# Low-dose 1,25-dihydroxyvitamin D<sub>3</sub> combined with arsenic trioxide synergistically inhibits proliferation of acute myeloid leukemia cells by promoting apoptosis

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Abstract. Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) has shown substantial efficacy in the treatment of patients with acute promyelocytic leukemia, a specific subtype of acute myeloid leukemia (AML). However, since not all patients can achieve remission after treatment, it is necessary to develop a novel method to overcome this problem. We investigated the anti-leukemic effect of low-dose 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) in combination with As<sub>2</sub>O<sub>3</sub> on the human AML cell lines HL-60 and K562. The cell viability was in reverse proportion to As<sub>2</sub>O<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration. In both HL-60 and K562 cells, after the combination treatment with  $As_2O_3$  and  $1,25(OH)_2D_3$ at a 10:1 ratio, the combination index (CI) values were <1 in all treatment groups. In the RT-PCR and western blot analysis, the combination treatment decreased Bcl-2 expression and increased Bax and caspase-3 expression more prominently than the single treatment. In the flow cytometric analysis performed in HL-60 cells, the proportion of late apoptotic cells was 4.9% in the control, 30.0% in cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>, 8.1% in cells treated with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, and 64.3% in cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub> plus 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>. In conclusion, low-dose 1,25(OH)<sub>2</sub>D<sub>3</sub> combined with As<sub>2</sub>O<sub>3</sub> synergistically inhibited proliferation of HL-60 and K562 cells. In addition, this combination activated the apoptosis pathway more prominently than the single-drug treatment.

## Introduction

Arsenic trioxide  $(As_2O_3)$  has shown substantial efficacy in the treatment of patients with acute promyelocytic leukemia

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(APL), a specific subtype of acute myeloid leukemia (AML) (1). Previous studies have demonstrated that  $As_2O_3$  influences various intracellular signaling pathways, which may result in the induction of apoptosis, the inhibition of growth and angiogenesis, and the promotion of differentiation (2). In clinical studies, the complete remission (CR) rate of  $As_2O_3$  treatment was substantial (3-5). However, at the same time, a significant portion of patients could not achieve CR in spite of the treatment using  $As_2O_3$  as well as all-*trans*-retinoic acid (ATRA) and conventional chemotherapy. Hence, it is necessary to investigate a novel treatment method overcoming the resistance of current treatments.

The 1,25-dihydroxyvitamin  $D_3$  (also known as 1,25(OH)<sub>2</sub> $D_3$ or calcitriol), the active form of vitamin D, is also known to regulate cell proliferation and differentiation as well as classical actions on calcium homeostasis (6-8). 1,25(OH)<sub>2</sub>D<sub>3</sub> was found to cause differentiation of myeloid leukemic cells and to prolong the survival of leukemic mice (9,10). In addition, previous studies have demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> causes differentiation of the chemo-naïve APL cell line HL-60 (11,12) and the ATRA-resistant APL cell line UF-1 (13). However, in a previous clinical study performed in patients with myelodysplastic syndrome, 1,25(OH)<sub>2</sub>D<sub>3</sub> single therapy resulted in hypercalcemia in half of the patients before concentrations necessary for sufficient anti-leukemic activity could be achieved (14). This hypercalcemic side effect has limited the clinical application of 1,25(OH)<sub>2</sub>D<sub>3</sub> single therapy to hematologic malignancies.

In this study, we aimed to elucidate the anti-leukemic effect of  $1,25(OH)_2D_3$  combined with  $As_2O_3$  on human AML cells. However, considering the dose-dependent hypercalcemic effect of  $1,25(OH)_2D_3$ , we used a low-dose of  $1,25(OH)_2D_3$ . Thus, the objective of this study was to elucidate the anti-leukemic effect of low-dose  $1,25(OH)_2D_3$  combined with  $As_2O_3$  on HL-60 and K562 cells.

#### Materials and methods

*Cell culture*. The HL-60 and K562 cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). HL-60 and K562 cells were cultured in RPMI-1640 medium containing

10% fetal bovine serum (FBS), 100 units of penicillin and 100  $\mu$ g/ml of streptomycin in a 37°C incubator under 5% CO<sub>2</sub>.

Measurement of cytotoxicity with trypan blue exclusion test. Inhibition of the proliferation rate of HL-60 and K562 cells was measured by the trypan blue exclusion test. The cytotoxic effect of As<sub>2</sub>O<sub>3</sub> on HL-60 and K562 cells was examined by treating 2.4x10<sup>5</sup> cells with 0, 0.5, 1.0, 1.5, 2.0 and 3.0  $\mu$ M As<sub>2</sub>O<sub>3</sub> for 24, 48 and 72 h. The cytotoxic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on HL-60 and K562 cells was examined by treating 2.5x10<sup>4</sup> cells with 0, 100, 200, 300, 400 and 500 µM As<sub>2</sub>O<sub>3</sub> for 24, 48 and 72 h. The effect of the combination treatment on HL-60 cells was evaluated by treating 1.2x10<sup>6</sup> cells for 24 h with the following combinations:  $0.5 \mu M As_2O_3$  plus 50 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1.0 µM As<sub>2</sub>O<sub>3</sub> plus 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1.5 µM As<sub>2</sub>O<sub>3</sub> plus 150 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, 2.0 µM As<sub>2</sub>O<sub>3</sub> plus 200 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> and 3.0 µM As<sub>2</sub>O<sub>3</sub> plus 300 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>. The effect of the combination treatment on K562 cells was evaluated by treating 1.2x10<sup>6</sup> cells for 24 h with the following combinations: 0.25 µM As<sub>2</sub>O<sub>3</sub> plus 25 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, 0.50 µM As<sub>2</sub>O<sub>3</sub> plus 50 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, 0.75 µM As<sub>2</sub>O<sub>3</sub> plus 75 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1.00 µM As<sub>2</sub>O<sub>3</sub> plus 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1.50  $\mu$ M As<sub>2</sub>O<sub>3</sub> plus 150 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>.

After treatment, we diluted the cells in complete medium, without FBS, to an approximate concentration of 1-2x10<sup>5</sup> cells/ml. Then, we added 0.1 ml of 0.4% trypan blue solution to 0.5 ml of the cell suspensions and mixed them thoroughly. These mixtures were incubated for 5 min at 15-30°C. The cell count was calculated using a hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and a microscope (CKX41; Olympus, Tokyo, Japan). Each experiment was performed in duplicate.

mRNA extraction and RT-PCR. HL-60 and K562 cells were treated for 24 h with the following combinations: control; 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>; 1.0 µM As<sub>2</sub>O<sub>3</sub>; 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> plus 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; and 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub> plus 3.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>. Total RNA was isolated from the cells using TRIzol reagent. The RNA pellets obtained were dissolved in diethylpyrocarbonate (DEPC)-treated H<sub>2</sub>O at concentrations of 0.5 to 1.0  $\mu$ g/ $\mu$ l, and then stored at -70°C. The quantity and quality of the RNA preparations were determined by measuring the absorbance at 260 and 280 nm. One microgram of total RNA was reverse-transcribed using a first strand cDNA synthesis kit with random primer  $p(dN)_6$  and the primers listed in Table I. The amplification conditions used were 35 cycles at -94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. All data were normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blot analysis.  $As_2O_3$ - or  $1,25(OH)_2D_3$ -treated HL-60 and K562 cells were prepared in the same way as described for the RT-PCR analysis. These cells were homogenized in 10 mM Tris (pH 7.4), 1 mM sodium vanadate (Na<sub>3</sub>VO<sub>4</sub>) and 1% sodium dodecyl sulfate (SDS). These homogenates were boiled for 5 min at 95°C and centrifuged at 13,000 x g for 15 min at 4°C. The pellet was discarded and the supernatant containing the protein was transferred to a clean tube. The total protein concentration was measured using the microbicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, Table I. Primers used for RT-PCR analysis of Bcl-2, Bax, caspase-3 and GAPDH genes.

Gene	Primer sequence			
Bcl-2	F: 5'-TTTGAGTTCGGTGGGGTCAT-3' R: 5'-TGACTTCACTTGTGGGCCCAG-3'			
Bax	F: 5'-TGGCAGCTGACATGTTTTCTGAC-3' R: 5'-TCACCCAACCACCCTGGTCTT-3'			
Caspase-3	F: 5'-GCAGCAAACCTCAGGGAAAC-3' R: 5'-TGTCGGCATACTGTTTCAGCA-3'			
GAPDH	F: 5'-CCACATCGCTCAGACACCAT-3' R: 5'-TGACAAGCTTCCCGTTCTCA-3'			

PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

USA) in accordance with manufacturer's instructions. Samples containing 30  $\mu$ g of protein, along with the molecular weight marker (BenchMark<sup>™</sup> Pre-Stained Protein Ladder; Invitrogen, Grand Island, NY, USA), were subjected to 10% SDS polyacrylamide gel electrophoresis under reducing conditions. The proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After nonspecific sites were blocked with 5% powdered skim milk in 0.05% Triton X-100/Tris-buffered saline (TBS-T) for 1 h, blots were incubated overnight with an IgG-purified rabbit polyclonal Bcl-2, Bax, or caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA) in a solution containing 5% powdered skim milk and 0.05% Triton X-100/TBS. The blots were then washed three times in TBS-T for 10 min each and incubated with a peroxidase-conjugated goat anti-rabbit IgG at a concentration of 1  $\mu$ g/ml in 5% powdered skim milk in 0.05% TBS-T. All samples were also blotted for  $\beta$ -actin (clone AC-15; Sigma-Aldrich, Dublin, Ireland) to normalize protein amounts.

Evaluation of apoptosis using flow cytometry. As<sub>2</sub>O<sub>3</sub>- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated HL-60 cells were prepared similarly as described in the RT-PCR analysis. The cells were washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of  $2x10^5$  cells/ml. A total of 100  $\mu$ l of the mixtures was then transferred to a 5-ml culture tube and then 5  $\mu$ l of fluorescein-conjugated Annexin V (Annexin V-FITC) and 5  $\mu$ l of propidium iodide (PI) were added to the culture tube. The cells were gently vortexed and incubated for 15 min at room temperature in the dark, and 150  $\mu$ l of binding buffer was added to each tube. Flow cytometry was then performed within 1 h.

Statistical analysis. The combined effects of  $As_2O_3$  and  $1,25(OH)_2D_3$  were analyzed by CalcuSyn software (Biosoft, Ferguson, MO, USA) using the Chou-Talalay method (15). This method is based on the median-effect equation for a dose-effect relationship  $f_a/f_u = (D/D_m)^m$ , where *D* is the dose,  $D_m$  is the IC<sub>50</sub>,  $f_a$  is the fraction affected by dose D,  $f_u$  is the unaffected fraction ( $f_u = 1 - f_a$ ) and *m* is a coefficient of the



Figure 1. Cytotoxic effects of  $As_2O_3$  and  $1,25(OH)_2D_3$  on HL-60 and K562 cells. HL-60 cells were treated with (A)  $As_2O_3$  and (B)  $1,25(OH)_2D_3$  at a variety of concentrations (0, 0.5, 1.0, 1.5, 2.0 and 3.0  $\mu$ M for  $As_2O_3$  and 0, 100, 200, 300, 400 and 500 nM for  $1,25(OH)_2D_3$ ). K562 cells were also treated with (C)  $As_2O_3$  and (D)  $1,25(OH)_2D_3$  at the same conditions. The cell viability was in reverse proportion to the concentration of  $As_2O_3$  or  $1,25(OH)_2D_3$ . K562 cells tended to be more sensitive to both  $As_2O_3$  and  $1,25(OH)_2D_3$  than HL-60 cells.

sigmoidicity of the dose-effect curve. The combination index (CI) was determined on the basis of the isobologram analysis for mutually exclusive effects:  $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$ , where  $(D_x)_1$  and  $(D_x)_2$  are the concentrations of As<sub>2</sub>O<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> which inhibit cell growth by x% and  $(D)_1$  and  $(D)_2$  are the drug concentrations in the combination treatments which inhibit cell growth by x%.  $(D_x)_1$  and  $(D_x)_2$  values can be determined by a rearrangement of equation  $D = D_m (f_a/(1-f_a))^{1/m}$ . The CI values of <1, 1 and >1 indicate synergism, an additive effect, and antagonism, respectively.

## Results

Synergistic cytotoxic effect of the combination treatment of  $As_2O_3$  and low-dose  $1,25(OH)_2D_3$ . As shown in Fig. 1, the viability of HL-60 and K562 cells was in reverse proportion to the concentration of  $As_2O_3$  (0, 0.5, 1.0, 1.5, 2.0 and 3.0  $\mu$ M) or  $1,25(OH)_2D_3$  (0, 100, 200, 300, 400 and 500 nM). K562 cells were more sensitive to both  $As_2O_3$  and  $1,25(OH)_2D_3$  than HL-60 cells.

The results of the combination treatment with both  $As_2O_3$ and low-dose 1,25(OH)<sub>2</sub>D<sub>3</sub> are summarized in Table II and III. In both HL-60 and K562 cells, the combination treatment with  $As_2O_3$  and 1,25(OH)<sub>2</sub>D<sub>3</sub> at a 10:1 ratio revealed a more prominent cytotoxicity compared to single treatments with either  $As_2O_3$  or 1,25(OH)<sub>2</sub>D<sub>3</sub>. Additionally, as shown in Fig. 2, in both HL-60 and K562 cells, the CI values of all combinations evaluated were <1, which suggests evident synergistic cytotoxic effects for  $As_2O_3$  and low-dose  $1,25(OH)_2D_3$ .

Expression of apoptosis-related genes (mRNA). The expression of apoptosis-related genes in HL-60 and K562 cells was analyzed using the RT-PCR method (Fig. 3). In both HL-60 and K562 cell lines, decreased Bcl-2 and increased Bax and caspase-3 expression was observed in either As<sub>2</sub>O<sub>3</sub>- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated cells compared to the control. In addition, combination treatment using both As<sub>2</sub>O<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> more prominently decreased Bcl-2 expression and increased Bax and caspase-3 expression. Additionally, the effect of the combination treatment was enhanced in proportion to the increased concentration of As<sub>2</sub>O<sub>3</sub>; 3  $\mu$ M As<sub>2</sub>O<sub>3</sub>-treated cells showed decreased Bcl-2 and increased Bax and caspase-3 expression when compared to the expression in 1  $\mu$ M As<sub>2</sub>O<sub>3</sub>-treated cells.

*Expression of apoptosis-related proteins*. Western blot analysis was performed to analyze the expression of apoptosis-related proteins in the HL-60 and K562 cells. As shown in Fig. 4, the results for both HL-60 and K562 cell lines were identical to those of the RT-PCR analysis (Fig. 3). The combination treatment enhanced the production of pro-apototic Bax and caspase-3 proteins and reduced the production of anti-apoptotic Bcl-2 protein. In addition, the effect of the combination

$As_2O_3$ ( $\mu M$ )	Fraction of cell death	1,25(OH) <sub>2</sub> D <sub>3</sub> (nM)	Fraction of cell death	Combination treatment		
				$ \frac{As_2O_3}{(\mu M)} $	1,25(OH) <sub>2</sub> D <sub>3</sub> (nM)	Fraction of cell death
).5	0.538	50	0.097	0.5	50	0.702
0.1	0.570	100	0.112	1.0	100	0.773
1.5	0.608	150	0.124	1.5	150	0.824
2.0	0.637	200	0.135	2.0	200	0.875
3.0	0.750	300	0.157	3.0	300	0.943

CI, combination index.

Table III. Combined effects of As<sub>2</sub>O<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> in K562 cells.

As <sub>2</sub> O <sub>3</sub> (μM)		1,25(OH) <sub>2</sub> D <sub>3</sub> (nM)	Fraction of cell death	Combination treatment			
	Fraction of cell death			$As_2O_3$ ( $\mu$ M)	1,25(OH) <sub>2</sub> D <sub>3</sub> (nM)	Fraction of cell death	CI
0.25	0.409	25	0.100	0.25	25	0.589	0.901
0.50	0.476	50	0.143	0.50	50	0.622	0.103
0.75	0.516	75	0.154	0.75	75	0.675	0.050
1.00	0.565	100	0.174	1.00	100	0.760	0.024
1.50	0.608	150	0.264	1.50	150	0.848	0.000

CI, combination index.



Figure 2. Fractional effect-CI plots of single As<sub>2</sub>O<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> treatments and combination treatment. The effect of the combination treatment was analyzed using CalcuSyn software in (A) HL-60 and (B) K562 cells. In all concentrations examined, the CI values of the combination treatment were <1, which corresponds to an evident synergistic cytotoxicity of As<sub>2</sub>O<sub>3</sub> and low-dose 1,25(OH)<sub>2</sub>D<sub>3</sub>.

treatment was enhanced in proportion to the increased concentration of As<sub>2</sub>O<sub>3</sub>.

Enhancement of  $As_2O_3$  and  $1,25(OH)_2D_3$ -induced apoptosis. HL-60 cells were labeled with Annexin V-FITC and PI, and analyzed using flow cytometry to differentiate whether the main cause of cell death was apoptosis or necrosis. As shown in Fig. 5, cell death was significantly increased after the combination treatment with As<sub>2</sub>O<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> in the HL-60 cells. The proportion of living cells (Annexin V-FITC- and PI-negative) was 92.6% in the control; 51.3% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; 86.4% in the cells treated with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>; and 2.0% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub> plus 100 nM  $1,25(OH)_2D_3$ . The proportion of early apoptotic cells (Annexin V-FITC-positive and PI-negative) was 1.3% in the control; 2.8% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; 4.0%

CI

0.179 0.167 0.129 0.075 0.019



Figure 3. Expression of apoptosis-related genes (mRNA): Bcl-2, Bax and caspase-3. In (A) HL-60 and (B) K562 cells, as compared to the control, decreased Bcl-2 and increased Bax and caspase-3 expression was observed in both the  $As_2O_3$ - and  $1,25(OH)_2D_3$ -treated cells. Combination treatment using both  $As_2O_3$  and  $1,25(OH)_2D_3$  more prominently decreased Bcl-2 expression and increased Bax and caspase-3 expression. The effect of the combination treatment was enhanced in proportion to the increased concentration of  $As_2O_3$ .



Figure 4. Expression of apoptosis-related proteins: Bcl-2, Bax and caspase-3. In (A) HL-60 and (B) K562 cells, as compared to the control, decreased Bcl-2 and increased Bax and caspase-3 expression was observed in both the  $As_2O_3$ - and  $1,25(OH)_2D_3$ -treated cells. In addition, prominently decreased Bcl-2 and increased Bax and caspase-3 expression was observed in the combination treatment group as compared to the single treatment groups. The effect of the combination treatment was enhanced in proportion to the increased concentration of  $As_2O_3$ .

in the cells treated with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>; and 0.2% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub> plus 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>. The proportion of late apoptotic cells (Annexin V-FITC- and PI-positive) was 4.9% in the control; 30.0% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; 8.1% in the cells treated with 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>; and 64.3% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub> plus 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>. The proportion of necrotic cells (Annexin V-FITC-negative and PI-positive) was 1.2% in the control; 15.8% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; 1.5% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>. The proportion of necrotic cells (Annexin V-FITC-negative and PI-positive) was 1.2% in the control; 15.8% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; 1.5% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub>; 1.5% in the cells treated with 1.0  $\mu$ M As<sub>2</sub>O<sub>3</sub> plus 100 nM 1,25(OH)<sub>2</sub>D<sub>3</sub>. In concordance with RT-PCR and western blot analysis, the

combination treatment resulted in a more prominent apoptotic cell death compared to the single-drug treatments. In contrast, the contribution of necrosis to cell death was relatively smaller compared to apoptosis in all treatment groups.

## Discussion

Previous studies have demonstrated that anti-leukemic activity of  $As_2O_3$  is mainly due to its ability to induce apoptosis via various mechanisms including downregulation of Bcl-2, upregulation of caspases, and generation of reactive oxygen species (ROS) (16-18). In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> was also found



Figure 5. Evaulation of apoptosis using flow cytometry. Apoptosis of HL-60 cells was evaluated using flow cytometry. HL-60 cells were labeled with Annexin V-FITC and PI. The combination treatment resulted in more prominent apoptotic cell death than that in the other treatment groups. In addition, the contribution of necrosis to cell death was relatively smaller when compared to apoptosis in all treatment groups.

to induce apoptosis of various types of cells including colon cancer, breast cancer, prostate cancer and normal adipocytes through the activation of the apoptosis pathway (19-23). In concordance with these results, our study found that treatment with  $As_2O_3$  and  $1,25(OH)_2D_3$  each inhibited the proliferation of HL-60 and K562 cells, increasing the production of proapoptotic Bax and caspase-3 proteins and decreasing the production of anti-apoptotic Bcl-2 protein. In addition, in the flow cytometric analysis using Annexin V-FITC and PI, the main cause of cell death induced by  $As_2O_3$  and  $1,25(OH)_2D_3$ was apoptosis, which suggests an evident pro-apoptotic effect of these drugs on AML cells.

In order to overcome the resistance of As<sub>2</sub>O<sub>3</sub>, which is an active drug against APL, we aimed to evaluate the additional benefit of  $1,25(OH)_2D_3$  in combination with As<sub>2</sub>O<sub>3</sub> on HL-60 and K562 cells. Due to the serious hypercalcemic side effect, the clinical application of  $1,25(OH)_2D_3$  single therapy to hematologic malignancies is limited in spite of its potent in vitro anti-leukemic activity (11,24). Considering this side effect, we chose a relatively low concentration of  $1,25(OH)_2D_3$ , which alone could not show sufficient anti-leukemic activity. Despite its low concentration,  $1,25(OH)_2D_3$  combined with As<sub>2</sub>O<sub>3</sub> showed an evident synergistic anti-leukemic effect on both HL-60 and K562 cells. To the best of our knowledge, this is the first study demonstrating the synergistic anti-leukemic effect of this combination treatment against AML cells. This combination treatment activated the apoptosis pathway of cells more prominently than the single treatments. Moreover, flow cytometric analysis showed that the combination treatment resulted in a more prominent apoptotic cell death compared to the single-drug treatments. The results of this study may provide evidence that low-dose 1,25(OH)<sub>2</sub>D<sub>3</sub> may be used for improving the therapeutic efficacy of  $As_2O_3$  for the treatment of patients with AML.

Since apoptosis was the dominant cause of cell death in this study, we mainly focused on the activation of the apoptosis pathway induced by  $1,25(OH)_2D_3$  and  $As_2O_3$ . However,  $As_2O_3$  is also known to induce caspase-independent necrotic cell death via the mitochondrial death pathway (25). In this study, the effect of necrosis was relatively small when compared to apoptosis, but it was also not negligible. The proportion of necrosis in the  $1,25(OH)_2D_3$ -treated cells (1.5%) did not appear to be different from that in the control group (1.2%). However, the proportion of  $As_2O_3$ -induced necrosis (15.8%) was substantial. In addition,  $1,25(OH)_2D_3$  profoundly increased the proportion of necrosis (33.5%) as well as apoptosis when combined with  $As_2O_3$ . This novel finding regarding the effect of  $1,25(OH)_2D_3$ on  $As_2O_3$ -induced necrosis also warrants further investigation.

It is known that both  $As_2O_3$  and  $1,25(OH)_2D_3$  influence intracellular calcium homeostasis of cells, resulting in induction of apoptosis (20,23,26-28). Since these drugs share a common pathway, it is speculated that the synergistic cytotoxicity of these agents would be attributed to intracellular calcium homeostasis and their association with apoptosis induction. Although we did not investigate the calcium signaling pathway in this study, this hypothesis should be validated in subsequent studies.

In summary, low-dose  $1,25(OH)_2D_3$  in combination with  $As_2O_3$  synergistically inhibited proliferation of the HL-60 and K562 cell lines. In addition, low-dose  $1,25(OH)_2D_3$  combined with  $As_2O_3$  more prominently activated the apoptosis pathway than a single treatment using either  $1,25(OH)_2D_3$  or  $As_2O_3$ . The main cause of cell death was also apoptosis. Our results suggest that low-dose  $1,25(OH)_2D_3$  could be applied to improving the therapeutic efficacy of  $As_2O_3$  against AML.

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