Cytotoxic Activity of Maytanprine Isolated from *Maytenus diversifolia* in Human Leukemia K562 Cells

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We examined the cytotoxic effect of maytanprine isolated from the methanol extract of *Maytenus diversifolia* on human leukemia K562 cells using a flow cytometer and compared its cytotoxicity with that of maytansine, a potent cytotoxic maytansinoid. Maytanprine at concentrations of 0.03 nM or more (up to 1 nM) attenuated cell growth with decreasing cell viability and increased the population of shrunken cells in a concentration-dependent manner. Complete inhibition of growth by maytanprine was observed at concentrations of 0.3 nM or more. The compound at 0.03 nM markedly decreased the population at G_0G_1 phase in the cell cycle, but only slightly decreased that in the G_2M phase, suggesting the possibility that it inhibits or delays cell division, and increased the population of cells with hypodiploidal DNA (apoptotic cells). The potency of maytanprine in inhibiting cell growth was greater than that of maytansine, although the inhibitory action of maytanprine was similar to that of maytansine. The results suggest that maytanprine exerts a potent inhibitory action on the growth of human leukemia K562 cells. *M. diversifolia* is one natural source of maytanprine, which is more cytotoxic than maytansine.

Key words cytotoxicity; human leukemia K562 cell; maytanprine; *Maytenus diversifolia*

Some compounds, such as camptothecin and vincristine, isolated from certain plants possess elegant structures and unique mechanisms of anticancer action.[1—3] Therefore random screening for compounds leading to candidates for anticancer agents is continually performed despite recent progress in molecular and cellular research on cancer therapy. In bioassay-guided fractionation using a flow-cytometric technique,[4,5] we succeeded in isolating maytanprine and maytansine from the whole part of *Maytenus diversifolia* in this study. Maytansine, first isolated from *Maytenus ovatus* and studied by Kupchan and coworkers,[6] is a well-investigated maytansinoid possessing very potent inhibitory action against the growth of cancer cells.[7] It acts as a mitotic inhibitor by interfering with the formation of microtubules in the cell nucleus.[8] However, the potency of maytansine in inhibiting the cell growth appears to be less than that of maytanprine.[7] The mechanism by which maytanprine inhibits cell growth is not well elucidated in spite of its extremely potent action. In the present study using a flow cytometer with fluorescent probes, the effect of maytanprine on human leukemia K562 cells was compared with that of maytansine.

MATERIALS AND METHODS

Cell Preparation Human leukemia K562 cells were cultured in 24-well Falcon tissue culture plates placed in a CO_2 incubator (Sanyo, Tokyo) at a temperature of 37 °C. Each well contained 2 ml of RPMI 1640 medium with glutamine (300 mg/l) and 10% fetal bovine serum (Sigma Chemical, St. Louis, MO, U.S.A.).

Analysis of Cytotoxicity Cytograms (forward-scatter intensity *versus* side-scatter intensity) were obtained from a programmed number (2000 or 2500 cells) of K562 cells with a flow cytometer (Cyto-ACE150, JASCO, Tokyo). Cell viability was estimated using the propidium iodide (Molecular Probes Inc., Eugene, OR, U.S.A.) staining method. Since propidium iodide, a dye highly impermeable to intact plasma membranes, is unable to stain living cells, K562 cells showing propidium fluorescence are dead or have compromised membranes. Propidium fluorescence was measured 2 min after adding propidium iodide to the medium to achieve a final concentration of 5 μM. The excitation wavelength for propidium was 488 nm. Emission was monitored at wavelength of 600±20 nm. Cell viability in this study indicates the percentage population of cells not stained with propidium to total cells. The number of cells counted during a programmed time (30 s) with a flow cytometer was correlated with those estimated by an erythrocytometer with a coefficient of 0.98.[4] Thus a flow cytometer with propidium iodide simultaneously estimated the effects of the drugs on the growth and viability of cells in the medium.[4,5]

To determine the distribution of DNA content in K562 cells, DNA was stained with propidium iodide. DNA staining solution contained 0.1% Triton X-100 (Sigma Chemical), EDTA 3 mM (Katayama Chemical, Osaka, Japan), RNase A 0.05 mg/ml (Sigma Chemical) and propidium iodide 100 μM in phosphate buffer solution.[9] The cells were suspended in DNA staining solution for at least 1 h at room temperature. Propidium fluorescence was measured in the cells using a flow cytometer. The excitation wavelength for propidium was 488 nm, and the emission was detected at 600±20 nm.

Data Presentation and Statistics Numerical values of experimental data are presented as the mean±standard deviation (S.D.) in this study. Statistical analysis was performed using Student's paired *t*-test and/or an overall test of significance using an *F*-ratio derived from one-way analysis of variance (ANOVA). A p value of <0.05 was considered significant.

RESULTS

Isolation of Maytanprine The whole part of *M. diversi-*

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folia (900 g), collected on Iriomote Island, Okinawa, Japan, were immersed in methanol for 7 d at room temperature and 104.8 g of methanol extract was obtained. Thereafter the hexane-, ethyl acetate-, butanol-, and water-soluble franctions of methanol extract, prepared by a method similar to that in a previous paper,[10] were evaporated to yield the respective contents (Fig. 1). The effects of the contents at a concentration of 10 μg/ml were tested on the growth of K562 cells to determine whether the extracts contained active substances. As shown in assay 1 in Fig. 1B, the ethyl acetate fraction exerted more cytotoxic action than other fractions. Therefore 20.2 g of the ethyl acetate fraction was subjected to open-column chromatography (Cosmosil 140 C18-OPN), which was eluted with 20% stepwise increases of methanol in water (10—90% per volume, as shown in fr. 2—fr. 6 in Fig. 1). As shown in assay 2 in Fig. 1, the eluate obtained after the elution with 70% methanol (Fr. 5) exerted the most cytotoxic action on the growth of K562 cells when the concentration was 10 μg/ml. The eluate was evaporated to yield the content (1.2 g), and then it was subjected to column chromatography (Merck Silica Gel 60) with 30% methanol–$CHCl_3$. Five fractions (fr. 5-1—fr. 5-5 in Fig. 1) obtained from fr. 5 were tested to determine which fraction exerted the cytotoxic action on K562 cells. Fr. 5-3 10 μg/ml completely inhibited the growth of K562 cells (assay 3 in Fig. 1). The content yielded from fr. 5-3 weighed 145.7 mg. Fr. 5-3 100 mg was subjected to HPLC (column: Develosil ODS-10/20) with 65% methanol in water (Fig. 2). Compounds obtained from two larger peaks, peak 1 (3.8 mg) and peak 2 (5.3 mg) as respectively indicated with asterisks (*) and (**) in Fig. 2, were subjected to isolation. Purification of the compounds was performed with ethyl acetate on silica gel TLC, affording maytansine (0.4 mg) and maytanprine (0.7 mg). Structural identification of maytanprine and maytansine were based on ^{1}H-NMR and FAB-MS. The chemical structures of these compounds are shown in Fig. 2. The purities of maytanprine and maytansine were estimated to be higher than 95% using NMR. As shown in Fig. 1, the hexane extract also exerted cytotoxic activity. However, in present study, we did not examine the active component(s) in the extract.

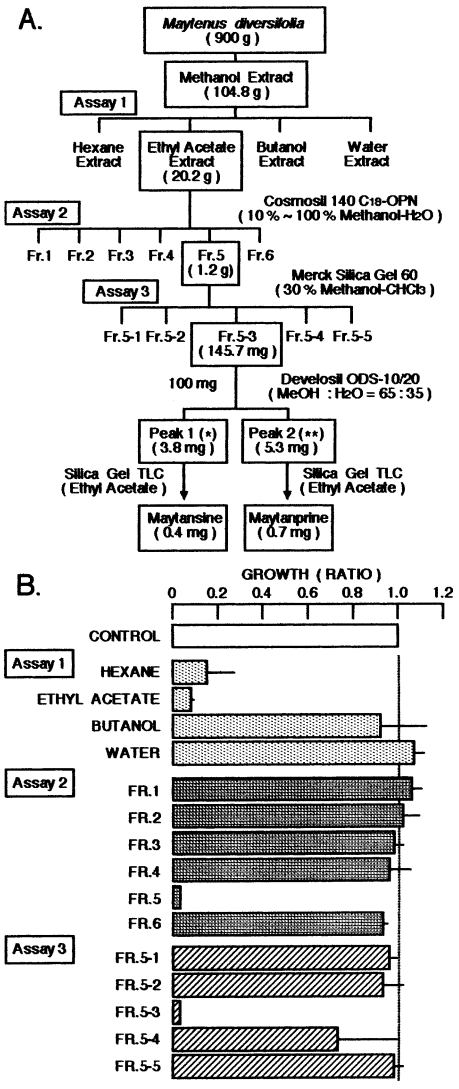

Fig. 1. Isolation of Cytotoxic Substances

(A) Procedure to isolate cytotoxic substances from the methanol extract of bulk *M. diversifolia*. (B) Cytotoxic assays of fractions of the ethyl acetate fraction in the methanol extract of bulk *M. diversifolia*. Column and bar represent mean and standard deviation of 4 experiments. The concentrations tested were 10 μg/ml for assay 1 and assay 2 and 1 μg/ml for assay 3.

Effect of Maytanprine on Cell Growth and Viability K562 cells became confluent for up to 72 h under control conditions. After 72 h of incubation of K562 cells in the presence and absence of maytanprine, the number of cells counted during a programmed time (30 s) with a flow cytometer under the control conditions was 3199, while it was 598 in the presence of maytanprine 0.03 nM (Fig. 3). These results indicate that the incubation of K562 cells with maytanprine 0.03 nM for 72 h markedtly inhibited the cell growth. The concentration–response relation for the maytanprine-in-

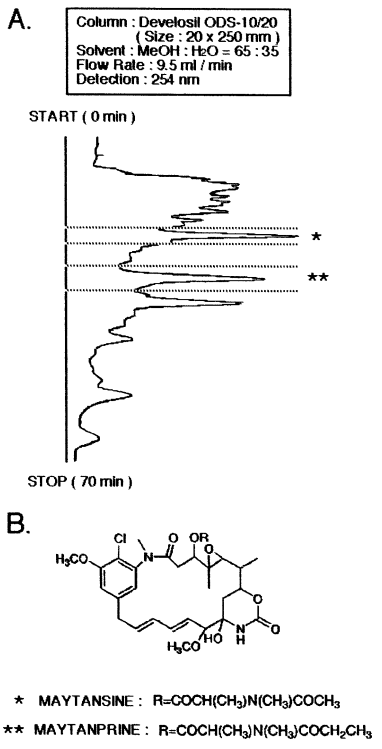

Fig. 2. Isolation of Maytanprine

(A) Isolation of maytanprine and maytansine from the most cytotoxic fraction (fr. 5-3) of the methanol extract from the whole part of *M. diversifolia*. (B) Chemical structure of maytanprine and maytansine.

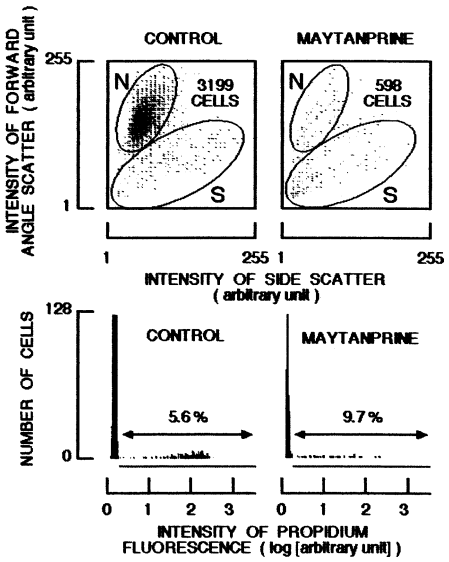

Fig. 3. Effects of Maytanprine on Cytogram and Histogram of Propidium Iodide Fluorescence of K562 Cells

Upper panel, Effects of maytanprine on cytogram obtained from human leukemia K562 cells. Cytograms were obtained from cells incubated with or without maytanprine (0.03 nM) for 72 h. The numbers in the cytograms, counted with a flow cytometer during a programmed time (30 s), are closely correlated with the cell density in culture medium. Lower panels, Effects of maytanprine on the histogram of propidium fluorescence obtained from K562 cells. Dotted line under the histogram indicates cells exhibiting propidium iodide fluorescence. Cells stained with propidium iodide were dead cells or cells with compromised membranes. Percentages indicate the percentage of the population of cells stained with propidium iodide.

duced inhibition of cell growth (Fig. 4) indicates that maytanprine exerts an all-or-none, rather than graded, inhibitory action against cell growth. Therefore the concentration of maytanprine to produce 50% inhibition of cell growth was not estimated. Complete inhibition of cell growth by maytanprine was observed when the concentration was 0.1 nM or more.

The number of cells measured with a flow cytometer consisted of living and dead cells.[4] As shown in Fig. 3, the percentage population of cells stained with propidium iodide (dead cells) was 5.6% under control conditions, while the 72 h incubation with maytanprine 0.03 nM increased that of dead cells to 9.7%. The results show that maytanprine 0.03 nM markedly inhibited cell growth while slightly decreasing viability. The concentration–response relation for the maytanprine-induced increase in the population of dead cells is shown in Fig. 4. Maytanprine at concentrations ranging from 0.03 nM to 1 nM increased the population of dead cells in a concentration-dependent manner.

It has been reported that maytansine, the most well-known maytansinoid, exerts potent inhibitory action against cell growth.[7] Therefore the effects of maytansine on the growth and viability of K562 cells were compared with those of maytanprine. K562 cells were incubated with maytansine at concentrations ranging from 0.01 nM and 1 nM for 72 h. Maytansine at 0.1 nM or greater markedly inhibited cell growth while slightly increasing the population of dead cells (Fig. 4). These results indicate that the potency of maytansine against K562 cells is less than that of maytanprine.

Effect of Maytanprine on Cell Population As shown in Fig. 3, there were two types of cells, normal-sized cells (gate N) and shrunken cells (gate S), in the cytogram.[9—12] The

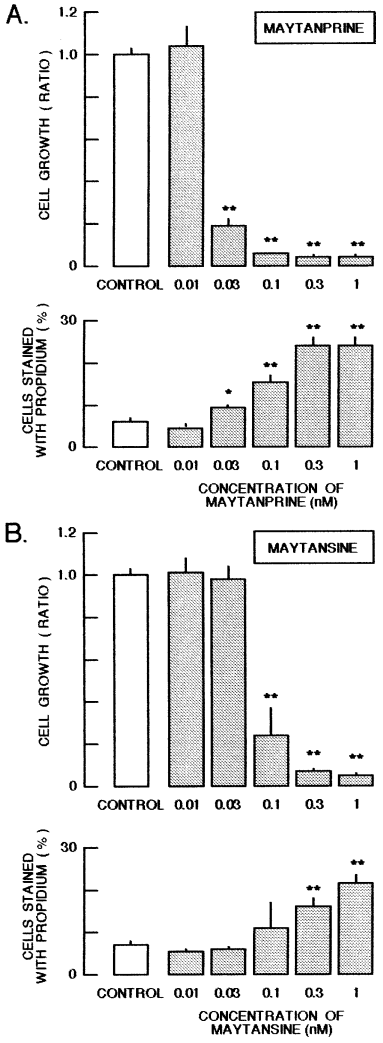

Fig. 4. Dose-Dependent Effects of Maytanprine (A) and Maytansine (B) on the Growth and Fatality of K562 Cells

Respective upper panels, Dose-dependent inhibition of growth of K562 cells by maytanprine and maytansine 72 h after the start of drug application. Respective lower panels, Dose-dependent effects of maytanprine and maytansine on the fatality of K562 cells 72 h after the start of drug application. The columns and bars indicate the mean value and S.D., respectively, of 4—11 experiments. Asterisks indicate a significant difference (* $p<0.01$, ** $p<0.005$) between the control group and test group.

shrunken cells are supposed to be dead cells and/or the cells with hypodiploidal DNA (apoptotic cells).[13—15] The percentage population of shrunken cells was concentration-dependently increased by treatment with maytanprine 0.03 nM or greater and by 0.1 nM or more in the case of maytansine (Fig. 5). Result suggests that both maytanprine and maytansine induce apoptosis in some cells of K562 cells.

To determine the possible mechanism of the inhibitory action of maytanprine on the growth of K562 cells, the effect of maytanprine 0.03 nM on the cell cycle of K562 cells was examined. The cells were incubated with maytanprine 0.03 nM for 72 h before examination. Under control conditions, the amplitude of the G_0G_1 peak as indicated by an asterisk (*) in Fig. 6 was much higher than that of the G_2M peak (**), while this was not the case in the presence of maytanprine 0.03 nM. Maytanprine markedly decreased the amplitude of the G_0G_1 peak, while that of the G_2M peak was slightly decreased (Fig. 6). Furthermore, the population of cells with hypodiploidal

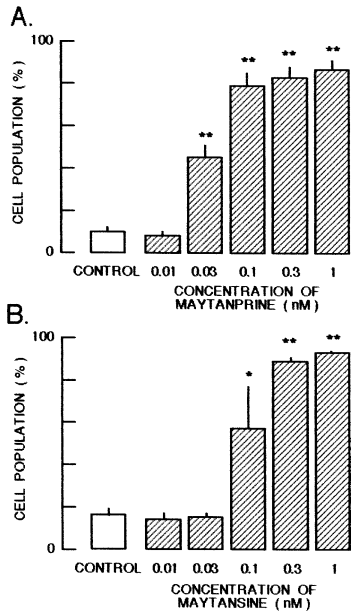

Fig. 5. Dose-Dependent Increase in Percentage Population of Shrunken Cells by Maytanprine (A) and Maytansine (B) 72 h after the Start of Drug Application

The columns and bars indicate the mean value and S.D., respectively, of 4—10 experiments. Asterisks indicate a significant difference ($*p<0.01$, $**p<0.005$) between the control group and test group.

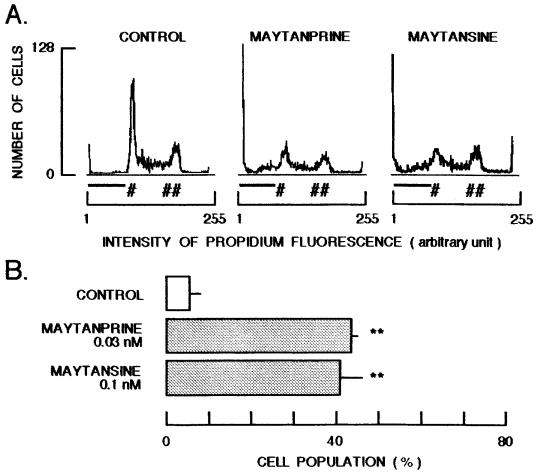

Fig. 6. Effect of Maytanprine on the Distribution of DNA Content (Cell Cycle) of K562 Cell

(A) Effects of maytanprine 0.03 nM and maytansine 0.1 nM on histogram of propidium iodide fluorescence. The cell cycle was analyzed in a programmed number of cells (2000 cells). Dotted lines under the histogram indicate the region of cells with hypodiploidal DNA. Asterisks under the histogram show the regions corresponding to the G_0G_1 phase (#) and G_2M phase (##). (B) Effects of maytanprine 0.03 nM and maytansine 0.1 nM on the population of cells with hypodiploidal DNA. The columns and bars represent the mean percentage population of cells with hypodiploid DNA±S.D., respectively, of 4 experiments. Asterisks indicate a significant difference ($**p<0.005$) between the control group and test group at the indicated times.

DNA was significantly increased by maytanprine 0.03 nM or maytansine 0.1 nM (Fig. 6). Maytanprine 0.01 nM and maytansine 0.03 nM did not inhibit cell growth (Fig. 4) and there was no change in the distribution of DNA content (cell cycle) in both cases.

DISCUSSION

The cell suspension medium contains not only living cells but also dead cells. It is necessary to estimate simultaneously the respective populations of living and dead cells if the effect of a toxic substance is examined in growing cells. The number of cells measured with a flow cytometer includes living and dead cells that are distinguishable by propidium iodide fluorescence. This allows simultaneously estimation of cell growth and viability.[4] Furthermore, in the present study, cell shrinkage was used as a parameter to evaluate cytotoxicity. The population of shrunken cells consisted of almost all dead cells and cells of which the membranes maintained selective permeability.[11,12] Cell shrinkage is one morphologic change during the early stage of apoptosis[16] and it appears to occur before cytochrome C release from mitochondria, caspase activation and DNA fragmentation.[13—15] Therefore flow-cytometric analysis is suitable for examining the cytotoxicity of chemicals in growing cells such as tumor cells.

Although maytansine, a reference compound in the present study, has shown no significant benefit in clinical trials as a cancer treatment,[17] this compound still attracts considerable attention from many investigators because of its very potent inhibitory action on the growth of cancer cells.[7] Therefore the effect of maytansine on cancer cells has been thoroughly investigated, while this is not the case for maytanprine. Maytanprine at concentrations of 0.03 nM or greater (up to 1 nM) markedly suppressed the growth of K562 cells with a concentration-dependent decrease in viability (Fig. 4) and increased the percentage population of shrunken cells (Fig. 5). The concentration–response relation for maytanprine-induced inhibition of cell growth was very steep (Fig. 4). The inhibition of cell growth by maytanprine preceded the increases in the populations of dead cells and shrunken cells (Figs. 4, 5). Maytanprine at 0.03 nM greatly decreased the population in the G_0G_1 phase and increased the population of cells with hypodiploidal DNA, while the population in the G_2M phase remained relatively unchanged (Fig. 6). These results suggest that maytanprine blocks mitosis and induces apoptosis, although the apoptosis (or cell death) induced by maytanprine may be not essential for the inhibition of cell growth. The potency of maytanprine in affecting the growth and viability of K562 cells was greater than that of maytansine (Figs. 4, 5) although there is only a slight difference in their chemical structure (Fig. 2). However, the ability of maytanprine to inhibit cell growth is similar to that of maytansine. Both compounds exert a growth-inhibitory activity and apoptosis-inducing activity on K562 cells at subnanomolar concentrations.

The concentration of maytansine in the original source plant is very low (2×10^{-5}% of dry weight).[7] In the present study, the amount of maytanprine obtained from 900 g of dry weight of *M. diversifolia* was calculated to be 1.02 g (1.13×10^{-4}%, 5.5-fold higher). Therefore *M. diversifolia*, a plant from Iriomote Island, Okinawa, Japan, is a useful source of maytanprine. It is concluded that subnanomolar concentrations of maytanprine exert very potent inhibitory action against the cell growth of human leukemia K562 cells *via* blockade of mitosis and its potency is greater than that of maytansine. However, because maytanprine exerts an all-or-none inhibitory action against cell growth, it may be difficult

to manage its cytotoxic action under *in vivo* conditions.

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