Molecular mechanism of synthesized chalcone as an anticancer agent in Leukemia Cell Line HL60

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Leukemia is a rare disease that causes deaths due to its fatal character. PI3K/Akt signaling pathway plays major role in cancer development. Chalcone, a precursor of flavonoids in plants, has been known to possess anticancer activity. This study aimed to evaluate the effect of chalcone derivatives on the PI3K/Akt signaling pathway in the HL60 cell line. The HL60 cell line was treated with two chalcone derivatives: (E)-1-(4-aminophenyl)-3-(2,3-dimethoxyphenyl)-prop-2-en-1-one (MTC4) and (E)-1-(4-aminophenyl)-3-phenylprop-2-en-1-one (MTC7), and compared to the anticancer drugs imatinib and isotretinoin. Measurements of Flt-3, caspase-3, STAT3, and cyclin D1 were performed using ELISA, whilst cell cycle analysis was performed with flow cytometry. Chalcone derivatives showed lower levels of Flt-3 compared to the leukemia drugs imatinib and isotretinoin. Both MTC4 and MTC7 significantly increased caspase-3 on HL-60, as also indicated by the higher apoptotic index compared to the control. MTC4 and MTC7 also decreased STAT3 on HL60. Moreover, MTC4 and MTC7 significantly decreased cyclin D1 levels on HL-60, as indicated by a decreased S phase as well as G2/M arrest, which were comparable with imatinib and isotretinoin. To conclude, MTC4 and MTC7 are promising anticancer drugs via inhibition of the PI3K/Akt signaling pathway, induction of apoptosis, and the blockade of cell cycle progression. Chalcone derivatives MTC4 and MTC7 may be potential compounds for treating leukemia disease.

Keywords: Chalcone, leukemia, anticancer, HL60

INTRODUCTION

Leukemia is a cancer of the white blood cells characterized by the widespread, rapid, and disorderly proliferation of leukocytes.(Pui et al. 2009) (Modak 2011) Leukemia is a rare disease; however, it exceeds the number of deaths caused by acute communicable diseases due to its fatal character.(Modak et al. 2011) In 2012, leukemia was suffered by approximately 352,000 people around the world and caused 265,000 deaths. (Stewart & Wild, 2014) Nowadays, chemotherapy is one of the most effective and potent strategies for the treatment of cancer. (Ge et al. 2009) However, the resistance of cancer cells to anticancer drugs remains a significant challenge to successful chemotherapy.(Liang et al. 2010) Thus the remarkable effect of phytochemicals from medicinal plants such as phenolic compounds has been more noticed as anticancer on several cancer cell lines and promoter for apoptosis. (Youness et al. 2017) Moreover, the current anticancer drugs have severe side effects in normal cells due to their non-specificity. Therefore, the discovery and development of new anticancer treatments with specific targets and novel mechanisms are urgently required.
MATERIALS AND METHODS

Tested Compounds

The methoxy amino chalcone derivatives were synthesized by Dr. Hery Suwito in the Department of Chemistry, Faculty of Mathematics and Natural Science, Airlangga University, Surabaya, Indonesia.

Cell Culture

The HL60 cell lines were obtained from Aretha Medika Utama, Biomolecular, and Biomedical Research Center, Bandung, Indonesia. The cell lines were maintained in Iscove's modified RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS) (Biowest S181H2), and 2% penicillin-streptomycin (Biowest L0022-100). The cell line was maintained 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 Trypan blue staining.

Measurement of Flt-3 on HL60

The concentration of Flt-3 was performed with an ELISA kit (ab176657). After pre-incubation of HL-60 treated with imatinib imaniitib (20.25 and 40.51 µM), isotretinoin (16.64 and 33.28 µM), MTC 4 (4.41 and 8.82 µM) and MTC 7 (22.38 and 44.76 µM), a 50 µL of sample was added to wells, and then 50 µL of the antibody was added to each well, and incubated for 1 hour at room temperature. Each well was washed with 350 µL wash buffer. Then, 100 µL of TMB Substrate was added to 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 recorded at 450 nm (MultiSkan Go
Thermoscientific). Untreated HL-60 served as a control.

**Measurement of Caspase-3 on HL-60**

The ELISA kit (ab181418) was used to determine Caspase-3 level on HL-60 cells. After pre-incubation of HL-60 with imatinib (20.25 and 40.51 μM), isotretinoin (16.64 and 33.28 μM), MTC 4 (4.41 and 8.82 μM) and MTC 7 (22.38 and 44.76 μM), a 50 μL aliquot of sample was added to the 96-well plate, followed by 50 μL of antibody solution; this was incubated for 1 hour at room temperature. Each well was washed with 3 x 350 μL Wash Buffer. One hundred μL of TMB Substrate was then added to each well and incubated for 10 minutes in the dark. Finally, 100 μL of stop solution was added to each well and absorbance was measured at 450 nm (MultiSkan Go Thermoscientific). HL-60 cells without treatment were served as control.

**Measurement of Apoptotic Index on HL-60**

Cells (2 x 10⁶ cells/well) were cultured in 24-well plates, and then treated with vehicle (0.1% DMSO) and 100 μL compound at the corresponding final concentration (imatinib 40.51 μM, isotretinoin 33.28 μM, MTC 4 8.82 μM and MTC 7 44.76 μM), before being incubated for 24 hr. Cells were harvested, washed using PBS, fixed in 70% ethanol overnight and stained with 50 μg/mL PI and RNase 20 mg/mL. Data acquisition and analysis were performed on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec), and data from 500,000 cells were collected for each data file. Cell cycle analysis was performed with MACS Quantify™ Software (Miltenyi Biotec).

**Measurement of STAT3 on HL-60**

The concentration of STAT 3 was performed based on ELISA kit (ab176666) protocol. After the pre-incubation of HL-60 treated with imatinib (20.25 and 40.51 μM), isotretinoin (16.64 and 33.28 μM), MTC 4 (4.41 and 8.82 μM) and MTC 7 (22.38 and 44.76 μM), 50 μL of all samples was added into wells, and then 50 μL of the antibody solution was added to each well, before being incubated at 1 hour room temperature. Each well was washed with 3 x 350 μL 1 x Wash Buffer PT. One hundred μL of TMB Substrate was then added to each well and incubated for 15 minutes in the dark. Finally, 100 μL of Stop Solution was added to each well and absorbance was recorded at 450 nm (MultiSkan Go Thermoscientific). Untreated HL-60 served as a control.

**Measurement of Cyclin D1 on HL-60**

After pre-incubation of HL-60 treated with imatinib (20.25 and 40.51 μM), isotretinoin (16.64 and 33.28 μM), MTC 4 (4.41 and 8.82 μM) and MTC 7 (22.38 and 44.76 μM), 100 μL sample was added to wells, incubated for 90 min at 37 °C, and then 100 μL Bio tnlabeled detection Ab was added to each well, before being incubated for 1 hour at 37 °C. The solution was discarded and washed with 300 μL wash buffer. One hundred μL HRP conjugate was added to each well and incubated for 30 minutes at 37 °C. Then, 90 μL substrate was added and incubated for 15 min at 37 °C in the dark. Finally, Stop Solution was added, and absorbance was measured at 450 nm (MultiSkan Go Thermoscientific).

**Cell cycle analysis**

Cells (2 x 10⁶ cells/well) were cultured in 24-well plates, and then treated with vehicle (0.1% DMSO), and 100 μL compound at an appropriate final concentration (imatinib 20.25 and 40.51 μM, isotretinoin 16.64 and 33.28 μM, MTC 4 4.41 and 8.82 μM, and MTC 7 5.59 and 11.19 μg/mL), before being incubated for 24 hr. Cells were harvested, washed using PBS, fixed in 70% ethanol overnight and stained with 50 μg/mL PI and 20 mg/mL RNase. Data acquisition and analysis were performed on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec), and data from 500,000 cells were collected for each data file. Cell cycle analysis was performed with MACSQuantify™ Software (Miltenyi Biotec).

**Statistical analysis**

SPSS (Version 16, SPSS Inc., Chicago, IL, USA) was used to perform one-way ANOVA in order to verify the results of different treatments, and Duncan post hoc was used to validate significant differences for all treatments. The results are displayed as means ± standard deviation with a 95% confidence level.

**RESULTS AND DISCUSSION**

Chemotherapy is one of the most potent and effective strategies to treat cancer. (Ge et al. 2009) The PI3K/Akt signaling pathway plays an important role in both normal and malignant hematopoiesis.(Martelli et al. 2010)(Polak & Buitenhuis, 2012) In the present study, all treatment showed significantly decreased Flt-3 on HL-60 cells compared to the control. Chalcone derivatives showed a lower level of Flt-3 compared to the leukemia drugs imatinib and isotretinoin. The lowest level of Flt-3 was obtained
for the treatment of MTC4 20.25 µg/mL (47.33 pg/mL) (Fig.2.). These results indicate that all chalcone derivatives decreased Flt-3 in leukemia cells. FLT3 is expressed at high levels in 70% to 100% of cases of AML and in a high percentage of ALL cases. (Birg et al. 1992) (Birg et al. 1994) (Carow et al. 1996) (Stacchini et al. 1996) There has been an intense focus on the development of FLT3 inhibitors because of the high frequency and poor prognosis of AML patients with mutant FLT3. To the best of our knowledge, there have been no studies reported on the ability of chalcone to decrease Flt-3 in cancer cells.

Figure 2. Flt-3 level on HL-60 cell line treated with imatinib (20.25 and 40.51 µM), isotretinoin (16.64 and 33.28 µM), Compound 4 (4.41 and 8.82 µM) and Compound 6 (22.38 and 44.76 µM).

Figure 3. Caspase-3 level on HL-60 cell line treated with imatinib (20.25 and 40.51 µM), isotretinoin (16.64 and 33.28 µM), Compound 4 (4.41 and 8.82 µM) and Compound 6 (22.38 and 44.76 µM).
However, many studies reported the effect of chalcone in decreasing Akt-signaling pathways, which have been associated with FLT3. (Mizuki et al. 2000)(Scheijen et al. 2004) Xu et al (2015) showed chalcone derivative, L2H17, inactivated NF-κB and Akt signaling pathways in colon cancer. (Xu et al. 2015) Akt is necessary for increased survival, proliferation, and leukemic transformation by Flt3-ITD, possibly by the inactivation of Foxo transcription factors. (Brandts et al. 2005) Mechanisms of Akt in cancer involved the following: survival of cells by blocking the function of pro-apoptotic proteins and processes; phosphorylation of Bad, (Datta et al. 1997)(del Peso et al. 1997) which promotes the release of Bad from heterodimers of Bcl-2 and Bcl-XL; phosphorylation of MDM2, (Ogawara et al. 2002)(Feng et al. 2004) stabilizing it and promoting its translocation to the nucleus, where it triggers p53 degradation; and phosphorylation of XIAP, an inhibitor of the caspase cascade, thus inhibiting its degradation.(Dan et al. 2004)

Activated Akt acts an essential survival factor by inhibiting apoptosis. (Hanada et al. 2004) In normal myeloid cells, ligand binding to wild-type FLT3 activates multiple signals including the PI3K/AKT, RAF/MAPK, and STAT pathways. In leukemic cells with FLT3-ITD, these pathways are constitutively activated. 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. (Scheijen et al. 2004) The PI3K/Akt signaling pathway plays an important role in both normal and malignant hematopoiesis. (Martelli et al. 2010)(Polak & Buitenhuis, 2012) Activated Akt is critical for leukemia cell survival and proliferation, (Chiarini et al. 2012) and is known to function as an essential survival factor by inhibiting apoptosis. (Hanada et al. 2004) These pathways are commonly targeted in cancer treatments.

Anticancer treatments are expected to induce apoptosis in cancer cells. Apoptosis is by far the best-characterized type of cell death, and is defined by morphologic modifications that promote executioner caspase activation. (Green, 1998) In this study, all treatments increased caspase-3 levels in the HL-60 cell line. The highest caspase-3 level was obtained in the treatment of 33.28 µM isotretinoin (0.4 ng/mL). All chalcone derivatives also showed high levels of caspase-3 which were comparable to those reported for isotretinoin and imatinib. Both MTC4 (4.41 and 8.82 µg/mL) showed caspase-3 levels of 0.36 ng/mL and 0.37 ng/mL, respectively, whilst MTC7 (22.38 and 44.76 µM) showed caspase-3 levels of 0.28 and 0.31 ng/mL, respectively (Fig. 3.). These results indicate that all treatments...
increased caspase-3 as an apoptotic marker in leukemia cells. These findings were confirmed by an apoptotic index, as shown in Fig. 4. All treatments of chalcone derivatives (MTC4 and MTC7) showed higher apoptosis levels compared to the control (8.19 and 4.55%, respectively). The highest apoptotic index was found in the treatment of 33.28 µM isotretinoin (10.10%). 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). (Shen et al. 2007) 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. (Green, 1998)

Signal transducer and activator of transcription-3 (STAT3), an oncogenic transcription factor, is often constitutively active in human cancer cells. (Bromberg et al. 1999) The measurement of STAT3 on HL-60 shows only four treatments that significantly decreased STAT-3 level in the HL-60 cell line. MTC4 of 4.41 µM was the only chalcone derivative that decreased STAT-3 level (22.23 µg/mL), and it was comparable to 40.51 µM imatinib, and 16.64 and 33.28 µM isotretinoin (28.09, 22.09, 20.97 µg/mL, respectively) (Fig. 5.). These findings are validated by a previous study which showed that a chalcone derivative, 4,3′,4′,5′-tetramethoxychalcone (TMOC), inhibits the phosphorylation of STAT3 and its upstream protein tyrosine kinase c-Src. (Qi et al. 2014) STAT3, an oncogenic transcription factor, is often constitutively active in human cancer cells. (Bromberg et al. 1999) 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. (Chan et al. 2004) (Yu et al. 2007)

Eukaryotic cells have developed control mechanisms that restrain cell-cycle transitions in response to stress. These regulatory pathways are termed cell-cycle checkpoints. (Paulovich et al. 1997) Cells can arrest transiently at cell-cycle check points to allow the repair of cellular damage. The cyclin D1/CDK4 complex is responsible for cell cycle progression in early G1 phase and is frequently overexpressed in various human carcinomas. (Wolter et al. 2001) (Bali et al. 2004) Cyclin D1 level on HL-60 treated with chalcone derivatives can be seen in Fig. 6, and the cell cycle is presented in Fig. 7.

Based on Fig. 6, all treatments decreased cyclin-D1 levels compared to the control (1.87 ng/mL). Chalcone derivatives, MTC4 and MTC7 in all treatments significantly decreased cyclin-D1. Cyclin-D1 level following treatment with MTC4 at 4.41 and 8.82 µM were 0.97 and 1 ng/mL respectively, whilst that of MTC7 at 22.38 and 44.76 µM were 1.04 and 1.03 ng/mL, respectively. In this study, MTC4 and MTC7 significantly decreased cyclin D1 level on HL-60. The cyclin D1/CDK4 complex is responsible for cell cycle progression in the early G1 phase and is frequently overexpressed in various human carcinomas. (Kusume et al. 1999) (Wolter et al. 2001) (Bali et al. 2004) MTC4 and MTC7 also arrested the G0/G1 and G2/M phase in HL-60 cells, which were comparable to imatinib and isotretinoin. These findings are validated with a previous study showing that chalcone inhibits the proliferation of T24 and HT-1376 cells by blocking cell cycle progression in the G0/M phase. (Shen et al. 2007) Another study investigated the effect of a synthetic chalcone derivative, 4,3′,4′,5′-tetramethoxychalcone (TMOC), which 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. (Qi et al. 2014) The p16 protein is a specific inhibitor of the CDK-cyclin D complex, preventing the phosphorylation of Rb and cell cycle reentry at G0/G1 phase. (Kusume et al. 1999) Eukaryotic cell cycle progression involves the sequential activation of cyclin-dependent kinases (CDK), whose activation is dependent on their association with cyclins. (Sancar et al. 2004) A complex formed by the association of Cdc2 (also known as Cdk1 or p34Cdc2) and cyclin B1 plays a major role at entry into mitosis. (Sancar et al. 2004) The phosphorylation of Tyr15 of Cdc2 suppresses the activity of the Cdk1/cyclin B1 kinase complex. Dephosphorylation of Tyr15 of Cdc2 is catalyzed by Cdc25 phosphatases, and this reaction is believed to be the rate-limiting step for entry into mitosis (De Souza et al. 2000).
As shown in Figure 7, all chalcone derivatives arrested G1 phase. G1 arrest results by 4.41 and 882 μM MTC4 were 39.54 and 41.24%, respectively, while those of 5.59 and 11.19 μM MTC7 were 41.02 and 41.11%, respectively. These results were comparable with those for 16.64 and 33.28 μM isotretinoin, showing G1 arrest results of 41.66 and 41.08%, respectively. Chalcone derivatives also decreased S phase. MTC4 at 4.41 and 8.82 μM decreased S phase by 29.22 and 33.55%, whilst MTC7 at 5.59 and 11.19 μM decreased S phase by 31.78 and 31.84%, respectively.

Figure 5. STAT-3 level on HL-60 cell line treated with imatinib (20.25 and 40.51 μM), isotretinoin (16.64 and 33.28 μM), Compound 4 (4.41 and 8.82 μM) and Compound 6 (22.38 and 44.76 μM).

Figure 6. Cyclin-D1 level on HL-60 cell line treated with imatinib (20.25 and 40.51 μM), isotretinoin (16.64 and 33.28 μM), Compound 4 (4.41 and 8.82 μM) and Compound 6 (22.38 and 44.76 μM).
Chalcone derivatives also arrested G2/M phase. However, 4.41 µM Compound 4 was the only chalcone that significantly arrested G2/M phase (41.21%) and was comparable to those for 16.64 and 33.28 µM isotretinoin (41.56 and 41.35%, respectively). Imatinib at 20.25 and 40.51 µM showed the highest arrest among treatments (50.14 and 47.30%, respectively). 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 block progression of the cell cycle at the G0/G1 or G2/M phases.40,42

CONCLUSION
From this study, we concluded that chalcone derivatives Compound 4 and 6 may be potential compounds for treating leukemia. The molecular mechanisms of chalcone derivatives in the leukemia cell line HL-60 involved: (i) inhibition of the PI3K/Akt signaling pathway, (ii) induction of apoptosis through the up-regulation of apoptotic markers, and (iii) the blockade of cell cycle progression by regulating cell cycle-related factors.

CONFLICT OF INTEREST
There is no conflict of interest in this study.

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AUTHOR CONTRIBUTIONS
NA designed and performed the experiment and also wrote the manuscript. MM, IA and JJ gave valuable insights related to this research. HS prepared the extract. All authors read and approved the final version.

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References


Martelli AM, Evangelisti C, Chiarini F, McCubrey JA. 2010. The phosphatidylinositol 3-
kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. Oncotarget 1: 89-93.