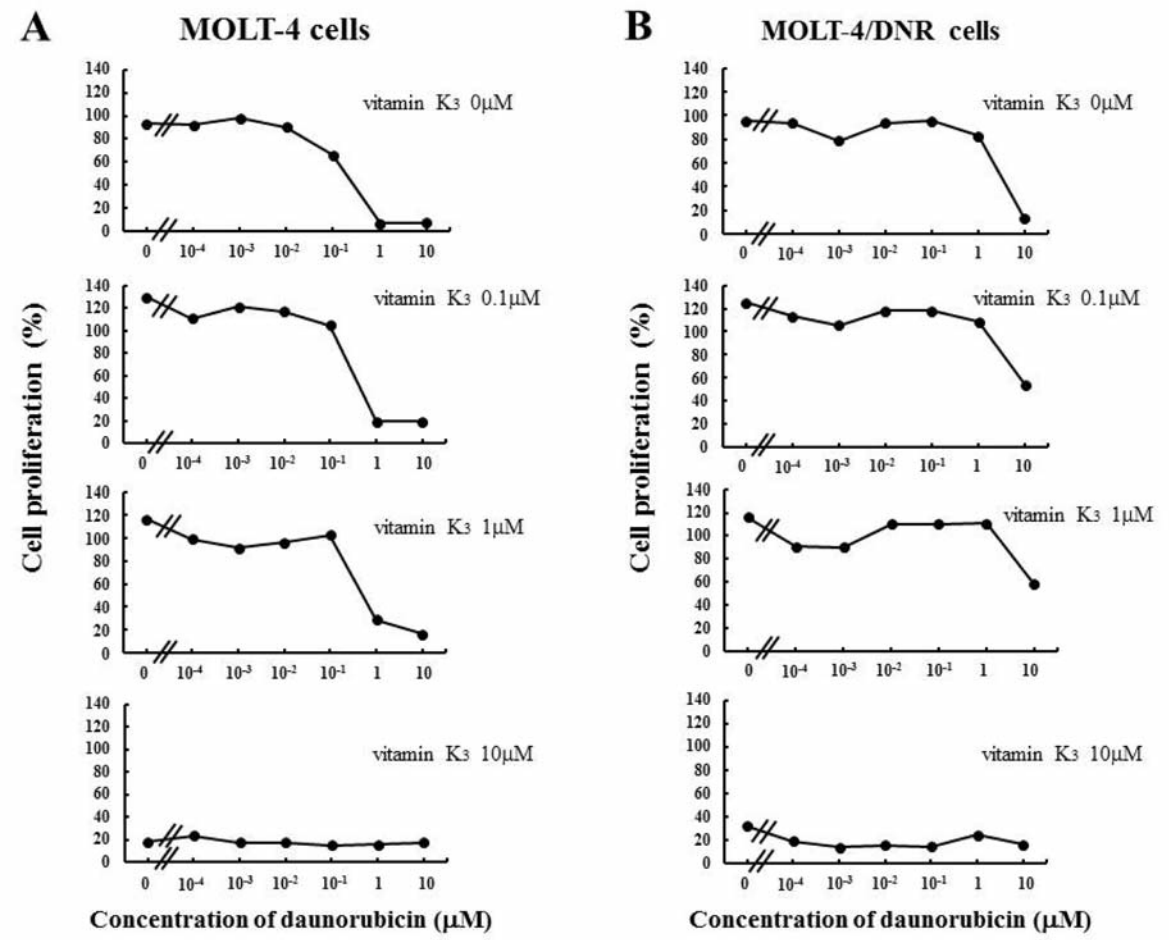

Figure 3. *Dose-response curves of daunorubicin in combination with serial concentrations of vitamin K_3 against the proliferation of MOLT-4 (A) and MOLT-4/DNR (B) cells. In each case, cells were treated with serial concentrations of daunorubicin in the absence (0 μM) or presence of 0.1-10 μM vitamin K_3 for 72 h, and cell proliferation was estimated by WST assay procedures.*

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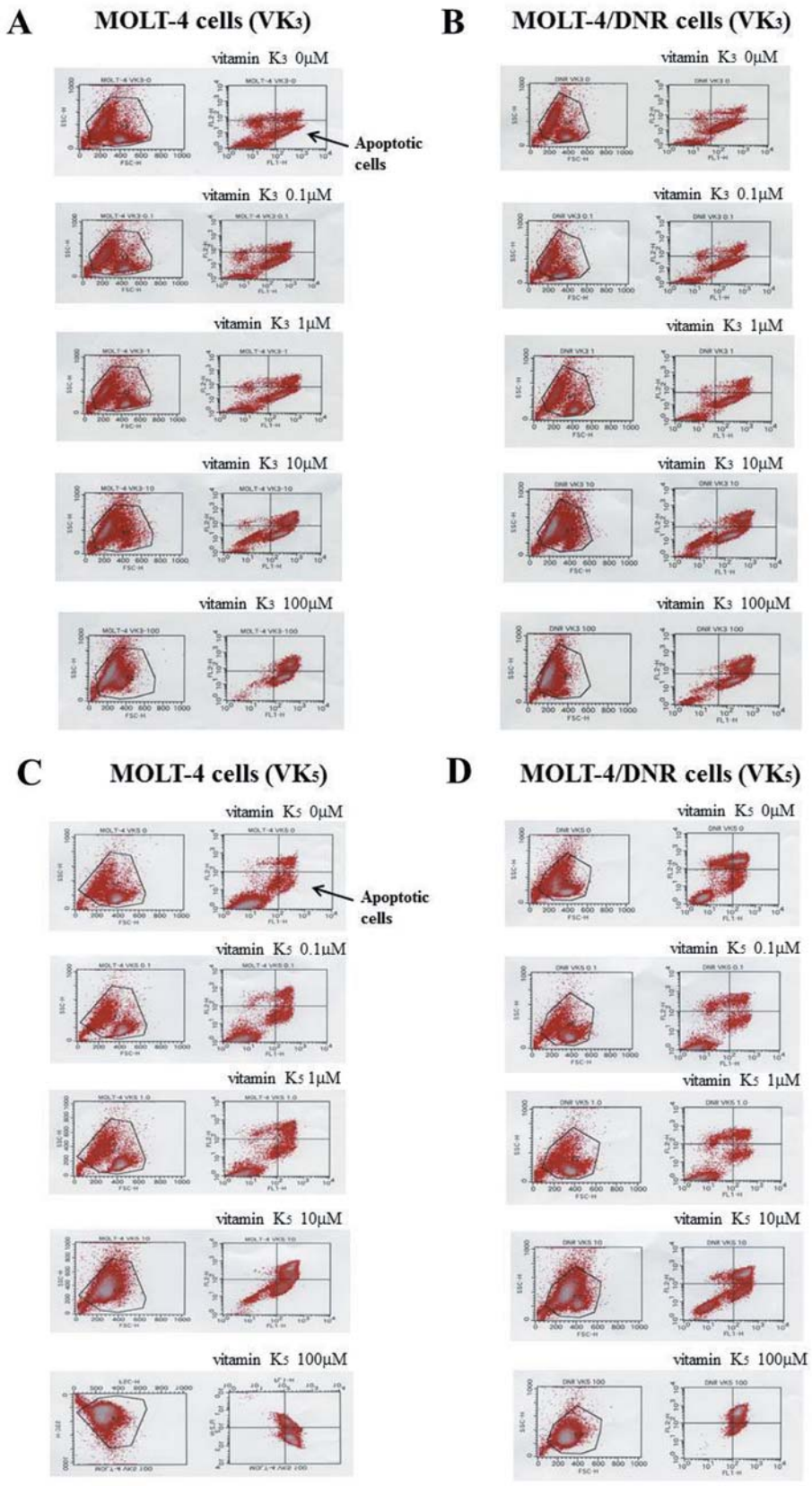

Figure 4. *Continued*.

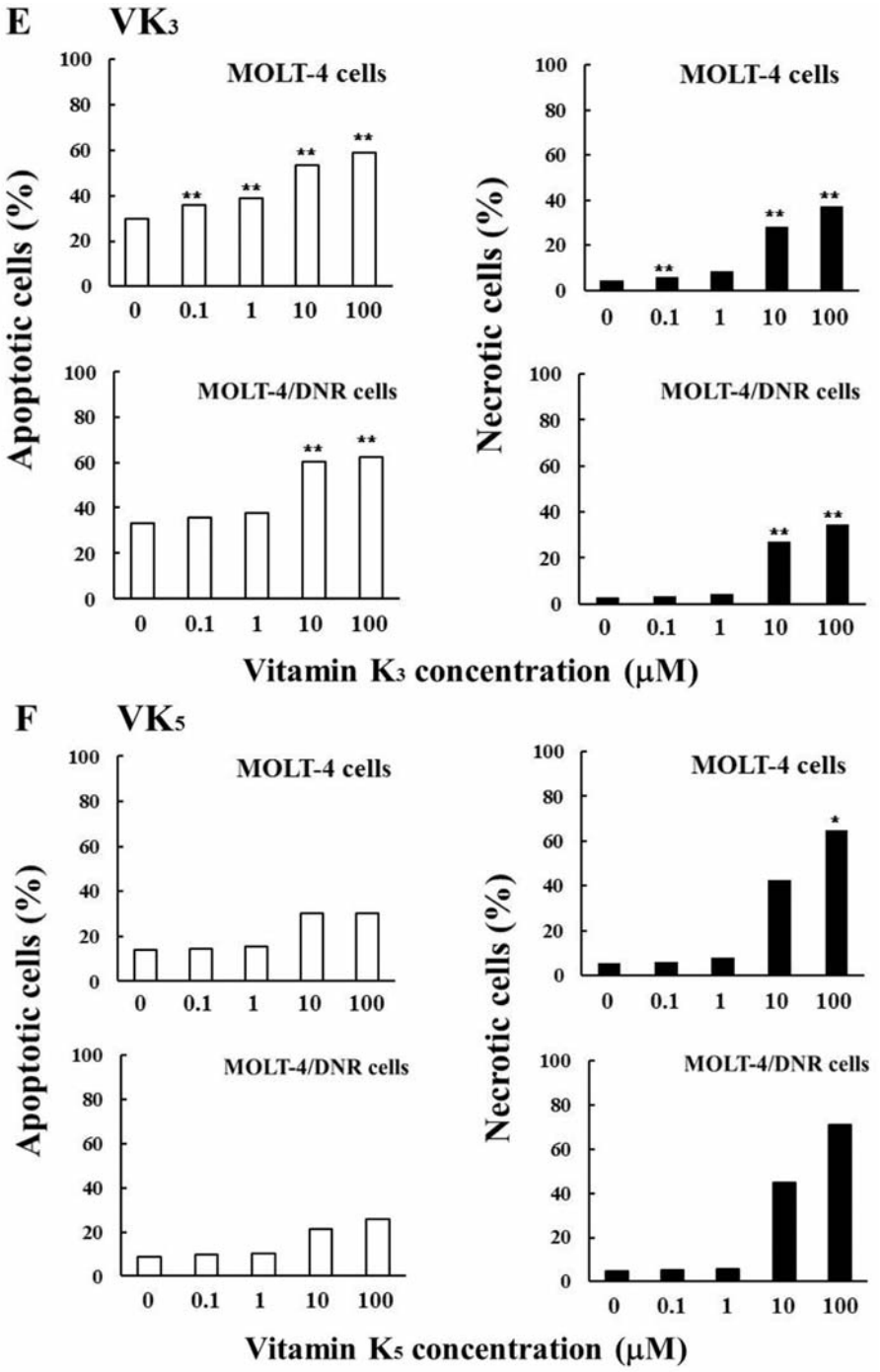

Figure 4. *Apoptotic cell induction by vitamin K derivatives in MOLT-4 and MOLT-4/DNR cells. Cells were treated with serial concentrations of vitamin K derivatives for 48 h, stained with Annexin V and propidium iodide, and analyzed with flow cytometry. Apoptotic cells were detected as Annexin V-positive and propidium iodide-negative cells, as shown in the lower-right area in each right-side figure (arrow in Figure A and B). A-D: Dot-plot analysis of apoptotic. The total cells shown in the left in each figure were gated as indicated by the surrounded area, and the cells in this area were analyzed as Annexin V-positive and propidium iodide-negative (apoptotic) cells (right-side in each Figure). Typical dot-plots were shown in each case. A and B: Effects of vitamin K_3 on MOLT-4 cells (left columns) and MOLT-4/DNR cells (right columns). C and D: Effects of vitamin K_5 on MOLT-4 cells (left columns) and MOLT-4/DNR cells (right columns). E and F: Mean percentages of apoptotic cells (left columns; open bars) and necrotic cells (right columns; closed bars) in MOLT-4 and MOLT-4/DNR cells after treatment by vitamin K_3 (E) and vitamin K_5 (F). The percentages of necrotic cells were calculated from the upper right area (Annexin V and propidium iodide double positive cells) in the dot plot data (insets A-D). Experiments were carried out for 5 times independently, and the differences in the percentages of apoptotic cells or necrotic cells treated by vitamin K derivatives, as compared to control (0 μM), were analyzed by Dunnett test. *$p<0.05$, **$p<0.01$.*

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