

Mechanism of cytotoxic activity of chalcone derivatives against K562 leukemia cell lines

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DOI: <http://dx.doi.org/10.19106/JMedSci004904201701>

ABSTRACT

Two chalcone derivatives i.e. (E)-1-(4-aminophenyl)-3-(2,3dimethoxyphenyl)-prop-2-en-1-one (Compound-1), and (E)-1-(4-aminophenyl)-3-phenylprop-2-en-1-one (Compound-2), has been proven to have potential cytotoxic activity. The aim of this study was to evaluate the effect of these compounds on PI3K/Akt signalling pathway in K562 cell lines. After incubation with the tested compounds, AKT, caspase-3, STAT3 and cyclin D1 concentrations were measured using ELISA. Furthermore, cell cycle was analysed using flowcytometry. Imatinib and isotretinoin were used as positive control, whereas cell culture without treatment was used as negative control. The AKT concentration after treatment with Compound-1 and -2 was significantly lower than that control, imatinib and isotretinoin ($p < 0.05$). The apoptotic indices after treatment with Compound-1 and -2 were significantly higher than control, however they were lower than imatinib and isotretinoin ($p < 0.05$). The caspase-3 concentration after treatment with Compound-1 at 5 and 10 $\mu\text{g}/\text{mL}$ and Compound-2 at 10 $\mu\text{g}/\text{mL}$ was significantly higher than that control and imatinib, however it was lower than isotretinoin ($p < 0.05$). The STAT3 concentration after treatment with Compound-1 and -2 was significantly lower than that control and isotretinoin at 50 $\mu\text{g}/\text{mL}$ ($p < 0.05$) and similar with imatinib ($p > 0.05$). The cyclin D1 concentration after treatment with Compound-1 and -2 was significantly lower than that control, imatinib and isotretinoin ($p < 0.05$). In addition, Compound-1 and -2 arrested G_0/G_1 and G_2/M phase in K562 cell lines, with comparable results to imatinib and isotretinoin. In conclusion, the mechanism of cytotoxic activity of Compound-1 and -2 are through the PI3K/Akt signalling pathway inhibition, apoptosis induction by upregulation of apoptotic markers, and inhibition of cell cycle progression by regulating cell cycle-related factors.

ABSTRAK

Dua turunan kalcon yaitu (E)-1-(4-aminofenil)-3-(2,3dimetoksifenil)-prop-2-en-1-one (Senyawa-1), and (E)-1-(4-aminofenil)-3-fenilprop-2-en-1-one (Senyawa-2), terbukti mempunyai aktivitas sitotoksik yang potensial. Tujuan penelitian ini adalah untuk mengkaji efek kedua senyawa tersebut pada jalur sinyal PI3K/Akt pada sel K562. Setelah

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diinkubasikan dengan senyawa uji, kadar AKT, caspase-3, STAT3 dan siklin D1 diukur dengan ELISA. Selanjutnya siklus sel dianalisis dengan *flowcyometry*. Imatinib dan isotretinoin digunakan sebagai kontrol positif dan kultur sel tanpa perlakuan sebagai kontrol negatif. Kadar AKT setelah perlakuan dengan Senyawa-1 dan -2 lebih rendah secara nyata dibandingkan dengan kontrol, imatinib dan isotretinoin ($p < 0.05$). Indeks apoptosis setelah perlakuan dengan Senyawa-1 dan -2 lebih tinggi secara nyata dibandingkan kontrol, tetapi lebih rendah dibandingkan imatinib dan isotretinoin ($p < 0.05$). Kadar caspase-3 setelah perlakuan Senyawa-1 dengan kadar 5 dan 10 $\mu\text{g/mL}$ dan Senyawa-2 dengan kadar 10 $\mu\text{g/mL}$ lebih tinggi secara nyata dibandingkan kontrol dan imatinib, tetapi lebih rendah dibandingkan dengan isotretinoin ($p < 0.05$). Kadar STAT3 setelah perlakuan dengan Senyawa-1 dan -2 lebih rendah secara nyata dibandingkan kontrol dan isotretinoin kadar 50 $\mu\text{g/mL}$ ($p < 0.05$) dan setara dengan imatinib ($p > 0.05$). Kadar siklin D1 setelah perlakuan dengan Senyawa-1 dan -2 lebih rendah secara nyata dibandingkan dengan kontrol, imatinib dan isotretinoin ($p < 0.05$). Senyawa-1 dan -2 terbukti menghambat siklus sel K562 pada fase G_0/G_1 dan G_2/M yang sebanding dengan imatinib dan isotretinoin. Dapat disimpulkan, mekanisme aktivitas sitotoksik senyawa-1 dan -2 melalui penghambatan jalur sinyal PI3K/Akt, induksi apoptosis melalui upregulasi penanda apoptosis dan penghambatan siklus sel melalui pengaruhnya terhadap factor-faktor yang berhubungan dengan pengaturan siklus sel.

Keywords : anticancer - chalcone derivatives – leukemia – K562 cell line - PI3K/Akt signaling

INTRODUCTION

Leukemia is a cancer of the white blood cells characterized by the widespread, rapid, and disorderly proliferation of leukocytes.^{1,2} Leukemia is a rare disease; however, it exceeds the number of deaths caused by acute communicable diseases due to its fatal character.² In 2012, leukemia was suffered by approximately 352,000 people around the world and caused 265,000 deaths.³ The PI3K/Akt signaling pathway plays an important role in both normal and malignant hematopoiesis.^{4,5} The activity of AKT is regulated by P13K, which brings AKT into the cell membrane upon PIP3 binding, which is activated by PDK1.⁶ Activated AKT is an essential survival factor which inhibits apoptosis through phosphorylation of several targets, including Bad, FoxO transcription factors, Raf-1 and caspase-9.⁷ AKT also activates the transcription activator (STAT3), which is critical for leukemia cell survival

and proliferation.⁸ Activation of STAT3 leads to tumor-promoting gene products, including cleavage caspase-3, cleavage poly ADP-ribose polymerase (PARP), cyclin D1, and survivin. Thus, these pathways are commonly targeted in cancer treatments.

Until now, chemotherapy is still one of the effective strategies for the treatment of cancer.⁹ However, the use of anticancer treatments as chemotherapy is limited. All of the current anticancer drugs have severe side effects in normal cells due to their non-specificity. In addition, the resistance of cancer cells to anticancer drugs remains a significant challenge to successful chemotherapy.¹⁰ Therefore, the discovery and development of new anticancer treatments with specific targets and novel mechanisms are urgently needed.

Chalcones are precursors of flavonoids, which are abundantly found in plants, and are reported to possess anticancer activity. Several chalcones have been isolated or synthesized, and their cytotoxicity on cancer cell lines

and their anticancer activity in cancer animal models have been reported.^{11,12} Furthermore, the molecular mechanisms of the anticancer action of chalcones have also been investigated. The chalcones exhibited anticancer activity through multiple mechanisms including cell cycle disruption, angiogenesis inhibition, tubulin polymerization inhibition, apoptosis induction and blockade of the nuclear factor-kappa B (NF-κB) signaling pathway.^{12,13}

We previously evaluated the cytotoxic activity of several chalcone derivatives on the K562 leukemia cell line, resulting in two chalcones showing the highest activity i.e. (E)-1-(4-aminophenyl)-3-(2,3 dimethoxyphenyl)-prop-2-en-1-one (Compound-1), and (E)-1-

(4-aminophenyl)-3-phenylprop-2-en-1-one) (Compound-2). In this study we reported the mechanism of cytotoxic activity of these compounds through their effect on the level of AKT, caspase-3, STAT3, cyclin D1, and cell cycle on K562 leukemia cell lines. Furthermore, imatinib and isotretinoin were used as positive control in this study.

MATERIALS AND METHODS

Tested compounds

The methoxy amino chalcone derivatives were synthesized by Suwito *et al.*¹⁴ The structure of these compounds is presented in FIGURE 1.

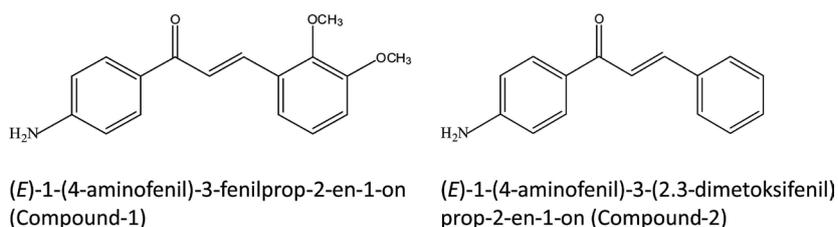


FIGURE 1. Chemical structure chalcone derivatives

Cell culture

The K562 cell lines were obtained from Stem Cells and Cancer Institute (SCI) Jakarta, Indonesia. The cell lines were maintained in iscove's modified dulbecco's medium (Biowest L0190-500) supplemented by 10% fetal bovine serum (FBS) (Biowest S181H2), and 2% penicilin-streptomycin (Biowest L0022-100) and incubated at 37°C in a 5% CO₂ atmosphere with 95% humidity. After 24 hours of incubation, the number of viable cells was counted using a haemocytometer with tryphan blue staining.

Measurement of AKT, caspase-3, STAT3 and cyclin D1 concentrations in K562 cell lines culture

The concentration of AKT was determined using an ELISA kit (ab176657). After pre-incubation of K562 cell lines treated with imatinib (10 and 50 µg/mL), isotretinoin (25 and 50 µg/mL), Compound-1 and -2 (5 and 10 µg/mL), 50 µL of sample was added to wells, and then 50 µL of the antibody was added into each well, and incubated for 1 hour at room temperature. Each well was washed with 350 µL of wash buffer. One hundred µL of TMB substrate was added into each well, and incubated for 15 minutes in the dark. The reaction was stopped by adding 100 µL of stop solution to each well. Absorbance was measured using Multiskan GO Microplate

Spectrophotometer at 450 nm. Untreated K562 cell lines served as control.

The similar methods were applied to determine the caspase-3 and STAT3 concentrations using different antibody i.e. ELISA kit ab181418 for caspase-3, ELISA kit ab176666 for STAT3 and biotinylated detection antibody for cyclin D1.

Cell cycle analysis

Cells (2×10^6 cells/well) were cultured in 24-well plates, and then treated with 0.1% DMSO, and 100 μ L tested compounds at an appropriate final concentration (10 and 20 μ g/mL for imatinib; 25 and 50 μ g/mL for isotretinoin; 5 and 10 μ g/mL for Compound-1 and -2, before being incubated for 24 hr. Cells were harvested, washed using PBS, fixed in 70% ethanol overnight and stained with 50 μ g/mL PI and 20 μ g/mL RNase. Data acquisition and analysis were performed on a MACSQuant Analyzer 10 flowcytometer (Miltenyl Biotec), and data from 500,000 cells were collected for each data file. Cell cycle analysis was performed with MACSQuantify™ Software (Miltenyl Biotec).

Statistical analysis

Data were presented as means \pm standard deviation (SD). SPSS (Version 16, SPSS Inc., Chicago, IL, USA) was used to perform one-

way analysis of variance (ANOVA) to analysis the different among group means. Duncan's multiple range test was used to validate significant differences for all treatments. A p value <0.05 was considered significantly different.

RESULTS

AKT concentration in K562 cell lines

AKT concentration on K562 cell lines of all treatments is presented in FIGURE 2. The AKT concentration of all treatments was significantly lower than control ($p < 0.05$) except on isotretinoin treatment at 50 μ g/mL which it was significantly higher ($p < 0.05$). The AKT concentration after treatment with Compound-1 at 5 μ g/mL (2.45 ng/mL) and Compound-2 at 10 μ g/mL (2.42 ng/mL) were lower than control (12.74 ng/mL). The similar results were observed after treatment with Compound-2 at 5 and 10 μ g/mL in which the AKT concentrations were 2.42 and 2.97 ng/mL, respectively. The lower AKT concentrations were also observed after imatinib treatment at 10 and 20 μ g/mL in which the AKT concentrations were 4.30 and 3.91 ng/mL, respectively. In contrast, the AKT concentration was higher (16.56 ng/mL) after treatment with isotretinoin at 50 μ g/mL. However, it was also higher (7.15 ng/mL) after treatment with isotretinoin at 25 μ g/mL.

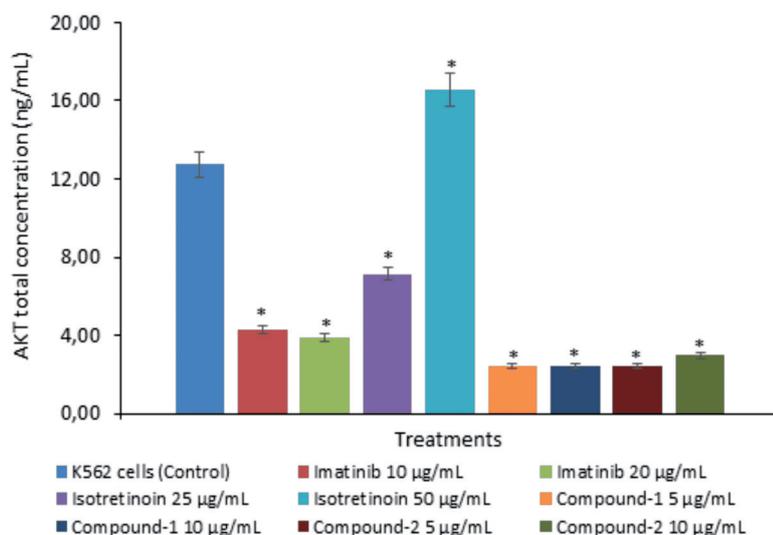


FIGURE 2. AKT total concentration on K562 cell lines after treatment with imatinib, isotretinoin, Compound-1 and -2. (*) indicates a significant difference between treatments compared to control ($p < 0.05$).

Apoptotic index and caspase-3 in K562 cell lines

Effects of chalcone derivatives on apoptosis in K562 cell lines as indicated in the caspase-3 concentration and apoptotic index are shown in FIGURE 3 and 4. The apoptotic indices in K562 cell lines of all treatments were significantly higher than control

($p < 0.05$). The highest apoptotic indices were observed after treatment with imatinib 20 µg/mL (6.89%) and isotretinoin 50 µg/mL (6.84%). The apoptotic indices after treatment with Compound-1 at 10 µg/mL, Compound-2 at 10 µg/mL and control were 3.89, 4.03 and 2.33%, respectively.

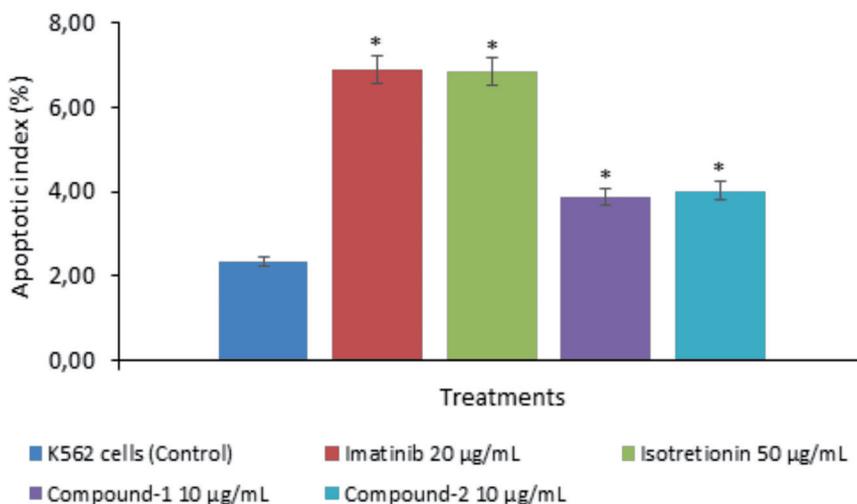


FIGURE 3. Apoptotic index on K562 cell lines after treatment with imatinib, isotretinoin, Compound-1 and -2. (*) indicates a significant difference between treatments compared to control ($p < 0.05$).

The caspase-3 concentrations on K562 cell lines after treatment with Compound-1 at 5 µg/mL (1.19 ng/mL), Compound-1 at 10 µg/mL (1.37 ng/mL) and Compound-2 at 10 µg/mL (0.66 ng/mL) were significantly higher than control (0.26 ng/mL) ($p < 0.05$). The similar results were observed after treatment

with imatinib at 10 and 20 µg/mL in which the caspase-3 concentrations were 2.97 and 2.83 ng/mL, respectively ($p < 0.05$). No significantly different in caspase-3 concentrations compare to control were observed treatment with isotretinoin at 25 µg/mL (0.24 ng/mL) and at 50 µg/mL (0.22 ng/mL) ($p > 0.05$).

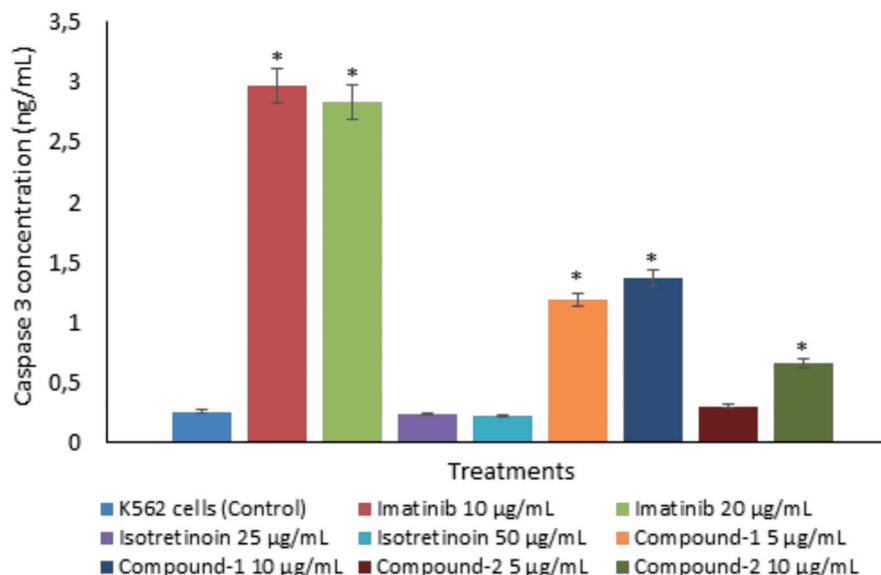


FIGURE 4. Caspase-3 concentration on K562 cell lines after treatment with imatinib, isotretinoin, Compound-1 and -2. (*) indicates a significant difference between treatments compared to control ($p < 0.05$).

STAT3 concentration in K562 cell lines

STAT3 concentration on K562 cell lines of all treatments is presented in FIGURE 5. The STAT3 concentration of all treatments was significantly lower than control ($p < 0.05$) except on isotretinoin treatment at 25 µg/mL which it was not significantly different ($p > 0.05$). The STAT3 concentrations after treatment with Compound-1 at 5 µg/mL (9.00 ng/mL), Compound-1 at 10 µg/mL (5.48 ng/mL), Compound-2 at 5 µg/mL (8.62 ng/mL)

and Compound-2 at 10 µg/mL (14.77 ng/mL) were significantly lower than control (37.56 ng/mL) ($p < 0.05$). The similar results were observed after treatment with imatinib at 10 and 20 µg/mL and isotretinoin at 50 µg/mL in which the STAT3 concentrations were 10.11, 7.83 and 21.51 ng/mL, respectively ($p < 0.05$). No significantly different in STAT3 concentrations compare to control was observed treatment with isotretinoin at 25 µg/mL (38.68 ng/mL) ($p > 0.05$).

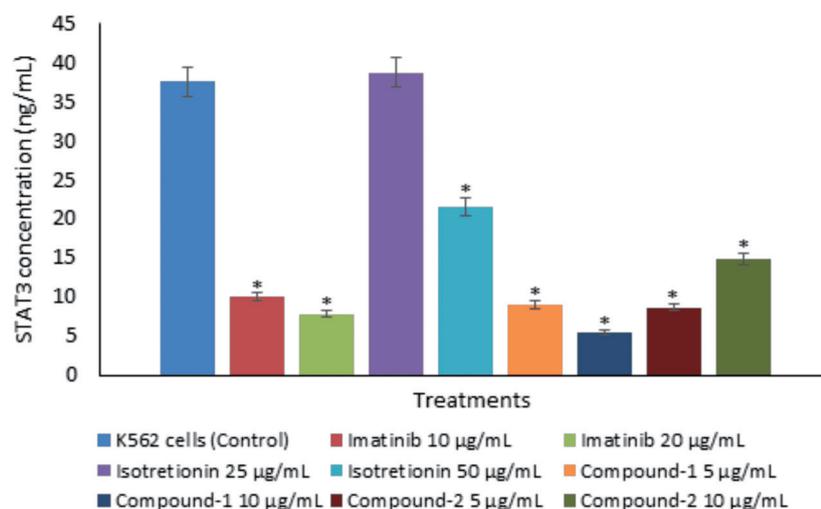


FIGURE 5. STAT3 concentration on K562 cell lines after treatment with imatinib, isotretinoin, Compound-1 and -2. (*) indicates a significant difference between treatments compared to control ($p < 0.05$).

Cyclin D1 concentration in K562 cell lines and cell cycle

Cyclin D1 concentration on K562 cell lines and its cell cycle of all treatments are presented in FIGURE 6 and 7. The cyclin D1 concentration of all treatments was significantly lower than control ($p < 0.05$). The cyclin D1 concentrations after treatment

with Compound-1 at 5 µg/mL (1.41 ng/mL), Compound-1 at 10 µg/mL (1.51 ng/mL), Compound-2 at 5 µg/mL (1.45 ng/mL) and Compound-2 at 10 µg/mL (1.47 ng/mL) were also significantly lower than imatinib 10 µg/mL (4.53 ng/mL), imatinib 20 µg/mL (3.43 ng/mL), isotretinoin 25 µg/mL (5.85 ng/mL) and isotretinoin 50 µg/mL (4.62 ng/mL) ($p < 0.05$).

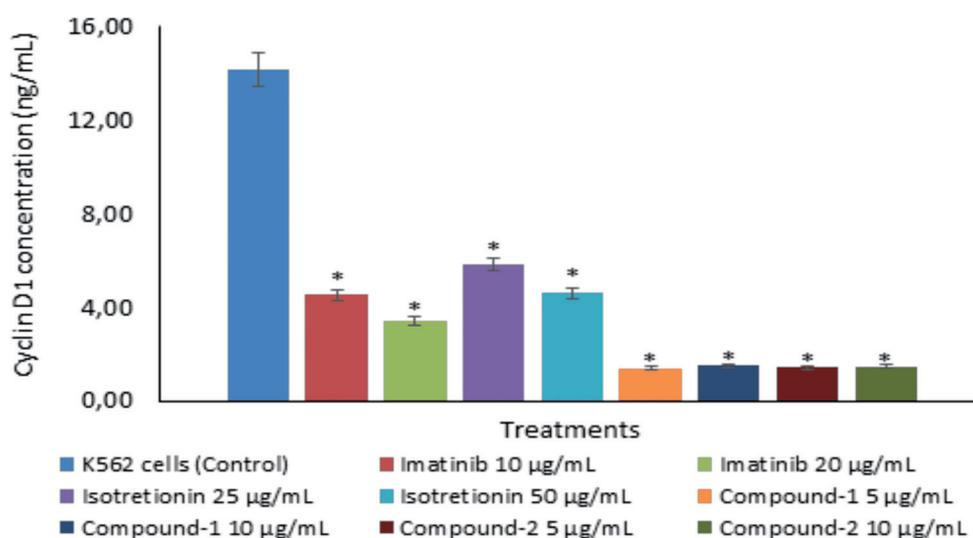


FIGURE 6. Cyclin D1 concentration on K562 cell lines after treatment with imatinib, isotretinoin, Compound-1 and -2. (*) indicates a significant difference between treatments compared to control ($p < 0.05$).

As shown in FIGURE 7, Compound-2 at 5 $\mu\text{g}/\text{mL}$ was the only compound that significantly arrested G_0/G_1 phase (32.42%), and it was comparable to imatinib of 20 $\mu\text{g}/\text{mL}$ (35.99%) and isotretinoin of 25 and 50 $\mu\text{g}/\text{mL}$ (32.15 and 33.87%, respectively). Both Compound-1 at 5 and 10 $\mu\text{g}/\text{mL}$ (21.73

and 22.01%, respectively) and Compound-2 at 5 and 10 $\mu\text{g}/\text{mL}$ (18.50 and 21.94%, respectively) significantly decreased S phase compared to control (32.52%). Compound-1 and -2 at 10 $\mu\text{g}/\text{mL}$ arrested G_2/M phase (37.21 and 41.07%, respectively), which were comparable to imatinib at 10 $\mu\text{g}/\text{mL}$ (38.45%).

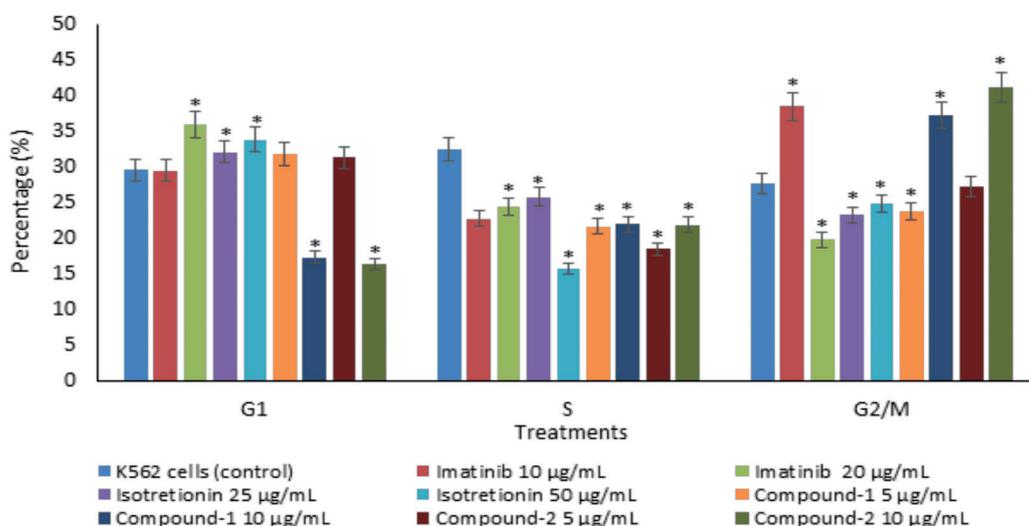


FIGURE 7. K562 cell cycle after treatment with imatinib, isotretinoin, Compound-1 and -2. (*) indicates a significant difference between treatments compared to control ($p < 0.05$).

DISCUSSION

Chemotherapy is one of the most potent and effective strategies to treat cancer.⁹ However, all of the anticancer therapies that are currently available have severe side effects on normal cells due to their non-specificity, leading to the development of new anticancer therapies with specific targets. Molecular mechanisms of cancer have been studied in drug discovery. The PI3K/AKT signaling pathway plays an important role in both normal and malignant hematopoiesis.^{4,5} Activated AKT is critical for leukemia cell survival and proliferation,⁸ and is known to function as an essential survival factor by

inhibiting apoptosis.⁷ These pathways are commonly targeted in cancer treatments.

Almost all treatments in the present study significantly decreased AKT levels on K562 cells, except isotretinoin at 25 $\mu\text{g}/\text{mL}$, which increased the AKT level. Both Compound-1 and -2 showed the most significant decrease among treatments. These findings are in accordance with previous studies that reported on chalcone derivatives as anticancer agents. Xu *et al.*,¹⁵ showed chalcone derivative, L2H17, inactivated NF- κ B and AKT signaling pathways in colon cancer. The role of AKT in cancer involves the following: survival of cells by blocking the function of proapoptotic

proteins and processes; phosphorylation of Bad,^{16,17} that promotes release of Bad from heterodimeric of Bcl-2 and Bcl-XL; phosphorylation of MDM2^{18,19} stabilizing it and promoting its translocation to the nucleus, where it triggers p53 degradation; and phosphorylation of XIAP, an inhibitor of caspase cascade, and thus inhibiting its degradation.²⁰

In this study, Compound-1 and -2 also significantly increased caspase-3 on K562, as confirmed by the higher apoptotic index compared to the control. Chalcones have also been reported to induce apoptosis in cancer cells. Referring to a previous study, chalcones (1,3-diphenyl-2-propenone) in a human diet rich in fruits and vegetables inhibit the proliferation of T24 and HT-1376 cells by inducing apoptosis. Chalcone increased the expression of Bax and Bak, but decreased the levels of Bcl-2 and Bcl-X(L) and subsequently triggered the mitochondrial apoptotic pathway (release of cytochrome c and activation of caspase-9 and caspase-3).²¹ Apoptosis is by far the best-characterized type of cell death and is defined by morphologic modifications (chromatin condensation, loss of mitochondrial membrane potential, plasma membrane asymmetry, overall cell shrinkage, blebbing of the plasma membrane, and detachment from the cellular matrix), all occurring before the loss of plasma membrane integrity. Those modifications occur due to executioner caspase activation.²²

The results of the present study showed that all treatments decreased STAT3 on K562. These findings are validated by a previous study which showed that a chalcone derivative, 4,3',4',5'-tetramethoxychalcone, inhibits the phosphorylation of STAT3 and its upstream protein tyrosine kinase c-Src.²³ STAT3, an oncogenic transcription factor, is often constitutively active in human cancer

cells.²⁴ Activated STAT3 may up-regulate the expression of genes such as apoptosis inhibitors (Bcl-xl, Bcl-2), cell cycle regulators (cyclin D1) and oncogenic transcription factors (c-myc) in tumorigenesis.^{25,26}

In this study, all treatments significantly decreased cyclin D1 levels on K562 cells. Compound-1 and -2 showed the highest decrease among treatments. The cyclin D1/CDK4 complex is responsible for cell cycle progression in early G₁ phase, and is frequently overexpressed in various human carcinomas.²⁷⁻²⁹ Compound-1 and -2 also arrested G₀/G₁ and G₂/M phase in K562 cells, with the results being comparable to imatinib and accutane. These findings are in agreement with a previous study that shows chalcone inhibits the proliferation of T24 and HT-1376 cells by blocking cell cycle progression in the G₂/M phase.²¹ Another study investigated the effect of a synthetic chalcone derivative, 4,3',4',5'-tetramethoxychalcone, that resulted in G₀/G₁ cell cycle arrest through the down-regulation of cyclin D1 and CDK4, and the up-regulation of p16, p21 and p27 proteins in A2780 cells.²³ The p16 protein is a specific inhibitor of CDK-cyclin D complex, preventing the phosphorylation of Rb and cell cycle reentry at G₀/G₁ phase.²⁷ Eukaryotic cell cycle progression involves sequential activation of cyclin-dependent kinases (CDK), whose activation is dependent on their association with cyclins.³⁰ A complex formed by the association of Cdc2 (also known as Cdk1 or p34Cdc2) and cyclin D1 plays a major role at entry into mitosis.³⁰ The phosphorylation of Tyr15 of Cdc2 suppresses the activity of Cdk1/cyclin B1 kinase complex. Dephosphorylation of Tyr15 of Cdc2 is catalysed by Cdc25 phosphatases, and this reaction is believed to be the ratelimiting step for entry into mitosis.³¹ Cell cycle progression is also regulated by the relative balance

between the cellular concentration of CDK inhibitors, such as members of the CDK-interacting protein/CDK-inhibitory protein (CIP/KIP) and inhibitor of CDK families, and that of cyclin-CDK complexes. The CIP/KIP family, including CIP/p21, and KIP/p27, bind to cyclin-CDK complexes and prevent kinase activation and subsequently blocking the progression of the cell cycle at the G₀/G₁ or G₂/M phases.^{30,32}

CONCLUSION

It can be concluded that (E)-1-(4-aminophenyl)-3-(2,3 dimethoxyphenyl)-prop-2-en-1-one (Compound-1), and (E)-1-(4-aminophenyl)-3-phenylprop-2-en-1-one (Compound-2) may be potential compounds to be developed as anticancer against leukemia. The molecular mechanisms of cytotoxic activity of these compounds in the K562 cell lines involved i) inhibition of the PI3K/Akt signaling pathway; ii) induction of apoptosis through the up-regulation of apoptotic markers; and iii) inhibition of cell cycle progression by regulating cell cycle-related factors.

ACKNOWLEDGMENTS

This research was supported by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for laboratory facilities, and research methodology. We would like to thank staff of Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung for their valuable assistance. We also would like to thank the Stem Cell and Cancer Institute, Kalbe Farma, Jakarta for the K562 cell lines.

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