이학석사 학위논문

KML001 and Doxercalciferol induce synergistic antileukemic effect in acute lymphoid leukemia cells

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Thesis of the Master's degree

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이 논문을 의학석사 학위논문으로 제출함

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협동과정 종양생물학 전공
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KML001 and Doxercalciferol induce synergistic antileukemic effect in acute lymphoid leukemia cells

by
Liu Yang

A thesis submitted to the Interdisciplinary Graduate Program
in partial fulfillment of the requirements for the
Degree of Master of Philosophy in Tumor Biology at
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Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor _____

Abstract

Introduction: KML001 (NaAsO2, Sodium metaarsenite, KOMINOX), a kind of arsenic compound, has shown promising efficacy in non-Hodgkin's lymphoma (NHL) both in vitro and in vivo. However, its antileukemic effect on acute lymphoid leukemia (ALL) was still not totally clear. Here, in our research, the antileukemic effect of KML001 on acute lymphoid leukemia (ALL) and its mechanism of action were investigated. And also, we tested the possibility of synergy of KML001 with Doxercalciferol, a vitamin D2 derivative.

Methods: To study the antileukemic effect of KML001 on ALL, we used MTT assay to test its cytotoxicity, flow cytometry to observe cell cycle arrest and cell apoptosis after treating two types of ALL cells, CCRF-CEM and Molt-4, with KML001. The synergistic effect of KML001 and Doxercalciferol on ALL cells was identified by MTT, flow cytometry. And finally, we did experiments like western blot, flow cytometry and intracellular calcium analysis to explore the possible reasons of this synergistic effect of two different drugs.

Results: KML001 inhibited cell proliferation in two types of ALL cell lines. 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 found that a combination of KML001 with Doxercalciferol showed a synergistic effect on ALL cell lines and this could be attributed to its different mechanism concerning apoptosis, cell cycle arrest and calcium relevant pathway.

Conclusions: Our findings demonstrated KML001 could be a promising antileukemic agent especially when it is combined with Doxercalciferol in ALL treatment.

Keywords: KML001, Doxercalciferol, acute lymphoid leukemia, CCRF-CEM, Molt-4, synergistic effect

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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]. And 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 up to 80% of leukemia in this group and 20% of leukemia in adults [2]. With the developments in theoretical knowledge and techniques, novel treatments in mounting 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 there are only one-third of children survived long-term after recurrences 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 has been known as one of the toxic metalloids and its oxidized form, arsenic trioxide (As_2O_3) 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 ($NaAsO_2$, Sodium metaarsenite, KOMINOX), a sodium salt of arsenous acid, is a water-soluble, therefore, orally bioavailable, trivalent arsenical compound. KML001 has shown a potent anticancer 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_{50}) 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 research of epidemiology [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 has been 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 to hematologic malignancies. Doxercalciferol, a low calcemic vitamin D2 derivative with the similar effect as 1, 25-dihydroxyvitamin D3, can be safely given to patients and was investigated in phase II studies of the myelodysplastic syndrome [17] and androgen-independent prostate cancer [18].

In our 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. And here, 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. YY 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 μg/ml streptomycin and incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Chemicals and antibodies. KML001 was obtained from Komipharm International (Siheung-Si, Gyeonggi-Do, South Korea). As_2O_3 was purchased from Sigma-Aldrich (Saint 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). Cells ($5x10^3$ cells/well) were seeded in 96-well microtiter plates (Falcon) and then incubated at 37˚C for 48 hours. After adding 10 μl of the CCK-8 solution to each well of the plate, the plate was incubated for at least 1 hour 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 over 2 hours at 4˚C and then stained with 50 μg/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 FACS Canto II (Becton-Dickinson, San Jose, CA, USA) equipped with an FACSDiva software 8.0.1 (Becton-Dickinson, San Jose, CA, USA).

Evaluation of apoptosis. Apoptosis was evaluated by FITC Annexin V apoptosis detection kit according to manufacturer's instructions. Cells were washed twice with cold PBS, then resuspended in the 1×binding buffer, containing 0.01 M Hepes/NaOH, 0.14 M NaCl and 2.5 mM $CaCl_2$, pH 7.4. Next, a total of 100 μl of the mixtures was transferred to a 5 ml culture tube and then incubated with 5 μl Annexin V-FITC and 5 μl propidium iodide for 15 minutes at room temperature in the dark. Subsequently, 400 μl of 1×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 μM Fluo-4 AM (Thermo Fisher Scientific, USA) and 0.1% Pluronic F-127 (Thermo Fisher Scientific, USA) in PBS for 60 minutes at 37˚C. After removing the medium in time, each well was washed with PBS and 200 μl 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 significance was determined by using Students' 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 vitro* models of human ALL, for 48 h with two different types of arsenic compounds, arsenic trioxide and KML001. Results showed that KML001 inhibited cell proliferation of two cell lines at a fairly lower concentration (more than 100 folds) than As_2O_3 [Fig. 1AB].

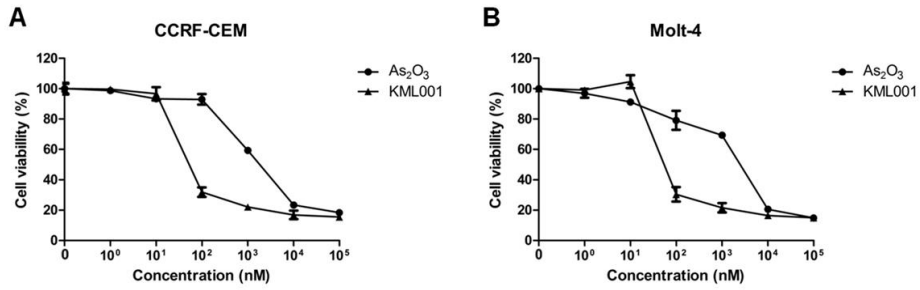

Figure 1. The superiority of KML001 compared with arsenite trioxide. CCRF-CEM cells and Molt-4 cells were treated with KML001 or arsenite trioxide at a variety of concentrations (0, 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 nM) (A and B), the data are presented as the mean values from three separate experiments±S.D..

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 two cell lines, decreased Bcl-xL and increased Bax were observed after 48 h 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/$p21^{WAF1}$ 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-CCEM 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.

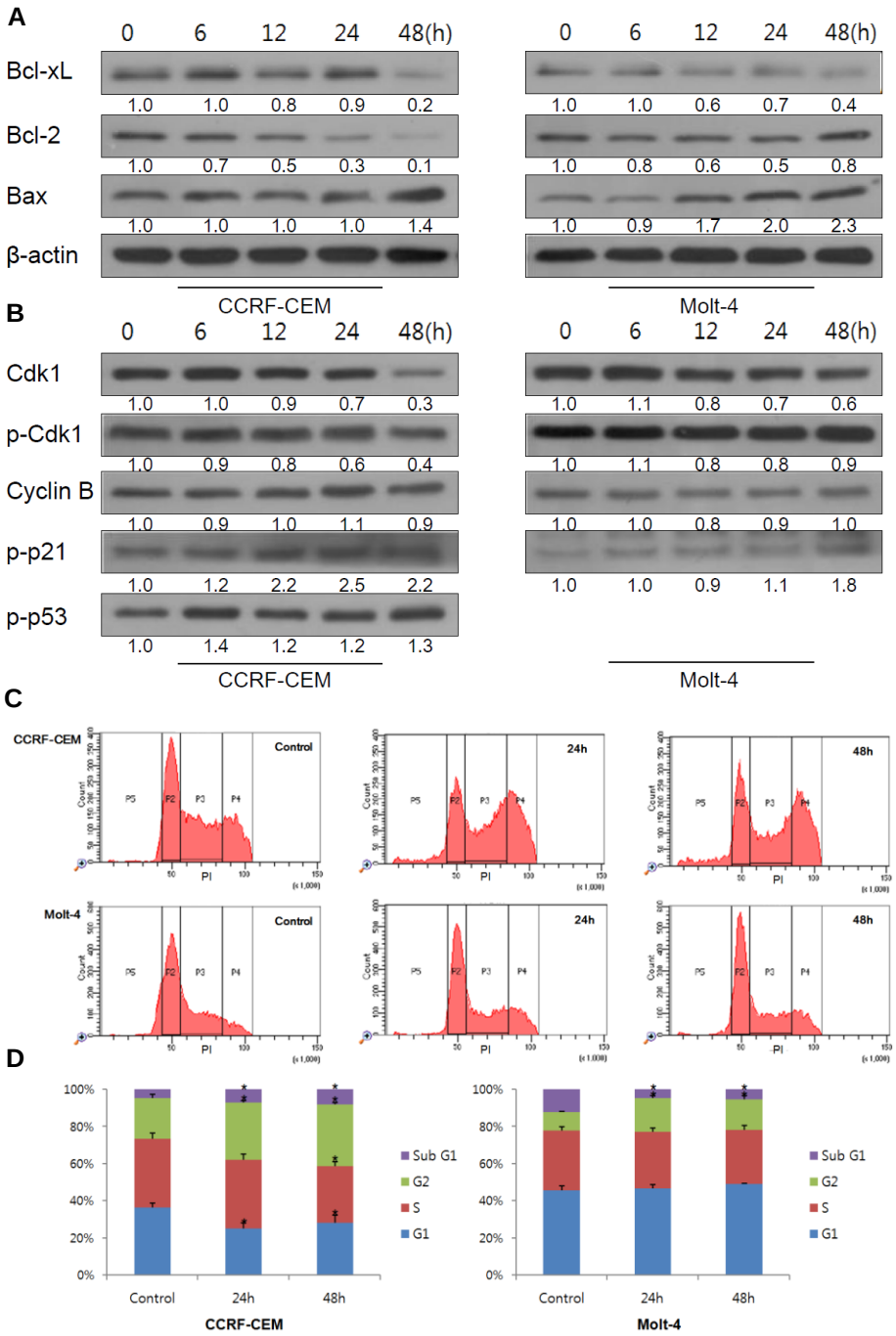

Figure 2. Antileukemic effects of KML001 on ALL cells. (A) Western blot analysis documents a time-dependent modulation of Bcl-2 family members expression by KML001. Antibody to β-actin served as a loading control. (B) Western blot analysis of expression of cell cycle related protein: Cdk1, p-

Cdk1, Cyclin B, p-p21 and p-p53. Image J has been used for quantify protein bands. (C) After treated with KML001 for 24 h 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±S.D..

Synergistic antileukemic effect of KML001 combined with Doxercalciferol. We also investigated whether KML001 and Doxercalciferol 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 Doxercalciferol at a ratio of 50 nM : 15.625 μM or 40 nM : 25 μM (KML001 : Doxercalciferol), repectively. CI and cell viability were then calculated. In both two cell lines, the combined treatments were synergistic, as indicated by CI below 1 (CCRF-CEM CI=0.741, Molt-4 CI=0.675).

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 Doxercalciferol [Fig. 3].

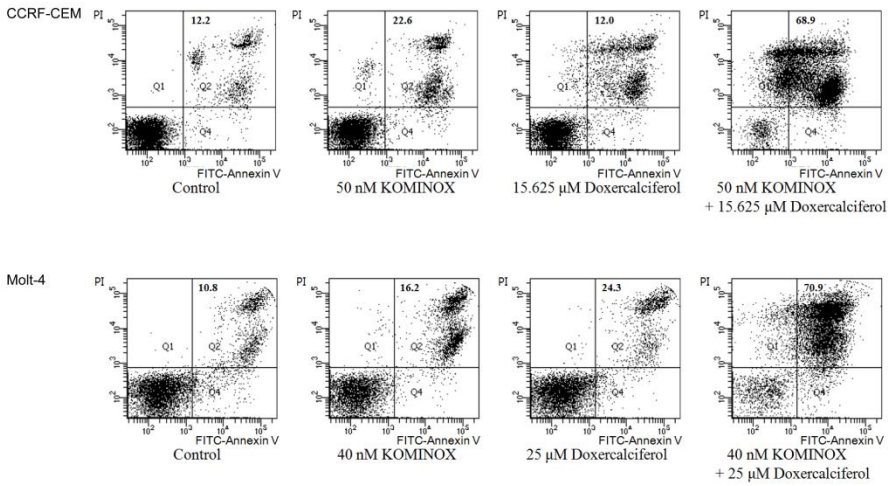

Figure 3. Synergistic antileukemic effect of KML001 combined with Doxercalciferol. Flow cytometric analysis of Annexin V-FITC/PI-stained CCRF-CEM and Molt-4 cells treated for 48 h with KML001 and Doxercalciferol either alone or combination. A significant increase of apoptosis cells was observed in groups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

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

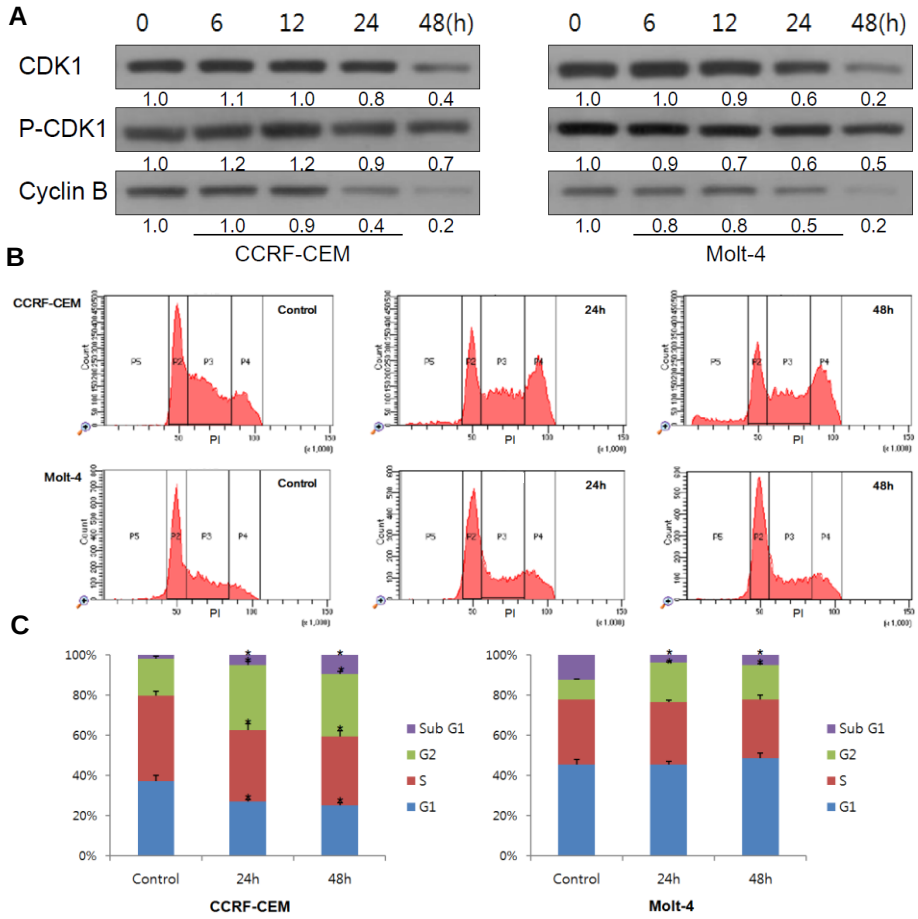

Figure 4. Doxercalciferol induces cell arrest at G2/M phase. (A) Western blot analysis of expression of cell cycle related protein: Cdk1, p-Cdk1, Cyclin B. Image J has been used for quantify protein bands. (B) After treated with Doxercalciferol for 24 h or 48 h or media as control, cells were stained 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±S.D..

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 vitro* ALL models. In results, 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 also 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 both 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 green signal, which indirectly reflects intracellular Ca^{2+} concentration, increased as time increases after treatment of Doxercalciferol while there was relatively less change observed in groups treated with KML001.

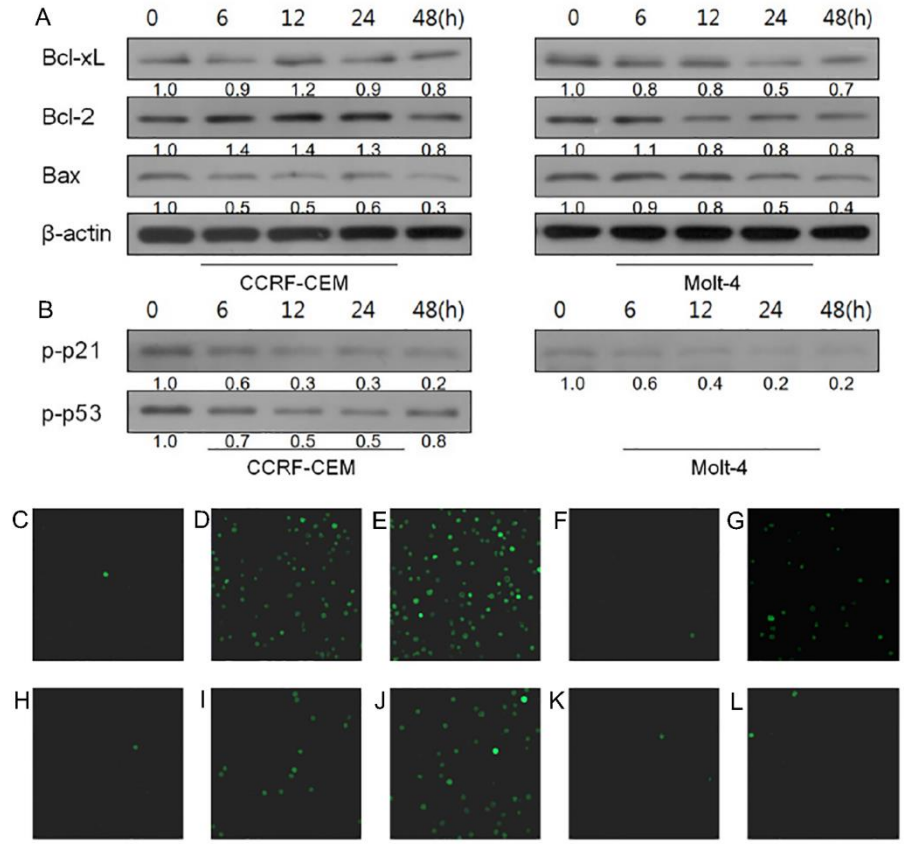

Figure 5. Mechanisms of action of Doxercalciferol-induced cell cycle and apoptosis. (A and B) Western blot analysis of Bcl-2 family members, p-p21 and p-p53 expression after treating cells with Doxercalciferol. Image J has been used for quantify protein bands. Intracellular calcium accumulation occurred in CCRF-CEM and Molt-4 cells. Images (green) reveal $[Ca^{2+}]i$ under a confocal microscope, with the depth of the color representing 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 24h and (E and J) for 48h; (F and K) Cells treated with KML001 for 24h and (G), (L) for 48h.

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 cannot survive after treatment due to problems like 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 did induce 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 check point. Down regulation 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 blot 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. And 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, treatment of Doxercalciferol 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 like telomere length shortening. However, Doxercalciferol induced cell apoptosis and cell cycle arrest in a totally different way, which may involve calcium related pathway but without activation of proteins like p53, p21 or Bcl-2 family involved pathway. Our study provided evidence that KML001 could be an effective anti-leukemic agent especially with Doxerclciferol in ALL treatment in the terms of cell level. Its availability on clinical application, however, should be further investigated.

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요약 (국문초록)

서론: 비소 화합물의 일종인 KML001 (NaAsO2, 메타아비산나트륨, KOMINOX)은 생체 내 및 생체 외 실험들을 통해 비호지킨 림프종 (NHL)에 효과적으로 작용하는 것으로 알려져 있지만 급성 림프구성 백혈병 (ALL)에서의 효과는 아직 자세히 밝혀지지 않았다. 본 연구에서는 KML001의 ALL에 대한 항백혈병 효과와 그 작용 기전, 그리고 KML001과 비타민 D2 유도체 인 Doxercalciferol의 시너지 효과 (synergy effect)의 가능성을 조사하였다.

방법: KML001의 ALL에 대한 항백혈병 효과를 조사하기 위해, ALL 세포주 인 CCRF-CEM 및 Molt-4에서 KML001을 처리한 후 MTT assay를 통해 세포 독성을 시험하였고, 유세포 분석(flow cytometry)을 이용해 세포 주기 정지 (cell cycle arrest) 및 세포 사멸 (cell apoptosis)을 확인하였다. 또한, KML001과 Doxercalciferol의 ALL 치료를 위한 시너지 효과를 확인하기 위해 MTT assay와 유세포 분석을 이용하였다. 두 약물의 시너지 효과의 작용기전을 증명하기 위해서는 웨스턴 블롯(western blot), 유세포 분석 및 세포 내 칼슘 분석을 수행하였다.

결과: KML001은 2 가지 유형의 ALL 세포주에 세포 증식을 억제하였다. ALL 세포는 KML001 처리 후 시간 경과에 따라 세포사멸을 증가시켰다. 다른 종류의 백혈병 세포주들에선 KML001에 의해 G0/G1기에서 정지가 일어나는 것과는 달리, ALL 세포주들에선 G2/M 기에서 세포주기 정지가 일어났다. Doxercalciferol은 세포사멸과 세포주기 정지, 그리고 칼슘 관련 신호전달체계에 KML001과는 다르게 영향을 주었으며, KML001과 함께 처리하였을 때 항백혈병 작용에 시너지 효과가 나타났다.

결론: 본 연구의 결과는 KML001 이 ALL 에서도 유망한 치료효과를 보였으며, 특히 Doxecalciferol 과 함께 작용할 때 더 큰 효과를 갖는다는 것을 증명하였다.

주요어: KML001, Doxercalciferol, 급성 림프구성 백혈병, CCRF-CEM, Molt-4, 시너지 효과

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