DOI Number: 10.37506/v11/i1/2020/ijphrd/194002

Curcumin and 6-Shogaol Increase Hemoglobin F Levels by Inhibiting Expression of STAT3 mRNA Gene in K562 Line Cell

Joko Setyono[1], Ahmad Hamim Sadewa[2], Edy Meiyanto[3], Mustofa.Mustofa[4]

[1]Department of Biochemistry, Faculty of Medicine, University of Jenderal Soedirman, Purwokerto, Indonesia, [2]Department of Biochemistry, Faculty of Medicine, Public Health and Nursing, University of Gadjah Mada, Jogjakarta, Indonesia, [3]Cancer Chemoprevention Research Center, Faculty of Pharmacy, University of Gadjah Mada, Jogjakarta, Indonesia, [4]Department of Pharmacology, Faculty of Medicine, Public Health and Nursing, University of Gadjah Mada, Jogjakarta, Indonesia

Abstract

One of the approaches for beta-thalassemia therapy is the induction of the Haemoglobin F (Hb F). Curcumin and 6-Shogaol are empirically known to induce HbF, but the signalling cascade has not been widely explained. This study aims to uncover the potential of Curcumin and 6-shogaol in inhibiting the expression of STAT3 mRNA gene. This study uses the K562 erythroleukemic line cell model with an experimental design post-test only with a control group. There are 5 groups, each group has 3 replications, named the control group without treatment, the positive control group with Hydroxyurea treatment (75 μM), the combination treatment group of Curcumin (2 μM) and 6-Shogaol (10 μM), the single curcumin (2 μM) treatment group and a single 6-Shogaol (10 μM) treatment group. Test samples were taken in 72-h and 96-h time series, then RNA extraction from the cell line was continued by cDNA synthesis. The expression of STAT3 mRNA gene was measured using the qRT-PCR technique; then, the Hb F level was measured by the ELISA method. Statistical analysis using ANOVA test with significance level $p < 0.05$. In the 72-h time series, there was a significant decrease in STAT3 Gena mRNA expression ($p < 0.05$). The lowest single curcumin group ($p < 0.01$) followed by a single 6-Shogaol group ($p < 0.05$) compared to the untreated control group, while the positive control group with hydroxyurea treatment and the Curcumin and 6-shogaol combination treatment groups are not significant. Hb F levels, there was an increase in 96-h time series ($p < 0.05$) respectively from highest to lowest in the curcumin group ($p < 0.05$), 6-shogaol ($p > 0.05$) compared to the control group, but in the positive control group ($p > 0.05$) and the combination group ($p > 0.05$) it is lower than the control group without any treatment.Curcumin and 6-shogaol increase Hb F levels through inhibition expression of STAT3 mRNA Gene on K562 cells. The results of this study could be the basis for further research in vivo to reveal the signalling pathway in Hb Finduction therapy.

Keywords: *Curcumin, 6-shogaol, STAT3 Gena mRNA, Hemoglobin F, K562 cells.*

Introduction

β-Thalassemia is a group of heterogeneous recessive autosomal hereditary genetic diseases associated with point mutation or small deletion resulting in the absence or reduction of β-globin chain protein synthesis, resulting in haemoglobin deficiency. There are alternative therapies that can be developed to overcome the severity of this disease by inducing Fetal Haemoglobin (Hb F). The globin-ϒ chain, which is similar to the globin-β chain, is produced during pregnancy when it joins with globin-α chain, is to form Fetal Hemoglobin ($\alpha_2\Upsilon_2$). Hence, one of the potential current therapeutic approaches to haematological disorders, including β-thalassemia, is the stimulation of induction of fetal haemoglobin production [1-4].

Corresponding Author:
Joko Setyono
Department of Biochemistry, Faculty of Medicine, University of Jenderal Soedirman, Purwokerto Indonesia
Tel.: +628121570458
e-mail: joko.setyono1907@unsoed.ac.id

One of the transcription factors that play a role in the production of Hemoglobin F is the phosphorylated STAT3 protein. This protein, which is the dominant-negative regulator, which acts in the 5'-untranslated globin-ϒ promoter region bound to AϒSTAT3, thus inhibiting the expression of globin-ϒ-dependent concentrations [5]. Therefore, it is necessary to have a potential inhibitor to STAT3 in the framework of globin-ϒ induction. Curcumin is a STAT3 inhibitor in the SH2 domain. Inhibition of the SH2 domain, not only disrupts activation but also dimerization of transcription factors [6]. Other active compounds of herbal ingredients that have molecular targets related to the regulation of Hemoglobin F are Shogaol. 6-shogaol is the most potent inhibitor of STAT3 activation when compared to analogues such as 6-gingerol, 8-gingerol and 10-gingerol [7]. The purpose of this study was to uncover the potential of Curcumin and 6-shogaol on the expression of STAT3 mRNA as one of the induction signals for Hemoglobin F.

Material and Method

1. **Provision of Curcumin and 6-shogaol and Hydroxyurea:** Curcumin (BioBasic, Canada) with > 95% of purity, 6-shogaol (Fortopchem, China) with >98% purity and Hydroxyurea (HU) (Sigma-Aldrich, USA) with approx >98% of purity. Curcumin, HU and 6-shogaol are dissolved in 100% dimethyl sulfoxide (DMSO) to reaches the final concentration of curcumin 271,000 µM, 6-Shogaol 361,000 µM, and HU 1,315,000 µM as a stock solution, then stored at -20ºC.

2. **K562 Cell Culture:** Erythroleukemia K562 cells obtained from CANCER CHEMOPREVENTION RESEARCH CENTER (CCRC) Faculty of Pharmacy in the University of Gadjah Mada was cultured with RPMI 1640 supplementary media (without phenol red) with 10% Fetal Bovine Serum (FBS) and 50 U/ml - 50 µg/ml penicillin-streptomycin (pen-strep). Culture was maintained under atmospheric humidity with 95% air/5% CO_2 at 37ºC with cell densities between 2 x 104 to 1 x 105 cells/ml.

3. **Cytotoxic test with MTT method assay 24 hours, 48 hours and 72 hours:** Cytotoxic tests were performed to obtain IC_{50} values following the protocol of CCRC Faculty of Pharmacy in the University of Gadjah Mada. The results of the IC_{50} values of each sample were taken from 24 hours, 48 hours and 72 hours incubation. The IC^{50} values obtained were 60 µM curcumin, 40 µM 6-shogaol and 300 µM HU, respectively.

4. **Combination Test:** K562 cells were distributed into 96 wells as much as 100 µL and incubated for 24 hours. Enter the Curcumin and 6-shogaol concentration series into the wells of 50 µL with five series of concentrations each consisting of ½ IC_{50}, ¼ IC_{50}, 1/8 IC_{50}, 1/16 IC_{50} and 1/32 IC_{50}. Incubation for 24 hours. Cells were counted by using a haemocytometer so that the number of living and dead cells in each well was obtained. The highest number of living cells is an indicator of the best combination dose, named Curcumin 2 µM, 6-shogaol 10 µM, and for HU it is determined by treatment of 75 µM (¼ IC_{50}).

5. **ELISA test to measure Hb F levels:** K562 cells that have been given the appropriate treatment in their groups are then carried out protein extraction by the procedure of the M-PER Kit (Thermo Scientific, USA). The lysate obtained was used for examination of Hb F levels measured by the Human HBF Cat ELISA kit. No: EH3213 (Fine Test, China) according to the manufacturer's instructions.

6. **Analysis of STAT3 Gena mRNA expression:** Total K562 RNA cells were extracted at different times depending on the treatment, using # RB100 (Geneaid, Taiwan), 1 µg of total RNA from each sample was carried out reverse transcripts to cDNA using the cDNA Synthesis Kit (Toyobo, Japan). Real-time PCR is done by machine (ABs) using the SensiFAST SYBR Lo-ROX Kit (Bioline, Germany). The relative mRNA levels of the target gene are normalized to the mean of the internal control gene, β-Actin.

   Primary mRNA with STAT3, forward: 5'-ATC ACG CCT TCT ACA GAC TGC-3 ', reverse: 5'-CAT CCT GGA TCT CTA CCA CT-3 '. β-ACTIN forward: 5 '-ACG GCC AGG TCA TCA CCA TTG-3', reverse: 5 '-GGC GTA CAG GTC TTT GCG GAT-3 TT'. The STAT3 gene expression between treated and untreated samples was calculated as $2^{-\Delta\Delta Ct}$ relatively to the reference gene, β-actin.

7. **Statistic analysis:** Data is displayed in mean ± SD and statistical analysis using one way ANOVA test followed by post hoc LSD. The test results are considered significant if $p < 0.05$ and 95% confidence intervals.

Findings: In this study, the impact of Curcumin, 6-shogaol and their combination on the interpretation of STAT3 mRNA expression were measured by the qRT-PCR method, and the results can be seen in Figure 1.

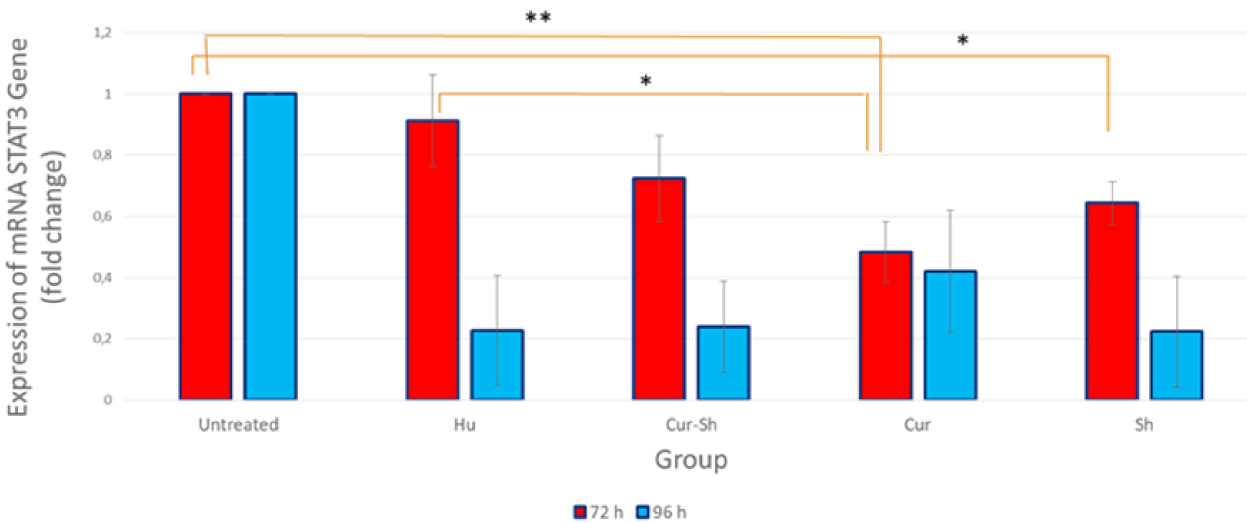

Figure 1: Differences in fold changes of STAT3 mRNA between groups after incubation of Curcumin (Cur), 6-Shogaol (Sh) and Hydroxyurea (Hu) on serial K562 cells for 72 and 96 h. Curcumin showed the strongest expression followed by 6-Shogaol compared with the control group without treatment (* p <0.05, ** p <0.01).

The results showed a decrease in STAT3 mRNA expression in all treatment groups in both the 72-h and 96-h time series compared to the untreated group. In the 72-h time series, the results are significantly different p <0.05. Then followed by post hoc LSD test, in the Curcumin treatment group, the lowest expression of STAT3 mRNA gene was 0.48 ± 0.1 (p = 0.008) which was significant compared to the control group without treatment. Next, in the 6-Shogaol treatment group, the expression of STAT3 mRNA gene (0.64 ± 0.07, p = 0.045) was significant compared to the control group without treatment. In the combination group of Curcumin with 6-shogaol, the expression of STAT3 mRNA gene (0.72 ± 0.14) was lower than the positive control group but higher than the curcumin and 6-shogaol group. In the 96-h time series, the results did not have significantly different (p = 0.098) between the treatment group and the control group without treatment. So, in the 96-h time series, there was a decrease in STAT3 mRNA expression, which was not significantly different.

The effect of Curcumin and 6- shogaol on haemoglobin F levels measured by the ELISA method can be seen from Figure 2. The results show that in a 96-h time series, Hb F levels were the highest in the curcumin group (1.6 ± 0.15; p = 0.015), then followed by the 6-shogaol group (1.11 ± 0.29). In the positive control group hydroxyurea and the combination of Curcumin + 6-shogaol, Hb F levels were consecutively (0.85 ± 0.16), (0.41 ± 0.01) lower than those in the untreated control group. The combination group of Curcumin with 6-shogaol was the group with the lowest Hb F level compared with the treatment group and the control group without treatment (p <0.05). In the 72-h time series, the Hb F level of all treatment groups was lower than the control group without treatment. Based on the one way ANOVA test, the 72-h treatment did not differ significantly (p> 0.05), but in the 96-h treatment, there was a significant difference (p <0.05). So, in the 96-h time series, the highest Hb F level occurred in the curcumin group, and the lowest Hb F level occurred in the combination group of Curcumin with 6-shogaol.

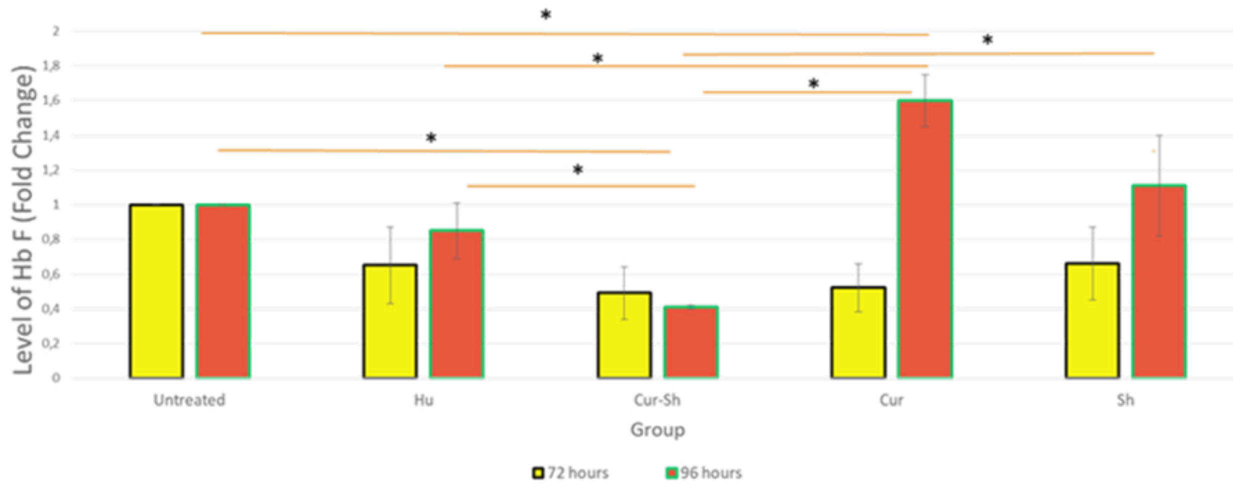

Figure 2: Differences in fold changes of HbF levels between groups after incubation of Curcumin (Cur), 6-Shogaol (Sh) and Hydroxyurea (Hu) on 72 and 96 h serial K562 cells. In the 96-h time series, Curcumin showed the highest effect on changes in HbF levels followed by 6-Shogaol compared to the control group without treatment (* p <0.0).

Reduced hexahydro-bisdemethoxy curcumin (HHBDMC) reduced curcuminoids were most effective in inducing gamma-globin mRNA (3.6 ± 0.4 fold) and Hb F (2.0 ± 0.4 fold) in erythroid primary precursor cells for seven days[8]. Curcumin works to reduce the expression of STAT3 genes so, the decrease in STAT3 gene expression will increase the production of Hb F. The role of 6-shogaol in Hb F induction through the activation of the p-p38 MAPK signal[9] whereas p38 MAPK is reduced by two weeks of curcumin activation[10].

Discussion and Conclusion

In beta-thalassemia patients there is an imbalance in the number of globin-α/globin-β chains, due to the lack or absence of globin-β synthesis, resulting in precipitation of free globin-α chains in erythroid precursors which results in the maturation and damage of erythrocyte cells, causing prolonged anaemia [11]. Therefore the best choice for the treatment of thalassemia patients is reactivation/induction of globin-ϒ, so that replaces globin-β to join globin-α to form fetal haemoglobin and ultimately there is no excess free globin-α chains [12]. Individuals with elevated Hb F levels (> 8.6%) show a reduction in symptoms and increase patient life expectancy; hence, the induction of Hb F has the potential as a therapy in beta-thalassemia patients. Of the current therapeutic options, Hb F induction through pharmacological agents is the most feasible therapeutic choice [8].

Other transcription factors that play a role in the production of haemoglobin F are the phosphorylated STAT3 protein. Therefore, it is necessary to have a potential inhibitor to STAT3 in the framework of gamma-globin induction. Pharmacological inhibitors targeting STAT3 can be done in 5 ways, namely inhibition of STAT3 DNA-binding domain, abrogation of the STAT3 N-terminal domain, suppression of the STAT3 SH2 domain, inhibition of the STAT3-importin interaction, and/or blockage of upstream kinase activity [13].

The active compounds of the following herb, Curcumin, have broad molecular targets related to various molecular and biochemical cascades interacting directly on the target protein and epigenetic modulation of the target genes. Curcumin, as an epigenetic agent, functionally in modulating multiple biological processes, occurs at low concentrations. Curcumin plays a role in the expression of genes through direct interaction with transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), epigenetic modulation through inhibition of DNA methyltransferase I (DNMT1), histone acetyltransferase (HAT), histone deacetylase complex (HDAC) [14]. Various molecular targets of Curcumin include inflammation, kinase activity (MAPK, PKA, JAK), transcription factors (CREB, STAT3, PPARϒ), enzyme activity (COX- 2, INOS, MMP), and others (VEGF, adiponectin, ROS) [15-16]. The 6-Shogaol are other active compounds of herbal ingredients that have molecular targets related to the regulation of Hemoglobin F. The 6-shogaol suppresses the expression of the products governed by STAT3. It was also reported that 6-shogaol caused the activation of JNK, p38 and ERK, as well as downregulating the expression of p38 MAPK, NF-κB and COX-2 [17]. Therefore, 6-shogaol can play a role in the induction of Hemoglobin F.

Hydroxyurea (100 μM) has a link between the regulator of globin-ϒ expression (MYB, BCL11A and KLF-1) with specific miRNA, and reveals the mechanism of Hb F production through inhibition of HU-induced miRNA [18]. Treatment with HU combined with HDAC2 knockdown increases gamma-globin expression. It was also reported that CD34 + cells treated with HU and MS-275 (HDAC inhibitors 1,2 and 3) had a relative induction of gamma-globin expression [19].

Curcumin is a decreasing expression of STAT3 mRNA gene and increases Hb F levels compared to 6-shogaol on K562 cells. While the combination of the two substances was not significant either in inhibiting STAT3 expression or HbF levels. The results of this study could be the basis for further research in vivo to reveal the signalling pathway in Hb F induction therapy ($\alpha_2\Upsilon_2$).

Conflict of Interest: Authors report no conflict of interest.

Source of Funding: It was funded by Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

Ethical Clearance: This study was approved by Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Gadjah MadaUniversity-DR.Sardjito General Hospital (Ref: KE/FK/1150/EC/2017).

References

1. Bianchi N, Chiarabelli C, Borgatti M, Mischiati C, Fibach E, Gambari, R. Accumulation of γ-globin mRNA and Induction of Erythroid Differentiation After Treatment of Human Leukaemic K562 Cells with Tallimustine. Br J Haematol. 2001;113:951-961
2. Fard AD, Hosseini SA, Shahjahani M, Salari F, Jaseb K. Evaluation of Novel Fetal Hemoglobin Inducer Drugs in Treatment of β-Hemoglobinopathy Disorders. Int J Hematol Oncol Stem Cell Res.2013;(3):47–54.
3. Makala LH, Torres CM, Clay EL, Neumert C, Pace BS. Fetal Hemoglobin Induction β-Hemoglobinopathies : From Bench to Bedside. J Hematol Transfus, 2014;2(2):1018
4. Dreuzy E, Bhukhai K, Leboulch P, Payen E. Current and Future Alternative Therapies for Beta-Thalassemia Major. BMJ.2016;39:24-38
5. Foley HA, Ofori-Acquah SF, Yoshimurai A, Critz S, Baliga BS, Pace BS. Stat3β Inhibit γ-Globin Gene Expression in Erythroid Cells. J Biol Chem.2002;277(18):16211-16219
6. Chai EZP, Shanmugam MK, Arfuso F, Dharmarajan A, Wang C, Kumar AP, et al. Targeting Transcription Factor STAT3 for Cancer Prevention and Therapy. Pharmacology & Therapeutics.2016;162:86-97
7. Kim S-M, Kim C, Bae H, Lee JH, Baek SH, Nam D, et al., 6-shogaol Exerts Anti-Proliferative and Pro-Apoptotic Effects Through the Modulation of Stat3 and MAPKs Signaling Pathways. Mol Carcinog.2014;54(10):1132-1146
8. Chaneiam N, Changtam C, Mungkongdee T, Suthatvoravut U, Winichagoon P, Vadolas J, et al. A Reduced Kurkuminoid Analog as A Novel Inducer of Fetal Hemoglobin. Ann Hematol.2013;92:379–386
9. Ramakhrisnan V, Pace BS. Regulation of γ-globin Gene Expression Involves Signaling Through the p38 MAPK/CREB1 Pathway. Blood Cells Mol Dis. 2011;47:12–22
10. Camacho-Barquero L, Villegas I, Sanche-Calvo JM, Talero E, Sanchez-Fidalgo S, Motilva V, et al. Kurkumin, a Curcuma Longa Constituent, Act on MAPK p38 pathway modulating COX-2 and iNOS expression in Chronic Experimental Colitis. Int Immunopharmacol. 2007;7(8):333–342
11. Sankaran VG. Targeted Therapeutic Strategies for Fetal Hemoglobin Induction. Hematology.2011:459–465
12. Bauer DE, Kamran SC, Orkin SH. Reawakening Fetal Hemoglobin: Prospects for New Therapies for The β-Globin Disorders. Blood. 2012;120(15):2945–2953
13. Chai EZP, Shanmugam MK, Arfuso F, Dharmarajan

A, Wang C, Kumar AP, et al. Targeting Transcription Factor STAT3 for Cancer Prevention and Therapy. Pharmacology & Therapeutics.2016;162:86-97

14. Fu S, Kurzrock R. Development of Curcumin as an Epigenetic Agent. Cancer. 2010;116:4670–4676
15. Sunagawa Y, Katanasaka Y, Hasegawa K, Morimoto T. Clinical Application of Kurkumin. PharmaNutrition. 2015;67:1 5
16. Setyono J, Harini IM, Sarmoko S, Rujito L. Supplementation of curcuma domestica extract reduces cox-2 and inos expression on raw 264.7 cells. Journal of Physics: Conf. 2019; Series, 1246 012059, IOP Publishing. doi:10.1088/1742-6596/1246/1/012059
17. Ha SK, Moon E, Ju MS, Kim DH, Ryu JH, Oh MS, et al. 6-Shogaol, a ginger product, modulates neuroinflammation: A new approach to neuroprotection. Neuropharmacology. 2012;63:211-223
18. Pulle GD, Mowia S, Novitzky N, Wonkam A., Hydroxyurea Down-regulates BCL11A, KLF-1 and MYB Trough miRNA-mediated Action to Induce γ-Globin Expression : Implications for New Therapeutic Approaches of Sickle Cell Disease. Clin Trans Med. 2016;5:1–15
19. Esrick EB, McConkey M, Lin K, Frisbee A, Ebert BL. Inactivation of HDAC1 or HDAC2 induces gamma globin expression without altering cell cycle or proliferation. Am J Hematol. 2015;90(7):624-628