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서울대학교 대학원

의학과 분자종양의학 전공

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# Evaluating the role of dual PI3K/Akt/mTOR pathway inhibitor in Acute Myeloid Leukemia

by

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# Abstract

Phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signal transduction pathway integrating signals from multiple receptor tyrosine kinases has been firmly established as a major determinant for cell growth, proliferation, and survival in a wide array of solid cancers. PI3K/Akt/mTOR pathway is frequently activated in acute myeloid leukemia (AML) cells and contributes to survival and drug-resistance of AML through various mechanisms. BEZ235 is one of the most promising dual inhibitor of PI3K and mTOR currently under clinical development in solid tumor area. In this study, the potential of BEZ235 was investigated as antileukemic agent using alone or with cytarabine arabinoside (AraC) as combination regimen.

AML cell lines KG-1, MV 4-11, THP-1 and HL60 were treated with AraC, BEZ235 and combination regimen with various mixed ratio. BEZ235 effectively inhibited leukemic cell growth with similar range of half maximal inhibitory concentration (IC50)

values among different cell lines. Apoptosis was induced gradually as BEZ235 concentration increased, but significant level of apoptosis was not shown even at higher concentration beyond IC50 value. Then, AraC-resistant MV4-11 and THP-1 cell lines were chosen to investigate interaction between BEZ235 and AraC. Using CalcuSyn software based on Chou and Talalay analysis, Combination Index (CI) value was calculated in each combination regimen. Moderate to strong synergism was shown and it was well maintained as combination ratio of AraC versus BEZ235 gradually decreased from 20:1 to 1,000:1. BEZ235 reduced resistance to AraC when it was added as combination regimen, and significance of combination effect changed according to AraC concentration. When antileukemic effect was compared among combination regimens with different schedules, synergism was maximized when BEZ235 was pretreated before AraC administration. This means BEZ235 sensitizes leukemic cells to apoptotic effect of AraC.

Genetic alteration in PI3K/Akt/mTOR pathway is an attractive target to investigate and dual pathway inhibitor BEZ235 has potential to maximize AML treatment through sensitization of

leukemic cells to conventional drug. Exact understanding for pathway to control leukemic cells and effective inhibition of key controlling pathways with innovative new drugs will be crucial to make huge progress in AML treatment.

Key words:

AML, PI3K/Akt/mTOR pathway, dual inhibitor, synergism

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| | |
|---|---|
| ALL | acute lymphoblastic leukemia |
| AML | acute myeloid leukemia |
| AraC | cytarabine arabinoside |
| CCK-8 | cell counting kit-8 |
| CI | combination index |
| ED | effective dose |
| FA | fraction of affected cells |
| IC50 | half maximal inhibitory concentration |
| mTOR | mammalian target of rapamycin |
| PI | propidium iodide |
| PI3K | phosphatidylinositol 3-kinase |

# Introduction

Phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signal transduction pathway integrates signals from multiple receptor tyrosine kinases to control cell proliferation and survival. Akt is activated at downstream of PI3K and can activate several effector proteins, including the serine/threonine kinase mTOR, which regulates both nutrient sensing and protein translation through phosphorylation of its substrates, p70S6 kinase and 4EBP-1. PI3K/Akt/mTOR signal transduction pathway has been firmly established as a major determinant for cell growth, proliferation, and survival in a wide array of solid cancers [1-3].

Acute myeloid leukemia (AML) is a clonal hematopoietic disorder caused by differentiation arrest and proliferation of specific stage of immature progenitor cells. Many studies have demonstrated that AML cells are characterized by recurrent mutations of genes involved in cell differentiation, survival, and proliferation. According to molecular basis of AML, two major types of genetic alterations are required to induce leukemic

transformation [4, 5]. Alteration in the activity of transcription factors that control hematopoietic differentiation is one necessary event for leukemogenesis. Transcription factor fusion protein such as Acute Myeloid Leukemia1/Eight Twenty One (AML1/ETO) or ProMyelocytic Leukemia/Retinoic Acid Receptor α (PML/RARα) is an example of this alteration, which blocks differentiation of myeloid cells [6]. Activation of receptor tyrosine kinase signaling pathway is another type of genetic alteration, which is related with disordered cell growth and up-regulation of cell survival genes. Likewise in solid cancers, recent studies have shown that PI3K/Akt/mTOR pathway, well-known key player in the regulation of cancer growth and metabolism, is frequently activated in AML cells and this pathway contributes survival and drug-resistance of AML [5, 7-9]. Many possible causes have been investigated for PI3K/Akt/mTOR pathway activation in AML. FLT3/ITD mutation, which occurs up to 30% of AML patients, ensures in stimulation of downstream signaling pathways including PI3K/Akt/mTOR [10, 11]. One report has shown that FLT3 kinase inhibitor results in diminished levels of phosphorelated Akt, but still there is a need for further

investigation. Other report has revealed that mutation of c-Kit is detected in about 20-30% of AML patients. Mutation in c-Kit is generally known for activating PI3K/Akt/mTOR pathway, but this result is not exactly matching with AML cell line studies [12, 13]. There have been also some reports indicating the possible causes of PI3K/Akt/mTOR pathway are due to autocrine/paracrine secretion of VEGF, IGF-1, FGF, and so on [14-17]. Whatever the main cause is, it is somewhat obvious that activation of PI3K/Akt/mTOR pathway plays an important role in AML pathogenesis and AML cell survival.

Based on knowledge about PI3K/Akt/mTOR pathway in cancers, the first pathway targeting compound, quercetin, was introduced in 1992 [18]. Following the first compound, wortmannin and LY294002 were identified as a potent PI3K inhibitor [19, 20]. Though all of them were impractical to use due to their toxicities and insolubilities, these agents opened the door to discover novel agents targeting PI3K/Akt/mTOR pathway. Akt inhibitors have been designed but some of them were terminated due to toxic side effects. Targeting another PI3K downstream molecule mTOR showed a great success. Currently two compound,

RAD001 and CCI-779 were commercially available in some solid tumor indications and investigation are still ongoing to optimize regimen or to develop new indications. [21]. Among many pathway targeting agents including pan-PI3K inhibitor, selective PI3K inhibitor, PI3K-isoform inhibitor, Akt inhibitor or new mTOR inhibitor, dual PI3K-mTOR inhibitor is one of the most interesting area to be investigated further. Inhibition of specific target molecule in pathway has shown to induce rebound activation of other molecule within the pathway. This is one of well known mechanism of resistance to mTOR inhibitors. Development of dual pathway inhibitors is based on the rationale that they are less likely to induce drug resistance by targeting two kinases at the same time [22].

BEZ235 (Novartis, Basel, Switzerland) is one of the most promising dual inhibitor of the PI3K and the downstream mTOR among many new agents currently under clinical development. It potently inhibits class I PI3K catalytic activity by competing at its ATP-binding site and also inhibits mTOR catalytic activity without targeting other protein kinases [23]. Like other PI3K/Akt/mTOR pathway targeting agents, BEZ235 was mainly investigated in

various solid tumors [24-29]. In those indications, BEZ235 showed sustained inhibition of mTORC1/ mTORC2 signal which resulted in effectively inducing cell apoptosis and tumor shrinkage. Importantly, BEZ235 also potentiated other agents' anticancer activities when it was added. One very recent publication reported BEZ235 increased tumor radiosensitivity by normalizing solid tumor vasculature to show broad translational importance to be combined with irradiation therapy [30]. Based on all these data, currently phase I/II clinical trials are ongoing to use BEZ235 alone or to combine with conventional agents in various solid tumors. Though recently it was reported that BEZ235 effectively inhibited the proliferation of multiple myeloma cells and it was synergistic with melphalan and doxorubicin [31], still investigation of antitumor potential of BEZ235 in hematologic field is still far behind comparing research in solid tumor area.

For a long time, the standard therapeutic approach for AML continues to be based on anthracycline and cytarabine arabinoside (AraC). Though survival has been prolonged due to progression in clinical research, leukemia is not the area where

new innovative drugs are being actively penetrated and delivering clinical benefit as they are leading huge advance in other cancer fields. Considering the importance of PI3K/Akt/mTOR pathway and the promising data of BEZ235 in other indications, BEZ235 can be an important new candidate for targeted therapy in AML. In this study, the potential of dual PI3K-mTOR inhibitor BEZ235 was investigated as monotherapeutic agent using alone and as part combination regimen combined with conventional cytotoxic agent AraC.

# Materials and Methods

## Cell lines and cultures

AML cell lines for experiment were selected based on previous report showing various AraC sensitivities between different cell lines [32]. According to this report, THP-1〔derived from a 1-year-old male with AML M5 and t(9;11)〕 was the most resistant against AraC with the highest IC80 value among 8 different leukemic cell lines and MV 4-11〔derived from a 10-year-old male with AML M5 and t(4;11)〕 followed the next. KG-1〔derived from a 59-year-old male with acute monocytic leukemia at relapse〕 was the most sensitive one with the lowest IC80 value. Above these 3 cell lines, HL60〔derived from a 36-year-old woman with acute promyelocytic leukemia〕 was selected to see whether pathway targeting agent showed different sensitivity in promyelocytic leukemia cell line, for which different treatment approach is applied based on unique sensitivity against all-*trans* retinoic acid. Four cell lines, THP-1, MV4-11, KG-1 and HL60 were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea)

or the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cell lines except MV4-11 were grown in RPMI 1640 medium (WelGene, Daegu, Korea) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), 100 U/mL penicillin and 100 ug/mL streptomycin (Gibco/Invitrogen, Grand Island, NY, USA) in 5% $CO_2$ humidified atmosphere at 37℃. MV4-11 cell line was grown in Iscove's Modified Dulbecco's Medium (IMDM; Gibco/Invitrogen, Grand Island, NY, USA) supplemented with same composition as described above.

## Materials

AraC was purchased from Sigma (St. Louis, MO, USA) and PI3K/mTOR pathway targeting agent, BEZ235 was kindly provided by Novartis (Basel, Switzerland). Cell viability after drug treatment was measured using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). FITC Annexin V Apoptosis Detection Kit was purchased from BD Pharmingen (San Diego, CA, USA).

## Cell Treatment and Cell viability Assays

To test the sensitivity of AraC and BEZ235 as monotherapy, cells were seeded onto 96-well plate at a density of $3x10^4$ cells per well. After 24 hours, quadruplicate wells were treated with varing concentrations of AraC (1-300,000 nM) and BEZ235 (0.1-3,000 nM), respectively, for 48 hours. Cell viability was assessed using CCK-8 proliferation assay kit as manufacturer's recommendation utilizing highly water-soluble tetrazolium salts. After adding 10 uL of CCK-8 solution to each well of the plate, absorbance at 450 nm was measured using microplate reader 3 hours later. The relative percentages of viable cells compared with untreated controls were calculated on each concentration of drug. Each experiment was carried out using 4 replicate wells for each drug concentration and carried out independently at least three times. Dose-response curve and half maximal inhibitory concentration (IC50) were calculated using PRISM software version 5.0 (GraphPad Software, San Diego, CA, USA). The IC50 values were defined as the concentrations that inhibited 50% of cell growth.

## Interaction Analysis

To determine synergistic, additive, or antagonistic effects between AraC and BEZ235, the Chou and Talalay analysis based on the median-effect principle was used [33-35]. Based on Chou and Talalay method, CalcuSyn software program (Biosoft, Cambridge, UK) calculated the combination index (CI), taking into account both potency［median dose (Dm) or IC50］and the shape of the dose-effect curve (the m value). The combination-index method is a mathematical and quantitative representation of a two-drug pharmacologic interaction. This method defines a CI values of 0.9 to 1.1 as nearly additive, 0.7 to 0.9 as moderate to slight synergism, 0.3 to 0.7 as synergism and <0.3 as strong synergism, whereas values >1.1 are considered as antagonism. To perform this analysis, AraC and BEZ235 were combined at various fixed ratio (20:1, 100:1, 1,000:1). For each concentration of combination regimen, the fraction affected and the corresponding CI values were calculated. Each experiment was carried out using 4 replicate wells for each drug concentration and carried out independently at least three times.

## Apoptosis Analysis

Apoptotic cell death was detected using a FITC Annexin V apoptosis detection Kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. To differentiate and quantitatively measure early and late apoptotic cell portions, both Annexin V-FITC and propidium iodide (PI) staining were performed. Cells were washed twice with cold PBS and then resuspended with binding buffer at a concentration of $1\times10^6$ cells/mL. Then, 100 uL of the solution ($1\times10^5$ cells) was transferred to a 5 mL culture tube with 5 μL Annexin V-FITC and 5 μL PI. Cells were incubated at room temperature in the dark for 15 minutes. Then 400 μL of binding buffer was added. Flow cytometric analysis was carried out within one hour. At least 10,000 events per sample were acquired.

## Statistics

Statistical analysis and nonlinear regression for dose-response curve fitting were performed using Prism software version 5.0 (GraphPad Software, San Diego, CA, USA). Statistical comparison of two groups was performed by Student *t* test and comparison

between three groups by one way ANOVA with Turkey's multiple comparison tests. To analyze two groups affected by two different factors, two-way ANOVA with Bonferroni multiple comparisons test was used. Mean values with standard deviation were shown in the figures. Data were confirmed by at least three independent experiments. *P* values below 0.05 were considered statistically significant. Interaction between AraC and BEZ235 was analyzed and classified using CalcuSyn software version 2.1 (Biosoft, Cambridge, UK).

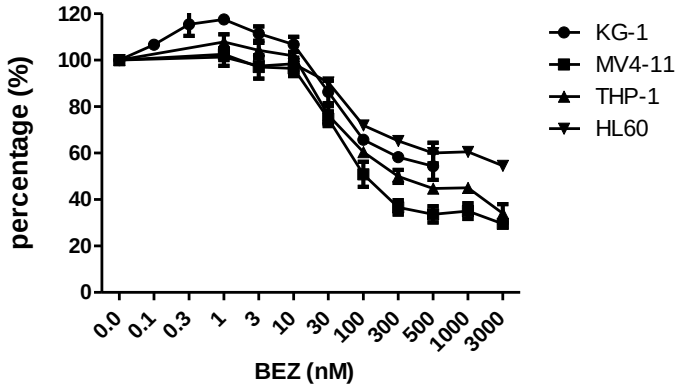
# Results

## BEZ235 inhibits cell growth in myeloid leukemia cells

The effect of BEZ235 on myeloid leukemia cell growth was assessed through measurement of cell viability using CCK-8 proliferation assay. KG-1, MV4-11, THP-1 and HL60 cell lines were incubated with increasing concentration of BEZ235 (0.1-3,000 nM) for 48 hours. Cell viability was presented as the relative percentage of viable cells compared with untreated control at each concentration of BEZ235. As shown in Figure 1A, no significant growth inhibition was shown till 10 nM of BEZ235 in all cell lines, but gradually cell growth was effectively inhibited in dose-dependent manner. Statistical analysis using one way ANOVA with Turkey's multiple comparison tests was performed. No statistically significant differences were shown between four cell lines (KG-1 vs. MV4-11/THP-1/HL60, MV4-11 vs. THP-1/HL60, THP-1 vs. HL60). Then, Dose-Response curve was plotted using Prism software (Figure 1B). IC50 values of BEZ235 were

calculated in each cell line. Four cell lines represent sigmoid pattern dose-response curve with similar range of IC50 values. IC50 values of KG-1, MV4-11, THP-1 and HL60 were 34.97 nM (95% Confidence intervals; 23.29 to 52.51), 46.40 nM (95% Confidence intervals; 33.76 to 63.77), 44.67 nM (95% Confidence intervals; 27.73 to 71.94) and 61.60 nM (95% Confidence intervals; 44.08 to 86.07), respectively.

(A)




(B)

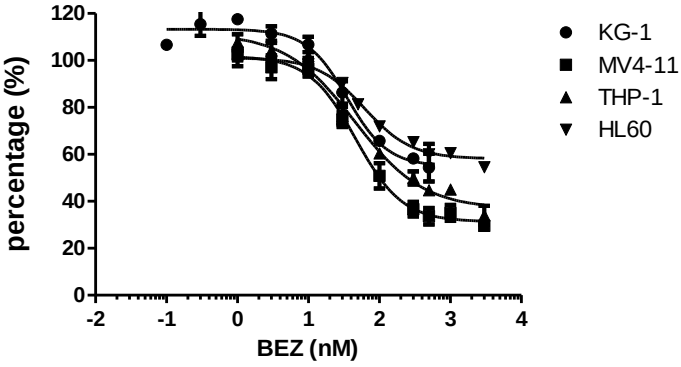



Figure 1. Effect of BEZ235 on growth of myeloid leukemia cells. Cell viability was presented as the relative percentage of viable cells compared with untreated control after 48 hours of incubation.

(A) Cell viabilities at each concentration of BEZ235 (B) plotted Dose-Response curve in log scale using Prism software. Mean values with standard deviations are shown in the figures. Data were confirmed by at least three independent experiments.

**BEZ235 induces apoptosis in myeloid leukemia cells**

Next, potency of BEZ235 to induce apoptosis was examined using alone. Three cell lines KG-1, MV4-11 and THP-1 were incubated with increasing concentrations of BEZ235 (0, 10, 100, 500, and 1,000 nM) for 48 hours. Apoptosis was measured by Flow Cytometric analysis using Annexin-V-FITC and PI staining. As one of the early event in apoptosis, the translocation of phosphatidylserine from the inner layer to the outer layer of the plasma membrane can be detected by staining of FITC Annexin V. The membranes of dead and damaged cells are permeable to PI, whereas viable cells with intact membranes exclude PI. So, viable cells are considered to be negative for both FITC Annexin V and PI. Cells in early apoptosis are FITC Annexin V positive but PI negative, and cells in late apoptosis are positive for both FITC Annexin V and PI. Figure 2-4 shows BEZ235 induces leukemic cell apoptosis gradually in all three cell lines, but no dramatic induction of apoptosis was shown even at much higher concentration of BEZ235 beyond IC50 value of each cell line. The percentages of viable cells (bottom left quadrant in FACS), early

apoptotic cells (bottom right quadrant in FACS), and late apoptotic cells (top right quadrant in FACS) were presented as bar graph (Figure 2E-4E), respectively.

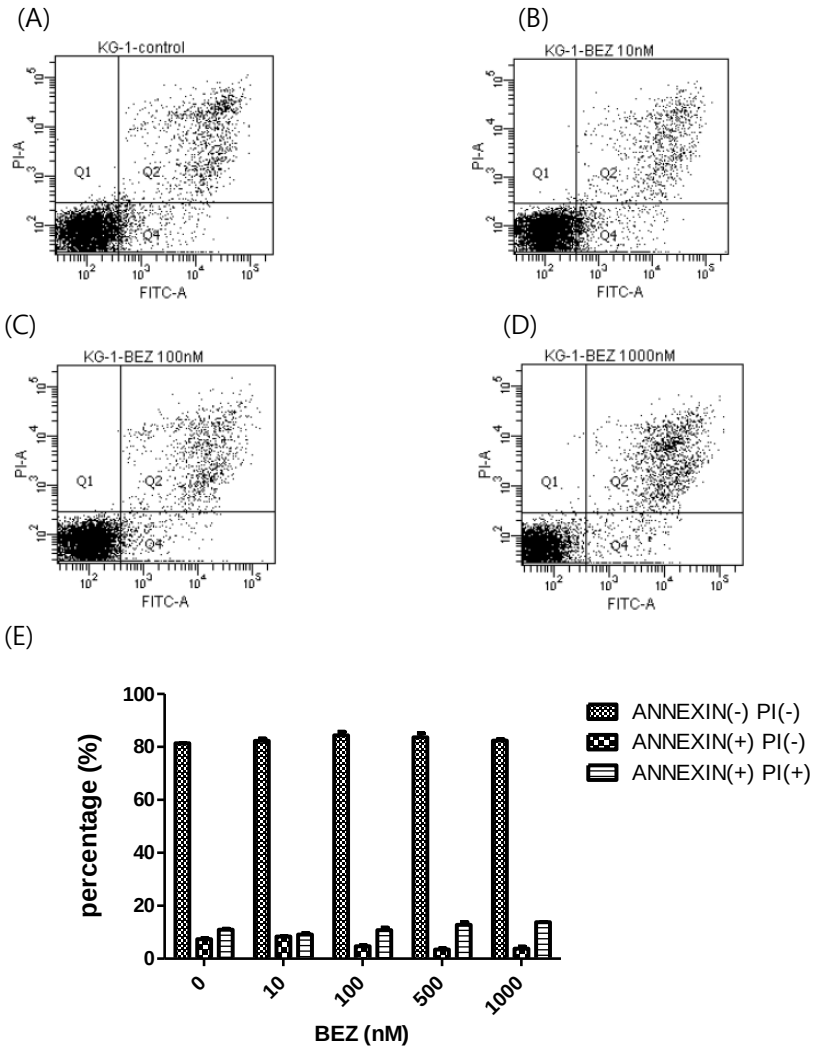



Figure 2. Apoptosis induction in KG-1. Flow cytometric analysis was done for Annexin V-FITC/PI staining. (A) At BEZ 0 nM, (B) At BEZ 10 nM (C) At BEZ 100 nM (D) At BEZ 1,000 nM (E) Bar graphs showing the percentage of viable, early apoptotic, and late apoptotic cells at each cencentration of BEZ235.

Mean values with standard deviations are shown in the figures. Data were confirmed by at least three independent experiments.

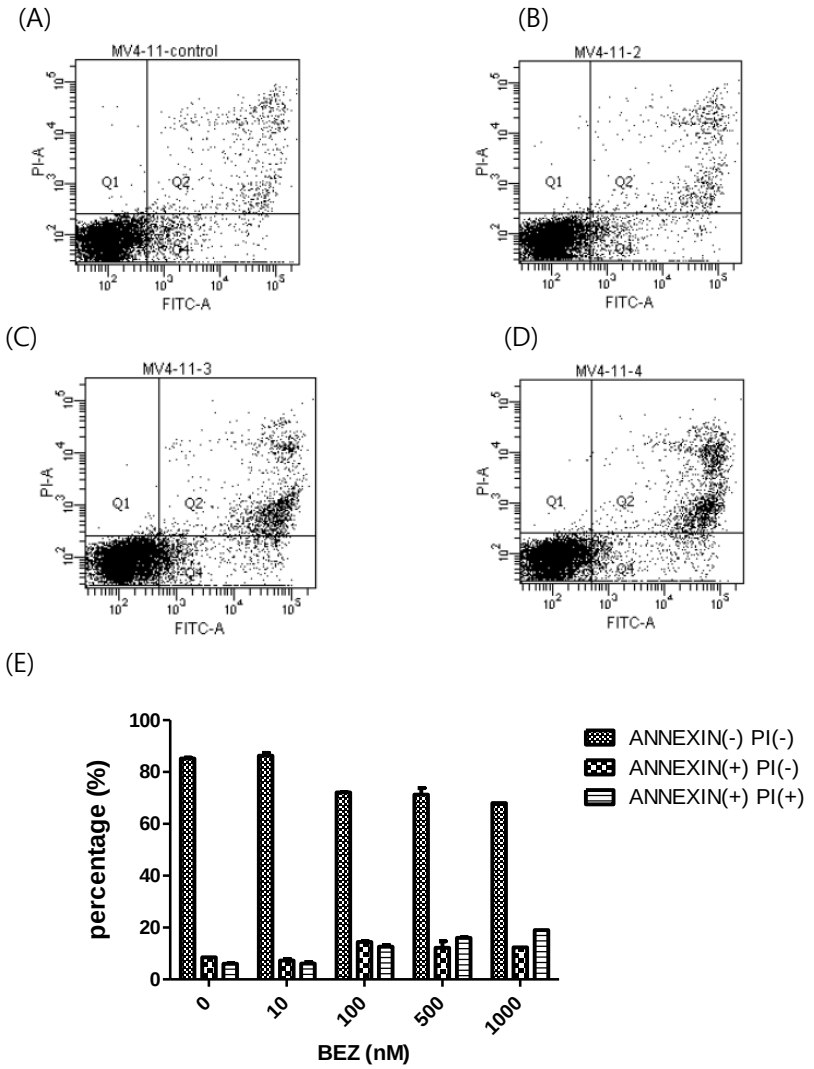



Figure 3. Apoptosis induction in MV4-11. Flow cytometric analysis was done for Annexin V-FITC/PI staining. (A) At BEZ 0 nM, (B) At BEZ 10 nM (C) At BEZ 100 nM (D) At BEZ 500 nM (E) Bar graphs showing the percentage of viable, early apoptotic, and late apoptotic cells at each cencentration of BEZ235. Mean values with standard deviations are shown in the figures. Data were confirmed by at least three independent experiments.

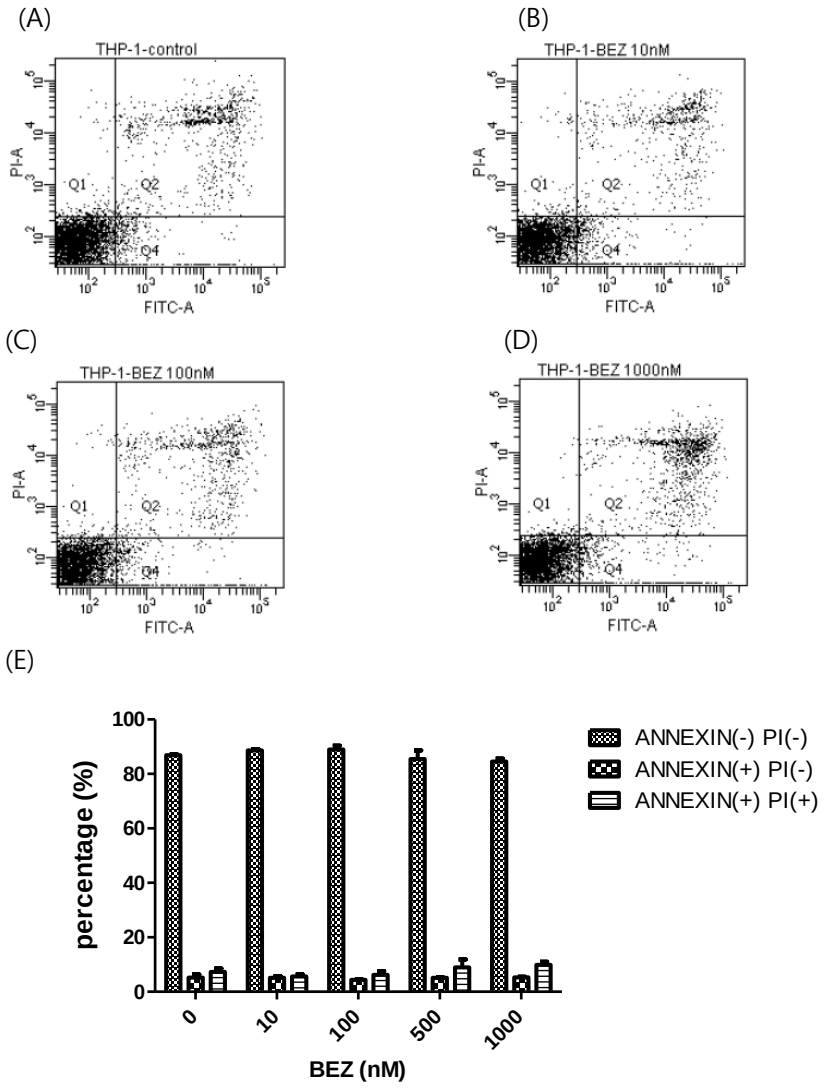



Figure 4. Apoptosis induction in THP-1. Flow cytometric analysis was done for Annexin V-FITC/PI staining. (A) At BEZ 0 nM, (B) At BEZ 10 nM (C) At BEZ 100 nM, (D) At BEZ 1,000 nM (E) Bar graphs showing the percentage of viable, early apoptotic, and late apoptotic cells at each cencentration of BEZ235. Mean values with standard deviations are shown in the figures. Data were confirmed by at least three independent experiments.

## Myeloid leukemia cells show different sensitivity to AraC

The effect of AraC on myeloid leukemia cell growth was assessed. KG-1, MV4-11, THP-1 and HL60 cell lines were incubated with increasing concentrations of AraC (1-300,000 nM) for 48 hours. Dose-Response curve was plotted using Prism software and statistical analysis using one way ANOVA with Turkey's multiple comparison tests was performed. As shown in Figure 5, all cell lines showed sigmoid pattern dose-response curves as AraC dose increased, but sensitivities to AraC were different cell by cell. Growth of HL60 cell line was most effectively inhibited in lower range of AraC concentration showing higher sensitivity. THP-1 was the most resistant cell line to AraC and MV4-11 followed the next. IC50 values of HL60, KG-1, MV4-11 and THP-1 were 143.9 nM (95% Confidence intervals; 121.9 to 169.9), 1,247 nM (95% Confidence intervals; 423.8 to 3670), 4,255 nM (95% Confidence intervals; 3,322 to 5,451) and 7,336 nM (95% Confidence intervals; 6,432 to 8,368), respectively. Statistically significant difference was shown between KG-1 and HL60 ($P<0.05$).

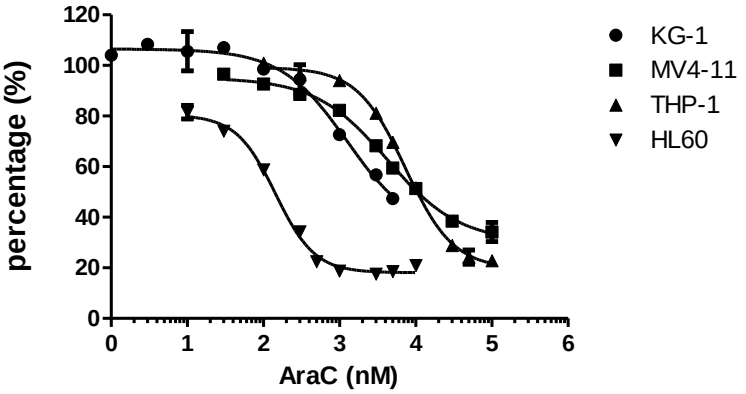



Figure 5. Effect of AraC on growth of myeloid leukemia cells. Cell viability was presented as the relative percentages of viable cells compared with untreated controls after 48 hours incubation. Dose-Response curve was plotted in log scale using Prism software. Mean values with standard deviations are shown in the figure. Data were confirmed by at least three independent experiments.

**BEZ235 exerts synergism with AraC**

To test drug interaction between AraC and BEZ235, cells were incubated with increasing concentration of AraC, BEZ235 and combination regimen with different fixed combination ratio for 48 hours. Cell viability data were analyzed using the CalcuSyn software to see whether interaction is synergistic, additive, or antagonistic in each regimen. Fraction of affected cells (FA) and CI value of each combination in KG-1, MV4-11 and THP-1 cell line were presented in Table 1. Moderate to strong synergism were shown with CI values <0.8 in most combination cases. As shown in Figure 6, BEZ235 exerts synergism with AraC (CI <0.9) through most range of fractional effects at 100:1 combination ratio in all three cell lines.

In the next step, drug interaction was analyzed for different combination regimen as 20:1, 100:1 and 1,000:1 ratio between AraC and BEZ235. This was designed to see difference in drug interaction as portion of BEZ235 was decreased to minimal as 1,000:1 ratio. MV4-11 and THP-1, two resistant cell lines to AraC, were selected as shown in Figure 5. CI-values were calculated at

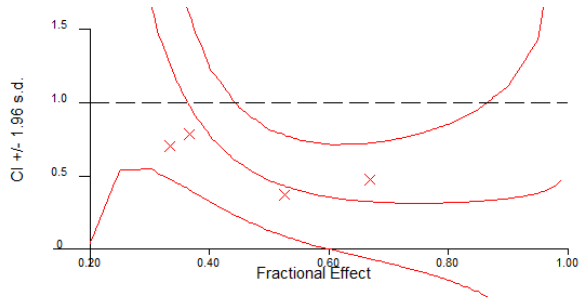
each dose level affecting 50% (ED50), 75% (ED75) and 90% (ED90) of cell lines (Table 2). In MV4-11 cell line, synergism became stronger as affected fraction increased in 100:1 combination ratio. Synergism was well maintained over broad range of concentration as BEZ235 portion decreased to 1,000:1 combination ratio. Interaction between AraC and BEZ235 was also synergistic in AraC-resistant THP-1 cell line. As BEZ235 combination ratio decreased from 20:1 to 1,000:1, synergism was stronger or well maintained through each dose level of ED50, ED75 and ED90.

Table 1. Interaction between AraC and BEZ235 as combination regimen

| | AraC (nM) | BEZ (nM) | FA | CI |
|---|---|---|---|---|
| **KG-1** | 3,000 | 30 | 0.291 | 0.965 |
| | 5,000 | 50 | 0.428 | 0.557 |
| | 10,000 | 100 | 0.539 | 0.510 |
| | 30,000 | 300 | 0.637 | 0.750 |
| **MV4-11** | 3,000 | 30 | 0.489 | 0.220 |
| | 5,000 | 50 | 0.515 | 0.316 |
| | 10,000 | 100 | 0.669 | 0.264 |
| | 30,000 | 300 | 0.748 | 0.480 |
| **THP-1** | 3,000 | 30 | 0.334 | 0.704 |
| | 5,000 | 50 | 0.368 | 0.784 |
| | 10,000 | 100 | 0.526 | 0.375 |
| | 30,000 | 300 | 0.670 | 0.478 |

Cell viabilities under various experimental conditions were determined by CCK-8 assay. Cell viability data was analyzed using the CalcuSyn software to see additive, synergistic, and antagonistic effects. Table shows CI-values obtained from fixed ratio (AraC:BEZ235=100:1) combination regimen in KG-1, MV4-11 and THP-1 cell line. CI; 0.9-1.1 additive, 0.7-0.9 synergistic, >1.1 antagonistic. FA; fraction affected.

(A)

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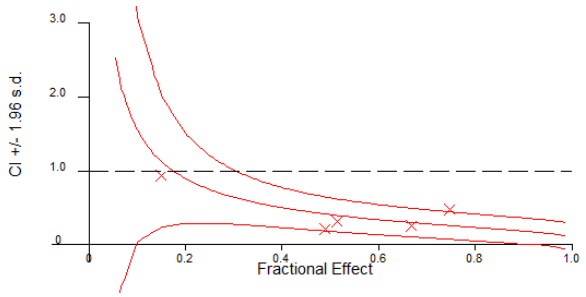


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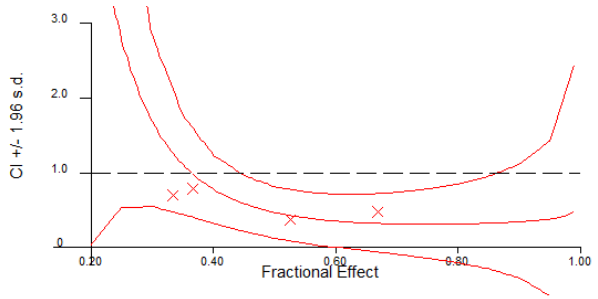



Figure 6. CI plot of AraC plus BEZ235 at a combination ratio of 100:1. (A) KG-1 (B) MV4-11 (C) THP-1 cells. CI was plotted using CalcuSyn software according to cytotoxic fractional effect. CI value below 0.9 means synergism. The figure shows result of one of measurement as an example. Upper and lower lines; 95% confidence intervals, dotted line; CI=1

Table 2. CI-values of different fixed ratio combination regimen

| | | CI | | |
|---|---|---|---|---|
| | | **ED50** | **ED75** | **ED90** |
| **MV4-11** | **AraC : BEZ =100 : 1** | 0.753 | 0.541 | 0.449 |
| | **AraC : BEZ =1,000 : 1** | 0.733 | 0.656 | 0.626 |
| **THP-1** | **AraC : BEZ =20 : 1** | 0.834 | 0.331 | 0.226 |
| | **AraC : BEZ =100 : 1** | 0.589 | 0.637 | 0.719 |
| | **AraC : BEZ =1,000 : 1** | 0.650 | 0.735 | 0.882 |

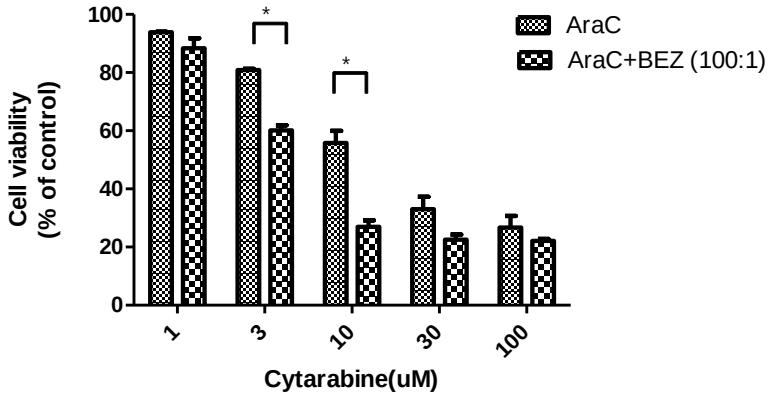
CI-values were calculated at each dose level affecting 50% (ED50), 75% (ED75) and 90% (ED90) of cell lines using CalcuSyn software. Mean values from at least two independent experiments were shown. ED; effective dose.

**BEZ235 reduces chemoresistance to AraC**

To test whether AraC resistance can be overcome by adding BEZ235, comparison was performed between AraC monotherapy and AraC plus BEZ235 combination regimen. THP-1 cell line was chosen because it showed the most resistant sensitivity to AraC in previous data (Figure 5). As shown in Table 2, THP-1 cell line showed sustainable synergism between AraC and BEZ235 in various combination ratios over ED50, ED75 and ED90 dose levels. Referring these data, 100:1 and 1,000:1 combination ratios (AraC versus BEZ235) were chosen to compare cell viabilities between AraC monotherapy and AraC plus BEZ235 combination regimen. THP-1 cells were treated with increasing concentration of AraC (1, 3, 10, 30 and 100 uM) with/without BEZ235 for 48 hours. Two-way ANOVA with Bonferroni multiple comparisons test was used to see how cell viability is affected by two factors, AraC concentration factor and BEZ235 combination factor, respectively, and whether these two factors interact with each other. In both 100:1 and 1,000:1 combination regimen, each of AraC concentration factor and BEZ235 combination factor

affected cell viability statistically significantly. Also these two factors interact significantly in both regimens, which mean BEZ235 combination effect is not all the same throughout different AraC concentration and significance of effect changes according to AraC concentration as shown in Figure 7. By adding BEZ235, AraC resistance was reduced comparing AraC monotherapy over all range of treatment concentration in both ratio of 100:1 (Figure 7A) and lower ratio of 1,000:1 (Figure 7B). Overcoming AraC resistance was more prominent at specific range of AraC concentration as shown statistically significant in Figure 7. When AraC reached high concentration as 100 uM, effect of BEZ235 combination decreased and statistical significance disappeared.

(A)




(B)

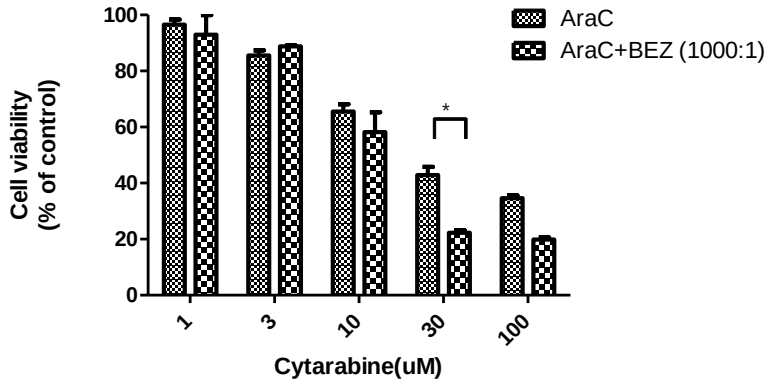



Figure 7. Comparison between AraC monotherapy and AraC plus BEZ235 combination regimen. Statistical comparison was performed using Prism software in each dose level of AraC (1, 3, 10, 30 and 100 nM) in 100:1 and 1,000:1 combination ratio, respectively. Mean values with standard deviations are shown in the figures. (A) AraC versus AraC+BEZ235 at 100:1 ratio. (B) AraC versus AraC+BEZ235 at 1,000:1 ratio. *$P$<0.05, compared with AraC monotherapy.

**Pretreatment with BEZ235 sensitizes leukemia cells to apoptotic effect of AraC**

Interaction between BEZ235 and AraC can be affected by administration order of two drugs. To see when BEZ235 should be administered to maximize antileukemic effect of AraC, cells were treated with different combination regimens and then cell apoptosis was detected by flow cytometry using Annexin V-FITC and propidium iodide (PI) staining. AraC-resistant THP-1 cell line was chosen. AraC 5,000 nM and BEZ235 50 nM were administered simultaneously or consecutively as follows:

(1) cells were treated with AraC only for 48 hours (presented as AraC)

(2) cells were treated with AraC and BEZ235 for 48 hours (presented as AraC/BEZ)

(3) cells were treated with BEZ235 for 24 hours, then AraC was added (BEZ235 pre-treatment; presented as BEZ-AraC)

(4) cells were treated with AraC for 24 hours, then BEZ235 was added (presented as AraC-BEZ)

Cell viability was measured at 48 hours after AraC was added in

all experimental conditions (1)-(4). Figure 8 A-D shows different patterns of cell apoptosis in experimental condition (1)-(4), respectively. As shown in Figure 8E, percentage of viable cell was decreased and apoptotic cell was increased when BEZ235 was administered with AraC simultaneously, compared with percentage in AraC monotherapy (AraC vs. AraC/BEZ). When BEZ235 was administered 24 hours before AraC, percentages of both early and late apoptotic cells were dramatically increased (AraC vs BEZ-AraC). No visible additional apoptotic effect was induced when BEZ235 was added to cells following AraC treatment, compared with AraC monotherapy (AraC vs. AraC-BEZ).

To define role of BEZ235 in BEZ235 pre-treatment regimen (3), which shows the most potent antileukemic effect among all AraC-based regimen, apoptotic effect of AraC (48 hours) and BEZ235 (72 hours) were measured and compared with untreated control, respectively. As shown in Figure 9A-F, percentages of early and late apoptotic cells were not significantly changed both in AraC monotherapy 48hr-regimen and BEZ235 monotherapy 72hr-regimen, compared with untreated controls,

respectively. But apoptosis was dramatically induced when cells were treated with AraC following BEZ235 pretreatment for 24 hours (Fig 9G-H; BEZ235 72 hours and AraC 48 hours). This means BEZ235 sensitizes leukemic cells to apoptotic effect of AraC when it is administered before AraC.

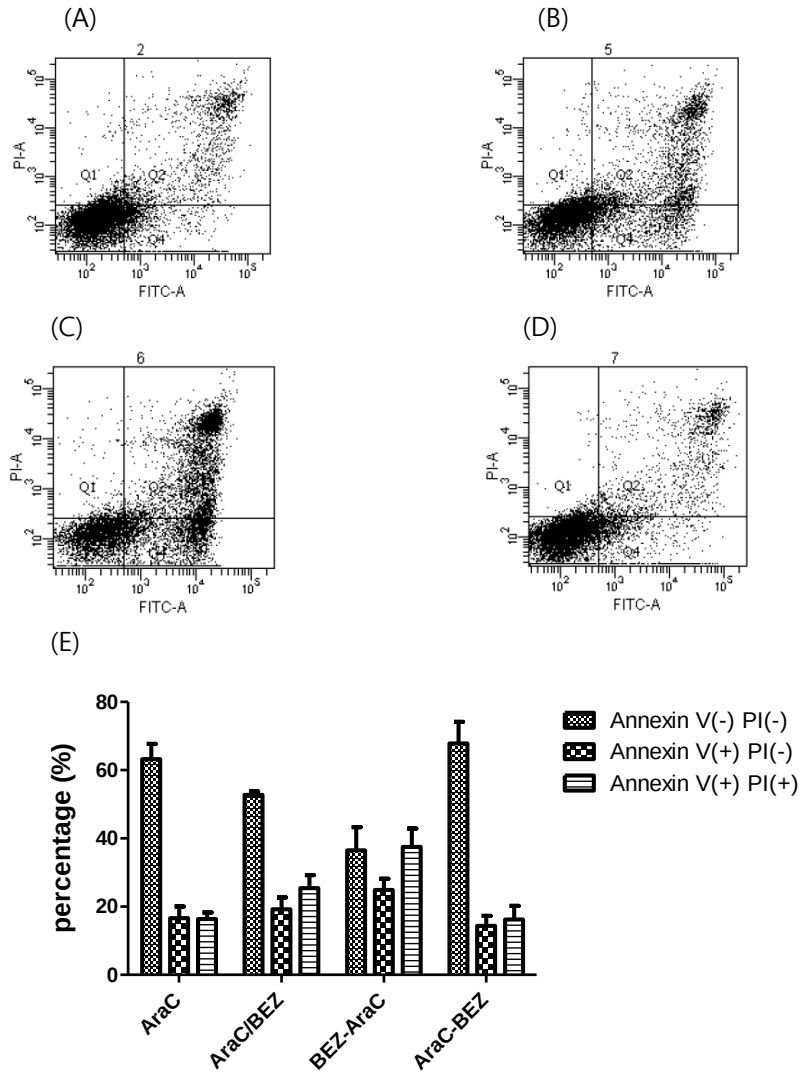
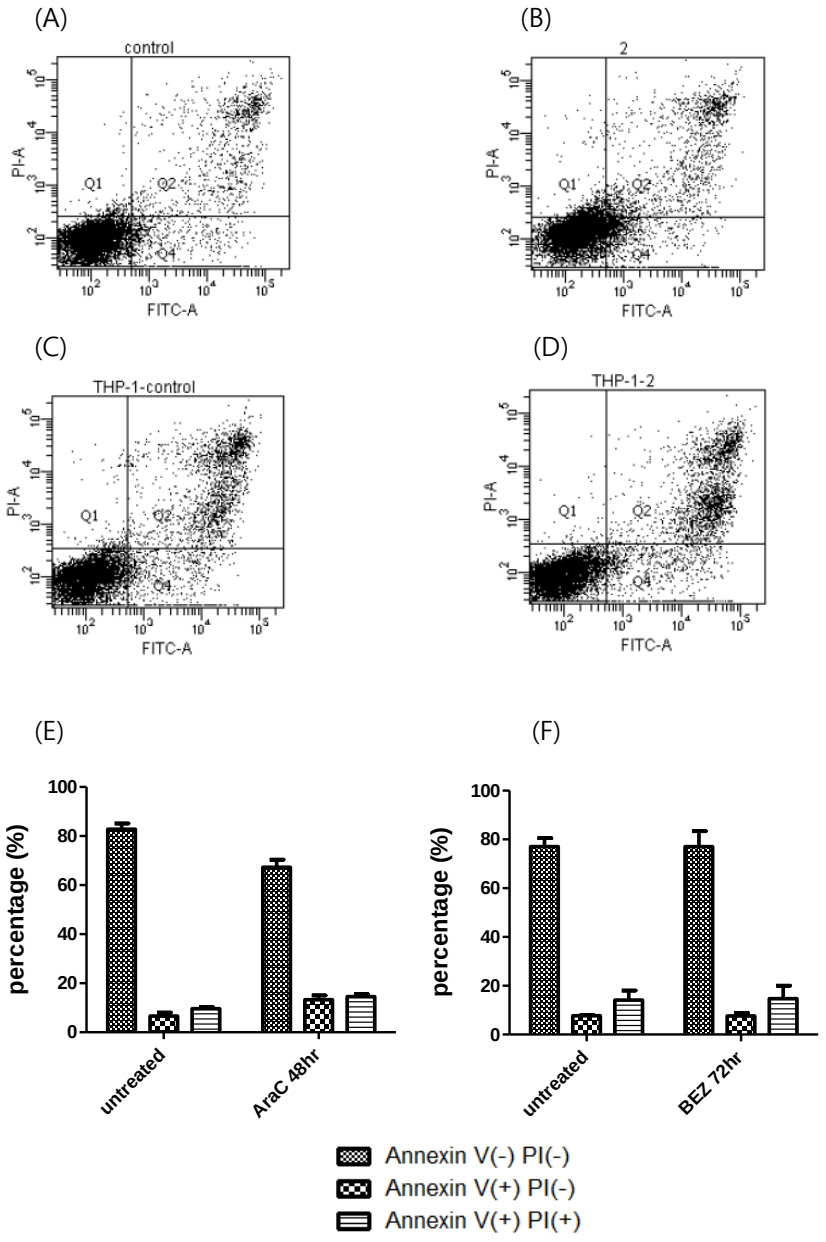
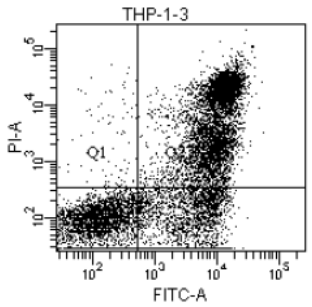



Figure 8. Different apoptotic effect according to combination schedule. THP-1 cell line was treated with AraC and AraC plus BEZ235 regimens with different schedule for 48 hours. Flow cytometric analysis was done for Annexin V-FITC/PI staining to measure appoptosis. (A) AraC only (B) AraC/BEZ235 simultaneously (C) BEZ235 pretreated 24 hours before AraC (D) BEZ235 added 24 hours later. AraC= 5,000 nM, BEZ235= 50 nM.


(A)
control
PI-A
FITC-A
Q1
Q2
Q4
(B)
2
(C)
THP-1-control
(D)
THP-1-2
(E)
percentage (%)
untreated
AraC 48hr
(F)
BEZ 72hr
Annexin V(-) PI(-)
Annexin V(+) PI(-)
Annexin V(+) PI(+)

(G)




(H)


Figure 9. Role of pretreated BEZ235 in AraC plus BEZ235 combination regimen. Apoptosis was measured for each component of BEZ pre-treated regimen (BEZ-AraC) in Figure 8. Flow cytometric analysis was done for Annexin V-FITC/PI staining to measure appoptosis.

(A) untreated control 48hr (B) AraC 48hr (C) untreated control 72hr (D) BEZ235 72hr (E) Bar graph; untreated control vs. AraC 48hr (F) Bar graph; untreated control vs. BEZ235 48hr (G) BEZ235 72hr/AraC 48hr (BEZ235 pre-treatment) (H) Bar graph; AraC 48hr vs. BEZ235 72hr vs. BEZ235 72hr/AraC 48hr (BEZ235 pre-treatment). AraC= 5,000 nM, BEZ235= 50 nM.

# Discussion

Despite a dramatic improvement in cancer treatment driven by many preclinical and clinical researches, major hurdle in successful cancer treatment is still drug resistance. Understanding the mechanism of drug resistance is a fundamental step to overcome this problem. Resistance to drug may come from characteristics of cancer cell itself before encountering chemotherapeutic agents, or it can be induced later during treatment. Several mechanisms of drug resistance have been revealed. Changes in membrane transporters to reduce drug accumulation, enhancement of repair mechanism for DNA damage, or multiple deficiencies in apoptosis induction take roles in drug resistance [36, 37]. Altered signaling pathways are also closely related with drug resistance. Besides its role in cancer development and progression, emerging data reveal that PI3K/Akt/mTOR pathway plays a key role in resistance to chemotherapy [38, 39]. Intrinsically Akt overexpression induces malignant transformation of cells and chemoresistance to drug [40]. Chemotherapy induced modulation of the PI3K/Akt/mTOR

pathway is closely related with drug resistance as well. There is an important report to show the ability of chemotherapy inhibiting Akt activity correlates well with the cytotoxic effects of these agents [39]. This was shown in many different cancer types. Anticancer agents like epidermal growth factor receptor (EGFR) targeting agent, BCR/ABL tyrosine kinase inhibitor, topoisomerase I inhibitor, and platinum agent inhibited Akt activity and increased cell death in breast, prostate, ovarian, epidermoid, head and neck, and lung cancers. Though most reports showed chemotherapy decreased Akt activity, some reported induction of Akt activity during chemotherapy [41]. In those reports, induction of Akt activity was mostly transient and followed by subsequent decrease. The basis for the different kinetics of Akt activation by chemotherapy is still unclear, but it is very obvious through many reports that PI3K/Akt/mTOR pathway has a key role in chemotherapy resistance. This means PI3K/Akt/mTOR pathway can be an attractive target to overcome drug resistance in cancer treatment.

In this study, the value of dual PI3K/Akt/mTOR pathway inhibitor BEZ235 in AML was evaluated. BEZ235 was effective in

inhibiting leukemia cell growth with similar low level of IC50 values among different AML cell lines, but apoptosis induction was gradual and not significant even at higher concentration of BEZ235 beyond IC50 values. This suggests BEZ235 has more like cytostatic effect rather than cytocidal effect to leukemia cells when it is used alone. More interesting data came from combination with AraC. AraC is currently the most important agent in most of standard therapeutic approaches for AML. If any new agent can induce synergism with AraC or restore sensitivity to AraC, it has tremendous value in clinical setting. To see the value of BEZ235 as combination regimen, cell viabilities were evaluated first in AraC monotherapy regimen and then in various BEZ235 plus AraC combination regimens. In the experiment to see sensitivity against AraC, cell lines represented very different sensitivities showing broad range of IC50 values among four cell lines (Figure 5). THP-1 cell line was the most resistant to AraC with the highest IC50 value, which was 5 times higher than KG-1 cell line and 20 times higher than the most sensitive HL60 cell line. Then combination experiments were performed in three cell lines, KG-1, MV4-11, and THP-1. In spite

of huge differences in sensitivity to AraC, all three cell lines demonstrated moderate to strong synergism when BEZ235 was added to AraC as combination regimen (Table 1 and Figure 6). It was notable that moderate to strong synergism was well maintained as combination ratio of AraC versus BEZ235 gradually decreased from 20:1 to 1,000:1 in two AraC-resistant cell line, MV4-11 and THP-1 (Table 2). Overcoming AraC resistance with BEZ235 was more clearly shown in Figure 7 through comparison between AraC monotherapy and AraC plus BEZ235 combination. In the past, other reports also showed value of PI3K/Akt/mTOR pathway inhibitors in combination with conventional cytotoxic agents. Though not fully developed due to its toxicity and insolubility, two PI3K inhibitors, wortmannin and LY294002 showed interesting data to increase cytotoxicity of anticancer drugs through combination *in vitro* and also *in vivo* [42-45]. Other reports also revealed inhibition of PI3K/Akt/mTOR signaling enhanced sensitivity to classical anticancer drugs in solid tumors [39, 41, 46-48]. In hematology field, there were some reports to show sensitization of leukemic cells to cytotoxic drug-induced apoptosis by PI3K/Akt/mTOR pathway inhibition.

Wortmannin and LY294002 were first reported to increase sensitivity to etoposide or doxorubicin in AML cells [49]. PI3K/Akt inhibitor Deguelin was also reported to enhance chemosensitivity of leukemic cells to etoposide and cytarabine [50]. Since 2008, further reports followed demonstrating the role of PI3K/Akt/mTOR pathway inhibition to overcome chemotherapy resistance majorly with mTOR inhibitors in AML [51-54] or in ALL [55, 56]. Recent report to investigate synergistic effect between two target agents, PI3K inhibitor and mTOR inhibitor, shows well that PI3K/Akt/mTOR pathway is attractive target in research of AML [57].

After BEZ235 was shown to reduce resistance to AraC as combination regimen, additional experiments were performed to investigate the way to maximize synergism and the role of BEZ235 in combination regimen. Most interestingly, pattern of combination interaction was different according to administration order of AraC and BEZ235 as shown in Figure 8. In case BEZ235 was administered 24 hours after AraC (AraC 48 hours, BEZ 24 hours), there was no difference in cell apoptosis compared with AraC monotreatment. Apoptosis was induced

most effectively when leukemic cells were pretreated with BEZ235 before AraC. In the next step, each component of combination regimen was analyzed separately to see the role of BEZ235 in combination regimen. As shown in Figure 9, both AraC treatment for 48 hours and BEZ235 treatment for 72 hours did not induce significant apoptosis compared with untreated controls, respectively. But combination regimen in which BEZ235 was administered 24 hours before treating AraC (BEZ235 72 hours, AraC 48 hours) showed profound difference in apoptosis induction. From this data, it was concluded that pretreatment with BEZ235 sensitized leukemic cells to apoptotic effect of AraC and maximized antileukemic effect in combination regimen with strong synergism.

As reviewed above, dual pathway inhibitor BEZ235 has huge potential to maximize antileukemic effect of conventional agent AraC through sensitization of leukemic cells. This also means genetic alteration in PI3K/Akt/mTOR pathway is an attractive target to investigate further in AML research. Interestingly, there have been reports showing overall survival of patients exhibiting Akt activation is significantly shorter in comparison with patients

with no Akt activation [58, 59]. Further investigation is necessary to conclude the relation between PI3K/Akt/mTOR pathway activation status and clinical outcomes. Exact understanding and effective control of target pathway using innovative new drug can result in huge progress in cancer treatment. To achieve this progress, it will not be enough to solely focus on one key signaling pathway. The reason of this complexity comes from broad cross-talk between key signaling pathways. Through many different mechanisms like negative feedback, cross-inhibition, cross-activation and pathway convergence, PI3K and its downstream pathways continuously interact with other signaling pathways and regulates each other. For example, Ras/Raf/MEK/ERK pathway is one of the most well known important interacting counterpart of PI3K/Akt/mTOR pathway [60-62]. Exact understanding of key signaling pathways behind cancer and interaction between different pathways will be the first step to look for new opportunities in the treatment of cancer. To bring meaningful data into the clinical benefit, it is also important to identify cancer subtype with specific genetic alteration and patients who most likely get benefit from pathway

inhibitors. Many innovative new targeting agents are coming, and many promising data are being generated as separate pieces of puzzle. It's time to investigate what to apply, where to apply and how to apply with those pieces of data to beat cancer.

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# 국문 초록

세포 표면의 다양한 receptor tyrosine kinases로부터 신호를 전달받고 이를 통합하는 Phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) 신호전달체계는 다양한 고형 종양에서 암세포의 성장과 증식, 그리고 생존에 핵심적인 역할을 수행하는 것으로 잘 알려져 있다. PI3K/Akt/mTOR 신호전달체계는 급성 골수성 백혈병 세포에서도 자주 활성화가 되어 있으며, 다양한 기전을 통하여 백혈병 세포의 생존과 항암제 저항성에 기여를 하고 있다. BEZ235 는 고형 종양 분야에서 현재 활발하게 개발 중인 가장 기대되는 dual PI3K/Akt/mTOR pathway inhibitor이다. 이에 본 연구는 급성 골수성 백혈병 세포에서 BEZ235 를 단독으로 투여하거나 AraC와 병용 투여 시 BEZ235 의 역할에 대하여 탐색하고자 하였다.

급성 골수성 백혈병 세포주인 KG-1, MV 4-11, THP-1, 그리고 HL60 세포에 각각 AraC와 BEZ235, 그리고 두 약제의 다양한 혼합 비율로 이루어진 병용 요법을 처리하였다. BEZ235를 단독으로 처리하였을 때 BEZ235는 각각의 다른 세포주에서 유사한 IC50값을 보이며 효과적으로 세포의 성장을 저해하였다.

반면 BEZ235의 농도가 증가함에 따라 점진적으로 apoptosis가 유도되었으나, 각 세포주의 IC50값을 초과하는 높은 농도의 범위에서도 뚜렷한 apoptosis의 증가는 보이지 않았다. 다음 단계로, AraC에 저항성을 보이는 MV4-11과 THP-1 두 가지 세포주에 대하여 BEZ235와 AraC 병용 요법 시 두 약제 간의 상호작용을 확인하기 위한 연구를 진행하였다. 각각의 병용 요법에 대하여 Chou and Talalay analysis에 근거한 CalcuSyn software를 사용하여 Combination Index (CI)값을 계산하였다. 병용 요법 시, 두 약제 간에 중등도 또는 그 이상의 synergism이 나타났으며, 이와 같은 synergism은 AraC에 대한 BEZ235의 혼합 비율이 20:1에서 1,000:1로 감소하면서도 잘 유지되었다. AraC-resistant MV4-11과 THP-1 세포주에서 BEZ235를 병용 요법으로 함께 투여하면 AraC에 대한 저항성이 크게 감소하였는데, 이와 같은 효과는 AraC의 농도 범위에 따라 각각 다르게 나타났다. 또한 AraC와 BEZ235 병용 요법을 각각 다른 스케줄로 투여하여 antileukemic effec를 비교해본 결과, AraC 투여 이전에 BEZ235를 전처치할 경우 두 약제의 synergism이 극대화되어 가장 큰 antileukemic effect가 나타났다. 이와 같은 결과는 AraC와의 병용 요법 시 BEZ235가 백혈병 세포를 감작시킴으로써 AraC에 대한 저항성을 극복하도록 하는 역할을

수행하는 것을 시사하고 있다.

백혈병에 있어 PI3K/Akt/mTOR 신호전달 체계는 추후 지속적인 연구가 필요한 중요한 표적이다. Dual pathway inhibitor인 BEZ235는 병용 투여 시 전통적인 항암제에 대해 백혈병 세포를 감작시킴으로써 급성 골수성 백혈병의 치료 효과를 극대화할 수 있는 가능성을 보여주었다. 치료의 표적이 되는 신호전달체계에 대한 정확한 이해와 더불어 혁신적인 신약을 이용한 신호전달체계의 효과적인 제어는 향후 급성 골수성 백혈병의 치료에 큰 진전을 가져오는데 있어 중요한 발걸음이 될 것이다.

중심 단어:

AML, PI3K/Akt/mTOR pathway, dual inhibitor, synergism

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