

# KML001 and doxercalciferol induce synergistic antileukemic effect in acute lymphoid leukemia cells

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**Abstract.** KML001 (NaAsO<sub>2</sub>, sodium metaarsenite, KOMINOX), a kind of arsenic compound, that has shown promising efficacy in non-Hodgkin's lymphoma (NHL) both *in vitro* and *in vivo*. In our study, the antileukemic effect of KML001 on acute lymphoid leukemia (ALL) and its mechanism of action were investigated. The results showed that KML001 inhibited cell proliferation in two types of ALL cell lines, CCRF-CEM and Molt-4. Exposure of ALL cells to KML001 induced apoptosis in a time-dependent manner. KML001 caused cell cycle arrest at G2/M phase instead of G0/G1 phase shown in other leukemia cells. In addition, we also tested the possibility of synergy of KML001 with doxercalciferol, a vitamin D2 derivative. Also, we found that a combination of KML001 with doxercalciferol showed a synergistic effect on ALL cell lines and this could be due to its different mechanism of action. Overall, our findings demonstrated KML001 could be a promising antileukemic agent especially when it is combined with doxercalciferol in ALL treatment.

## Introduction

Acute lymphocytic leukemia (ALL), also called acute lymphoblastic leukemia, is a kind of lymphoid neoplasm. Lymphoid neoplasms have been divided into two major categories: neoplasms derived from B- and T-lineage lymphoid precursors and those derived from mature B, T or NK cells in recent WHO classification (1). Also, ALL belongs to the first group which is B/T-precursor-stage lymphoid cell malignancies that block

lymphoid differentiation and drive aberrant cell proliferation and survival. In addition, ALL is the most common leukemia in pediatrics, among which there are ≤80% of leukemia in this group and 20% of leukemia in adults (2). With the developments in theoretical knowledge and techniques, novel treatments in increasing number such as chemotherapy, steroids, radiation therapy and intensive combined treatments have been adopted for ALL treatment. Nevertheless, relapse among ALL patients is still the leading problem causing children's death. Despite the high cure rate in children with ALL, patients accounting for 10-20% are forecasted to relapse and outcomes of salvage therapy have been disappointing and only one-third of children survive long-term after recurrence of disease (3). In addition, the outcomes of adults with ALL are usually markedly worse than those of pediatric ones. Therefore, novel methods and effective drugs are urgently needed.

Arsenic is known as one of the toxic metalloids and its oxidized form, arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) has been successfully used as a medicine for patients with acute promyelocytic leukemia (APL) via intravenous injection. However, the anticancer effect of arsenic trioxide has not been proven in solid tumors or other hematologic malignancies (4). KML001 (NaAsO<sub>2</sub>, sodium metaarsenite, KOMINOX), a sodium salt of arsenous acid, is water-soluble, therefore, orally bioavailable, trivalent arsenical compound. KML001 has shown a potent antitumor effect on various human cancers, including solid tumors such as prostate cancer, ovarian cancer and hematologic malignancy like acute myeloid leukemia (AML) (5-7). In addition, its half maximal inhibitory concentration (IC<sub>50</sub>) has been found much lower than arsenic trioxide in various NHL cell lines, which may make it more suitable for clinical applications (8).

Based on epidemiological research (9,10) and *in vitro* studies (11-13), vitamin D derivatives (VDDs) have been considered as a promising anticancer drug. As one of the most typical VDDs, 1, 25-dihydroxyvitamin D3 is known to control cell proliferation and differentiation as well as calcium related actions in leukemia cells (14-16). However, the side effect of hypercalcemia has limited its clinical application in hematologic malignancies. Doxercalciferol, a low calcemic vitamin D2 derivative with similar effect as 1, 25-dihydroxyvitamin D3, can be safely given to patients and was investigated

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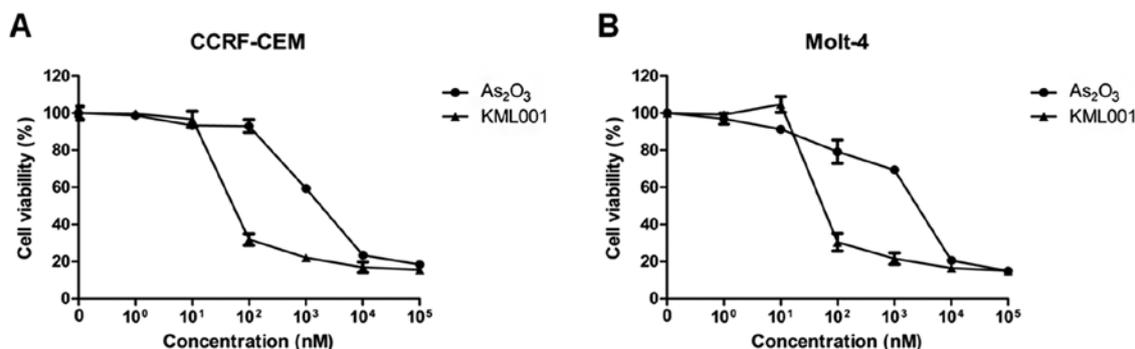


Figure 1. The superiority of KML001 compared to arsenite trioxide. CCRF-CEM cells and Molt-4 cells were treated with KML001 or arsenite trioxide at a variety of concentrations (0, 10<sup>0</sup>, 10<sup>1</sup>, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> and 10<sup>5</sup> nM) (A and B), the data are presented as the mean values from three separate experiments  $\pm$  SD.

in phase II studies of the myelodysplastic syndrome (17) and androgen-independent prostate cancer (18).

In this study, the antileukemic effect of KML001 on ALL was investigated, which was focused on apoptosis and cell cycle. In addition, the combination effect of KML001 with doxercalciferol was studied. We provided evidence of synergistic effect of KML001 and doxercalciferol in ALL cell lines and possible explanations of this synergy.

## Materials and methods

**Cells and cell culture.** Human acute lymphoid leukemia cell line CCRF-CEM and Molt-4 were kindly presented by Dr Y.Y. Lee (Hanyang University, Seoul, Korea). The cells were cultured in tissue flasks or plates in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Chemicals and antibodies.** KML001 was obtained from Komipharm International (Siheung-Si, Gyeonggi-Do, Korea). As<sub>2</sub>O<sub>3</sub> was purchased from Sigma-Aldrich (St. Louis, MO, USA). Doxercalciferol was purchased from Selleck Chemicals (Houston, TX, USA). Antibodies of Cdk1, p-Cdk1, cyclin B, p-p53, p-p21, Bcl-2, Bax, Bcl-xL were from Abcam (Cambridge, UK).

**Growth inhibition assay.** Cell viability was determined by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA). Cells (5x10<sup>3</sup> cells/well) were seeded in 96-well microtiter plates (Falcon) and then incubated at 37°C for 48 h. After adding 10  $\mu$ l of the CCK-8 solution to each well of the plate, the plate was incubated for  $\geq$ 1 h in the incubator. Finally, absorbance at 450 nm was measured by Multiskan Spectrum microplate reader (Thermo LabSystems, USA).

**Western blot analysis.** Cells were lysed with lysis buffer [25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS with Halt™ Protease, Phosphatase Inhibitor Cocktail and Benzonase® Nuclease] on ice for 20 min. Briefly, protein samples were resolved in SDS-polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and then probed with antibodies overnight. Finally, the blots were

developed using the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, USA).

**Cell cycle distribution.** Cells were fixed with 66% ethanol for >2 h at 4°C and then stained with 50  $\mu$ g/ml of propidium iodide (PI) containing 550 U/ml of RNaseA (ab139418 PI Flow Cytometry kit for cell cycle analysis (Abcam, MA, USA). The DNA content of the cells was gated and analyzed using a FACSCanto II (Becton-Dickinson, San Jose, CA, USA) equipped with an FACSDiva software 8.0.1 (Becton-Dickinson).

**Evaluation of apoptosis.** Apoptosis was evaluated by FITC Annexin V apoptosis detection kit according to the manufacturer's instructions. Cells were washed twice with cold PBS, then resuspended in the 1X binding buffer, containing 0.01 M HEPES/NaOH, 0.14 M NaCl and 2.5 mM CaCl<sub>2</sub>, pH 7.4. Next, a total of 100  $\mu$ l of the mixtures was transferred to a 5-ml culture tube and then incubated with 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l propidium iodide for 15 min at room temperature in the dark. Subsequently, 400  $\mu$ l of 1X binding buffer was added to each tube and then cells were immediately analyzed by flow cytometry. In analysis process, Annexin V-positive or PI-negative cells were considered as early apoptotic, both Annexin V and PI-positive cells were considered as late apoptotic, and Annexin V-negative but PI-positive cells were considered as necrotic.

**Intracellular calcium analysis.** Cells treated with KML001, doxercalciferol or medium (control) for 48 h were washed three times with the PBS and then incubated with 4  $\mu$ M Fluo-4 AM (Thermo Fisher Scientific) and 0.1% Pluronic F-127 (Thermo Fisher Scientific) in PBS for 60 min at 37°C. After removing the medium in time, each well was washed with PBS and 200  $\mu$ l PBS was used to cover all cells in the trough. Finally, we used laser confocal scanning microscopy (LCSM) to measure the fluorescence intensity (FI) of the cells at an excitation wavelength of 488 nm.

**Statistical analysis.** The combined effects of KML001 and doxercalciferol were analyzed by CalcuSyn software (Biosoft Ferguson, MO, USA) using the Chou-Talalay method (19). The combination index (CI) values of <1, 1 and >1 represent synergy, additive effect, and antagonism, respectively. All experiments have been repeated at least 3 times. Statistical

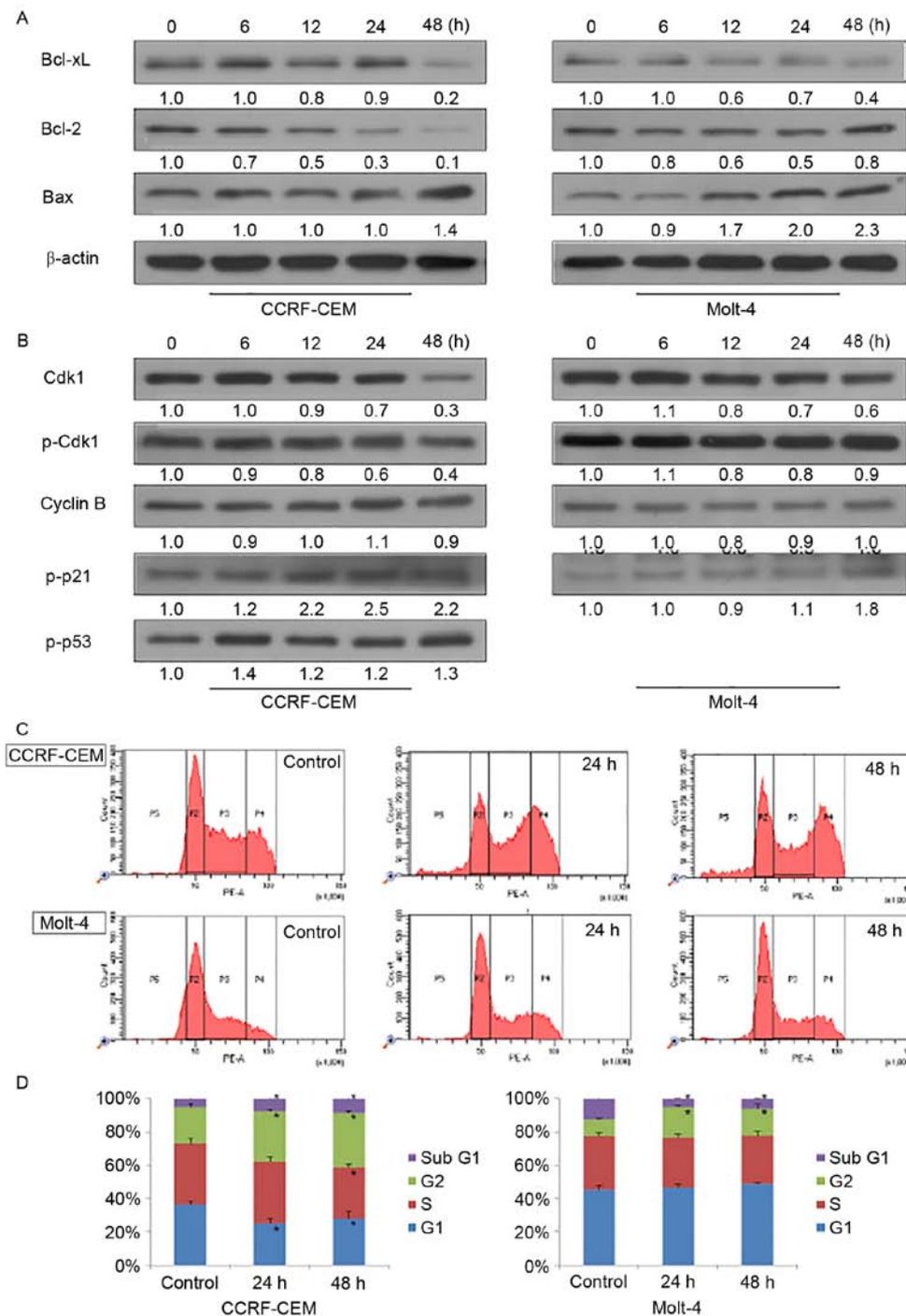


Figure 2. Antileukemic effects of KML001 on ALL cells. (A) Western blot analysis documents a time-dependent modulation of expression of Bcl-2 family members by KML001. Antibody to  $\beta$ -actin served as a loading control. (B) Western blot analysis of expression of cell cycle related protein: Cdk1, p-Cdk1, cyclin B, p-p21 and p-p53. ImageJ was used for quantify protein bands. (C) After treated with KML001 for 24 or 48 h or media as control, cells were stained with PI solution followed by analyses of cell cycle distribution using flow cytometry. (D) Quantitative analysis of results of C, the data are presented as the mean values from three separate experiments  $\pm$  SD.

significance was determined by using Student's t-test. P-value <0.05 was considered statistically significant.

**Results**

*The superiority of KML001 compared with arsenite trioxide.* The current experiments were initiated by treating CCRF-CEM and Molt-4 cells, in *in vitro* models of human ALL, for 48 h with two types of arsenic compounds, arsenic

trioxide and KML001. Results showed that KML001 inhibited cell proliferation of the cell lines at a fairly lower concentration (>100-fold) than  $As_2O_3$  (Fig. 1).

*Antileukemic effects of KML001 on ALL cells.* To further investigate the mechanism of action of KML001, western blot analysis was performed for apoptosis-related proteins in CCRF-CEM and Molt-4 cells (Fig. 2A). In both cell lines, decreased Bcl-xL and increased Bax were observed after 48-h

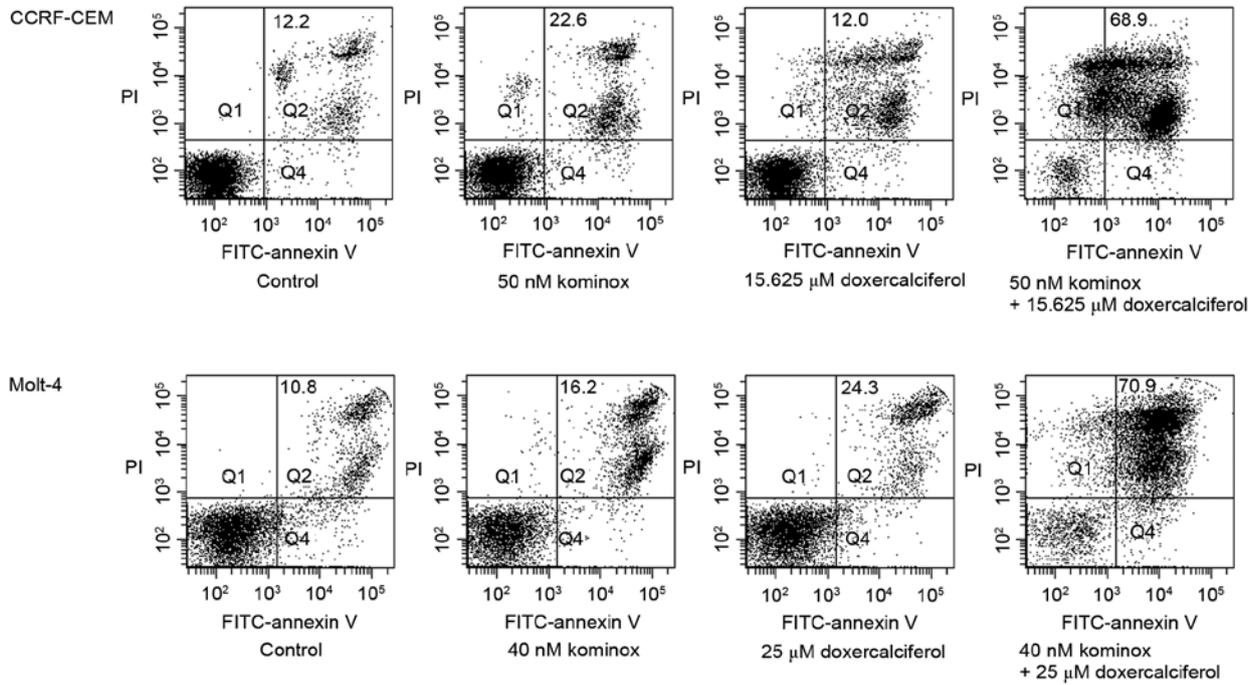


Figure 3. Synergistic antileukemic effect of KML001 combined with doxorubicin. Flow cytometric analysis of Annexin V-FITC/PI-stained CCRF-CEM and Molt-4 cells treated for 48 h with KML001 and doxorubicin either alone or combination. A significant increase of apoptotic cells was observed in groups treated with the drug combination with respect to single treatments.

Table I. MTT assay for synergistic effect of KML001 and doxorubicin.

Cell lines	KML001 (nM)	Doxorubicin (μM)	Fraction of cell death	CI
CCRF-CEM	10	3.125	0.183	1.529
	20	6.25	0.367	1.242
	40	12.5	0.556	1.195
	50	15.625	0.723	0.741
	60	18.75	0.775	0.682
	80	25	0.801	0.786
Molt-4	5	3.125	0.082	1.226
	10	6.25	0.107	1.906
	20	12.5	0.280	1.394
	40	25	0.683	0.675
	80	50	0.809	0.782
	160	100	0.777	1.824

CI, combination index.

treatment of KML001. Expression of anti-apoptotic protein Bcl-2 decreased markedly in CCRF-CEM cell line while its expression increased a little in the Molt-4 cell line.

Cdk1/cyclin B1 complex, the critical regulator of G2/M checkpoint, plays important roles in mitosis and mitotic catastrophe (20,21). As shown in Fig. 2B, the expression of Cdk1 kept decreasing after treatment of KML001 while cyclin B1 expression did not show any change. Lack of Cdk1 may cause

cell arrest at G2/M phase. To confirm this point, we conducted cell cycle analysis by propidium iodide staining of the treated cells followed by flow cytometry (Fig. 2C). As shown by the G2/M ratios in both cell lines (Fig. 2D), KML001 did induce cell cycle arrest both in CCRF-CEM and Molt-4 cells. Previous studies suggest that p53 and p21 were involved in both cell cycle arrest and cell apoptosis (22,23). Here, we found p53/p21<sup>WAF1</sup> pathway was involved in KML001-induced G2/M phase arrest. Fig. 2B indicates that p-p21 expression increased in a time-dependent manner in CCRF-CEM and Molt-4 cells after KML001 treatment. In addition, increased p53 phosphorylation was observed in CCRF-CEM cells but not in Molt-4 cells which are p53-deficient.

*Synergistic antileukemic effect of KML001 combined with doxorubicin.* We also investigated whether KML001 and doxorubicin could cause synergistic effects in terms of reduced cell viability in ALL cell lines. CCRF-CEM and Molt-4 cell lines were incubated for 48 h with one drug alone or with a combination treatment of KML001 and doxorubicin at a constant ratio of 1:312.5 or 1:625 (KML001:doxorubicin). CI and cell viability were then calculated. As shown in Table I, in the two cell lines, the combined treatments were synergistic, as indicated by CI <1.

To further evaluate its synergy, flow cytometry analysis was performed. Also, we observed a significant increase of late apoptotic cells (double-positive for Annexin V and PI) after combination treatment compared with single treatment of KML001 or doxorubicin (Fig. 3).

*Doxorubicin induces cell arrest at G2/M phase.* Western blot analysis and flow cytometry were performed to know whether doxorubicin induces cell cycle arrest in ALL

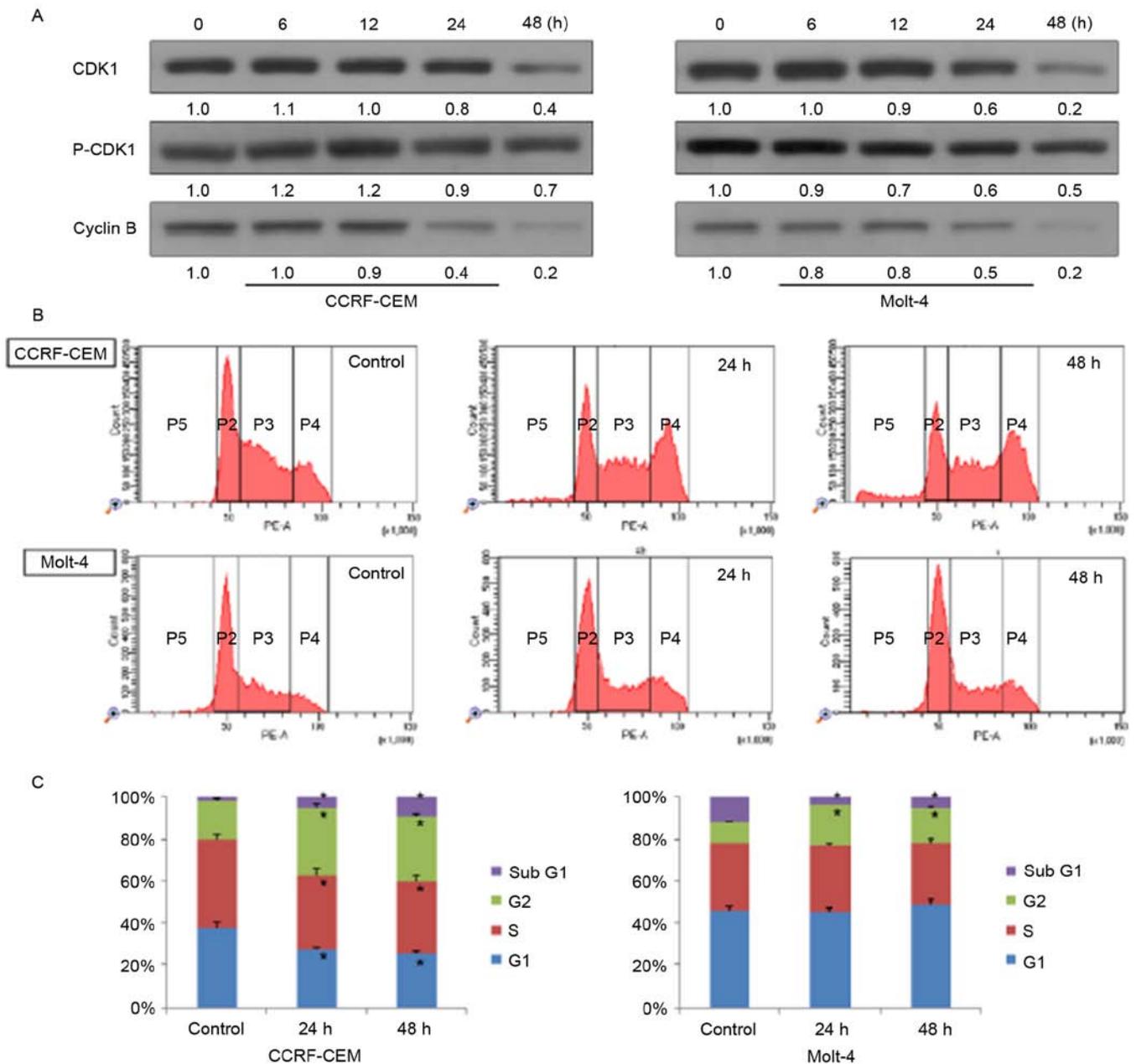


Figure 4. Doxercalciferol induces cell arrest at G2/M phase. (A) Western blot analysis of expression of cell cycle-related proteins: Cdk1, p-Cdk1, cyclin B. ImageJ was used to quantify protein bands. (B) After treated with doxercalciferol for 24 or 48 h or media as control, cells were stained with PI solution followed by analyses of cell cycle distribution using flow cytometry. (C) Quantitative analysis of results of B, the data are presented as the mean values from three separate experiments  $\pm$  SD.

cells. As shown in Fig. 4A, expression of Cdk1 and cyclin B decreased in a time-dependent manner after treatment, which indicates that doxercalciferol may also lead to G2/M phase arrest of these two cell lines. Flow cytometry analysis indicates that doxercalciferol was able to induce cell cycle arrest at G2/M phase in CCRF-CEM and Molt-4 cells (Fig. 4B and C).

*Doxercalciferol induces cell apoptosis and cell arrest via different mechanism of action.* To further identify mechanisms underlying this synergy, we also tried to understand mechanisms of action of doxercalciferol in ALL models *in vitro*. The pro-apoptotic protein Bax and anti-apoptotic protein Bcl-xL did not change as we expected (Fig. 5A). Considering the late

apoptotic cells observed in flow cytometry results, doxercalciferol may cause cell apoptosis without the involvement of Bcl-2 family pathway.

In addition, we tried to understand mechanisms of doxercalciferol-induced G2/M phase arrest by studying intracellular signaling pathway through western blot analyses. However, doxercalciferol neither induce p53 activation nor p21 activation in the two cell lines (Fig. 5B).

Finally, to investigate whether doxercalciferol-induced apoptosis is associated with  $Ca^{2+}$ , the effect of doxercalciferol on intracellular calcium ion concentration in ALL cells was observed by using LSCM after staining with the fluorescent probe, Fluo-4/AM. As shown in Fig. 5C-L, the depth of the

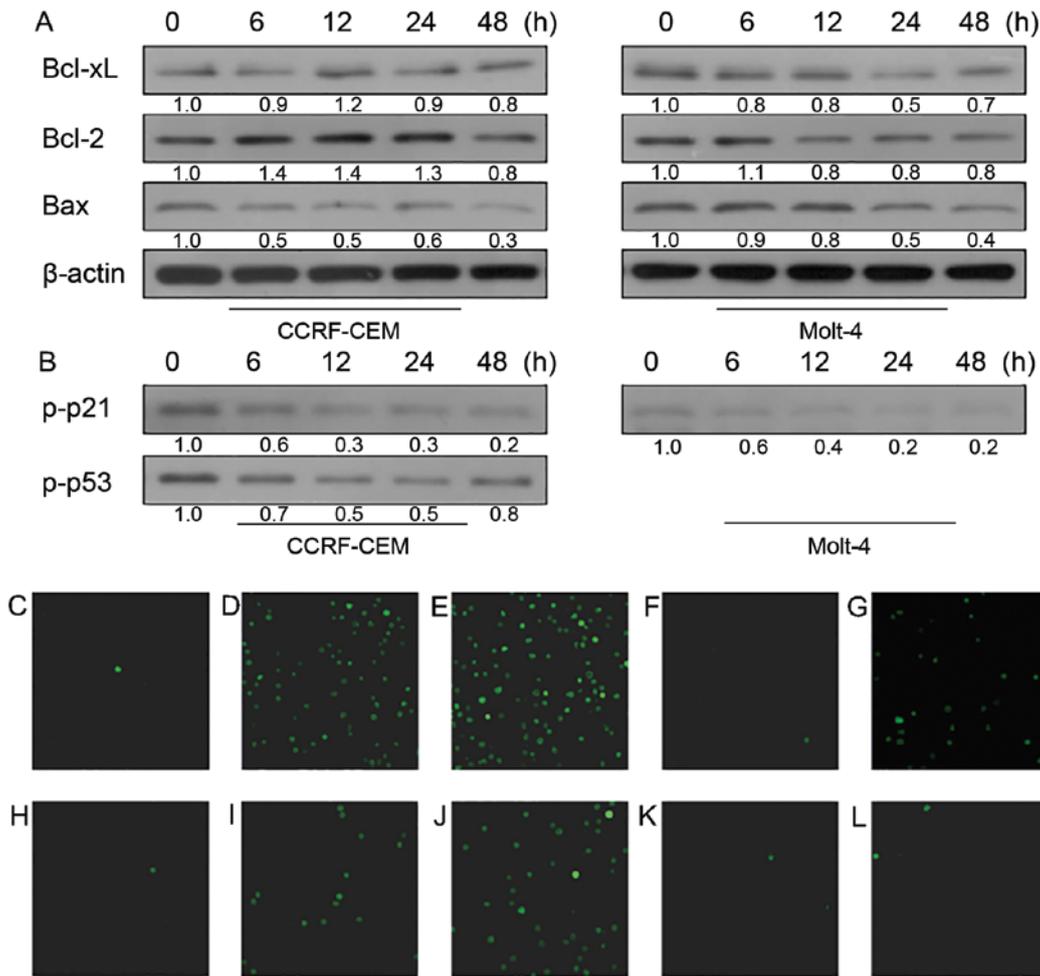


Figure 5. Mechanisms of action of doxercalciferol-induced cell cycle and apoptosis. (A and B) Western blot analysis of Bcl-2 family members, p21 and p53 expression after treating cells with doxercalciferol. ImageJ was used to quantify protein bands. Intracellular calcium accumulation occurred in CCRF-CEM and Molt-4 cells. Images (green) reveal  $[Ca^{2+}]_i$  under a confocal microscope, with the depth of the color represents the fluorescence intensity (FI), which indirectly reflects  $[Ca^{2+}]_i$ . (C and H) Control for CCRF-CEM and Molt-4 cell line respectively. (D and I) Cells treated with doxercalciferol for 24 h and (E and J) for 48 h. (F and K) Cells treated with KML001 for 24 h and (G and L) for 48 h.

green signal, which indirectly reflects intracellular  $Ca^{2+}$  concentration, increased as the time increased after treatment of doxercalciferol while there was relatively less change observed in groups treated with KML001.

## Discussion

Acute lymphoblastic leukemia occurs when primitive or immature lymphoid cells grow uncontrollably, disturbing normal hematopoiesis. Until now, it is still one of the most aggressive hematologic malignancies. With the progress and availability of combination chemotherapy, radiotherapy or hematopoietic cell transplantation, the outcomes of ALL patients have improved markedly. However, there are still many who cannot survive after treatment due to problems such as relapse, drug resistance or side effects like toxicity. Here, we evaluated antileukemic activity of KML001, a kind of arsenic compound alone and KML001 combined with doxercalciferol in ALL cells.

Previous studies have demonstrated that antileukemia activity of KML001 is mainly due to its ability to induce apoptosis and cell cycle arrest via regulation of MAPK and

PI3K pathways or telomere shortening (6,24). Also, our study showed that KML001 induced apoptosis both in CCRF-CEM and Molt-4 cell lines. Gene expression of Bcl-xL, Bax, Bcl-2, members of Bcl-2 family, has been known to control the apoptotic machinery (25). KML001 increased expression of pro-apoptotic protein Bax, and suppressed the expression of anti-apoptotic protein Bcl-xL in both cell lines.

Cdk1/cyclin B1 complex is one of the pivotal signaling molecules driving cell progression, which is involved in G2/M checkpoint. Downregulation of Cdk1 or cyclin B1 expression may lead to cell cycle arrest at G2/M phase. In our experiments, constant decreasing expression of Cdk1 in western blotting plus flow cytometry results identified that treatment with KML001 induced cell cycle arrest mainly at G2/M phase rather than at G0/G1 phase which was shown in AML research (6). To investigate the specific mechanism of KML001-induced G2/M phase arrest, we checked the expression of p-p53 and p-p21 by western blot analyses. Results showed a constant increase of expression of p-p53 or p-p21 after treatment of KML001. The activation of p21 and p53, which are the main regulator of CDK1, might be precedent to the change of Cdk1 after KML001 treatment in CCRF-CEM and Molt-4 cells.

In addition, KML001 may cause cell arrest and apoptosis in Molt-4 cells through non-p53 involved pathway since these cells are p53-deficient. This difference may be associated with the reality that Molt-4 cell line was created from a relapsed patient but CCRF-CEM cell line was not.

In order to overcome the possible resistance of KML001, we tried to evaluate the additional benefit of doxercalciferol in combination with KML001 *in vitro* models. As far as we know, this is the first study demonstrating the combination effect of KML001 with doxercalciferol against ALL cells. Also, in our results, the combinatorial treatment of KML001 with doxercalciferol showed a synergistic antileukemic activity in CCRF-CEM and Molt-4 cell lines, which suggests that the different mechanisms might work in ALL cells co-treated with KML001 and doxercalciferol.

Vitamin D and vitamin D analogs exert its anticancer effects mainly through anti-proliferation, pro-differentiation and pro-apoptotic effect (26). Unlike KML001 treatment, doxercalciferol treatment induced increment of intracellular calcium ion level. Furthermore, doxercalciferol induced apoptosis and G2/M phase cell cycle arrest but without the involvement of Bcl-2 family pathways or activation of p53 and p21.

Collectively, KML001 suppressed Cdk1 via p53 and p21 activation. These changes led to cell cycle arrest at G2/M phase in ALL cells, and its apoptosis mechanisms also includes p53, p21 activation and other mechanisms such as telomere length shortening. However, doxercalciferol induced cell apoptosis and cell cycle arrest in a totally different way, which involves calcium related pathway but without activation of proteins p53, p21 or Bcl-2 family involved pathway. This study provided evidence that KML001 could be an effective anti-leukemic agent especially with doxercalciferol in ALL treatment in the terms of cell level. Its availability on clinical application, however, should be further investigated.

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