

Screening for Novel Hemoglobin F Inducer Compounds

Yanisa Wassanatip* Dr.Pornthip Chaichompoo**

Dr.Saovaros Svasti***

ABSTRACT

β -Thalassemia is an inherited disorder that is characterized by an imbalanced of α - and β -globin chain synthesis. Defection of β -globin chain synthesis leads to accumulation of unbound α -globin chains in erythroid cells which is the major cause of pathology. Reactivation of γ -globin chain production to combines with the excess unbound α -globin chains form fetal hemoglobin (HbF) can ameliorate disease severity. HbF inducers used nowadays are chemical agents and most of them are high toxicity and low specificity. This study aimed to screen 52 compounds extracted from Thai medicinal plants for novel efficacy HbF inducer with low toxicity by used reporter cell lines. The results showed that 2 compounds, code 355 and 358, significantly enhance γ -globin gene expression with low cytotoxicity.

Keywords: β -Thalassemia, Hemoglobin F, Thai natural compounds

* Student, Master of Science Program in Biochemistry, Faculty of Science, Mahidol University

** Lecturer, Department of Pathobiology, Faculty of Science, Mahidol University

*** Associate Professor, Thalassemia Research Center, Institute of Molecular Biosciences and Department of Biochemistry, Faculty of Science, Mahidol University

Introduction

β -Thalassemia is an inherited disorder that caused by the defection in β -globin chain synthesis that lead to the reduced (β^+) or absent (β^0) production of β -globin chains while α -globin chain is continued normal synthesized. The excess unbound α -globin precipitate in erythroid precursors and mature red blood cells cause ineffective and short red cells survival. The pathology of thalassemia is due to the excess unbound α -globin chains includes chronic anemia, iron overload, splenomegaly, bone change, osteoporosis and many affected of various organs such as cardiac dysfunction and heart failure (Cao & Galanello, 2010).

Current therapies for β -thalassemia patients are blood transfusion supplemented with iron chelator and stem cell transplantation. In severe anemic patients, regular blood transfusion is necessary (Rund & Rachmilewitz, 2005). However, blood transfusion has a high rate of allo-immunization and be a cause of iron-overload. So, iron chelator such as desferioxamine is required for the patients (Borgna-Pignatti, 2007). The stem cell transplantation is the only curative treatment but it has the limitation too. It needs the human leukocyte antigen match donors and high cost (Musallam, Taher, & Rachmilewitz, 2012). An alternative treatment for β -thalassemia is use fetal hemoglobin (HbF, $\alpha_2\gamma_2$) inducer to stimulate γ -globin chain production which can assemble with unbound α -globin chains to form HbF to reduce the excess unbound α -globin chains (Gambari & Fibach, 2007; Perrine et al., 2010).

Some HbF inducing agents such as 5-azacytidine, cisplatin and hydroxyurea have been reported. However, there are disadvantages of these compounds due to the low efficiency and high toxicity (Bianchi, Zuccato, Lampronti, Borgatti, & Gambari, 2009; El-Beshlawy, Hamdy, & El Ghamrawy, 2009). These lead to the searching for new HbF inducing agents especially from the natural product from Thai medicinal plants. Studies about HbF inducer from Thai medicinal plants revealed that labdane diterpenes from *Curcuma comosa* and curcuminoid compounds from *Curcuma longa* can induce HbF synthesis and γ -globin gene expression (Chaneiam et al., 2013; Changtam et al., 2010). Although, these compounds are low toxicity, the potential of them are not effective as equal as chemical agents. This is the causes why another study still has to perform to find better HbF inducing agents from natural products. In this study, we focus on screening 52 compounds extracted from Thai medicinal plants for HbF inducing property.

Objective of the study

To screen compound extracted from Thai medicinal plants for new HbF inducing agents.

Methodology

Compounds

Fifty-two compounds were extracted from Thai medicinal plants and purified by high performance liquid chromatography (HPLC) then prepared as stock solutions by dissolved in 100% DMSO. The final concentration of solution is 10 mM and stored at 4°C in dark. The compounds were kindly provided by Prof. Apichart Suksamran, Department of Chemistry, Faculty of Science, Ramkhamhang University, Bangkok.

Cell maintenance

A reporter cell line, K562:: $\Delta^{\text{G}}\gamma\text{-}^{\text{A}}\gamma$ EGFP cells, containing green fluorescence protein (EGFP) coding sequence in-frame replacement of the $\text{G}\gamma$ - and $\text{A}\gamma$ -globin coding sequence in the human α -globin cluster was used for screening of γ -globin gene inducer (Vadolas, Wardan, Orford, Williamson, & Ioannou, 2004). These cells were cultured in RPMI 1640 supplement with 20% fetal bovine serum (Sigma) and incubated at 37°C in 5% CO₂ incubator. The cells were subcultured every 3-4 days in a ratio of 1x10⁵ cells/ml.

Cell treatment

Before treatment, cells were transferred to 24 well-plate at final concentration 1.5x10⁵ cells/ml, total volume 2 ml. Cells were treated with the 52 compounds at three concentrations (10, 20 and 30 μ M). EGFP expression was analyzed by flow cytometer after 5 days of treatment. The untreated cells and DMSO were used as the negative control, 5 μ M cisplatin (Pfizer) and 10 μ M hemin (Sigma) as positive control.

EGFP expression and cell viability analysis

K562:: $\Delta^{\text{G}}\gamma\text{-}^{\text{A}}\gamma$ EGFP cells treated with and without the compounds were measured EGFP expression and cell viability after 5 days of treatment. The 1x10⁵ cells were collected and measured EGFP expression by FACScan flow cytometer (BD Biosciences) and Cell Quest software (BD Biosciences). The mean fluorescent intensity (MFI) of EGFP expression was measured and used for calculation of the fold change response to treated and untreated cells. Cell viability was analyzed by propidium iodide (PI) staining method. Percentage of PI stained cells as dead cells were used to calculate the cell viability. Flow cytometric analysis of the EGFP expression and cell viability were shown in Fig. 1. The MFI of HbF productions and cell viability were calculated as following:

$$\text{Fold change of EGFP expression} = \frac{\text{Mean fluorescent intensity of treated live cells}}{\text{Mean fluorescent intensity of untreated live cells}}$$

$$\text{Cell viability (\%)} = \frac{\text{Total number of cells} - \text{Number of fluorescence positive cells}}{\text{Total number of cells}} \times 100$$

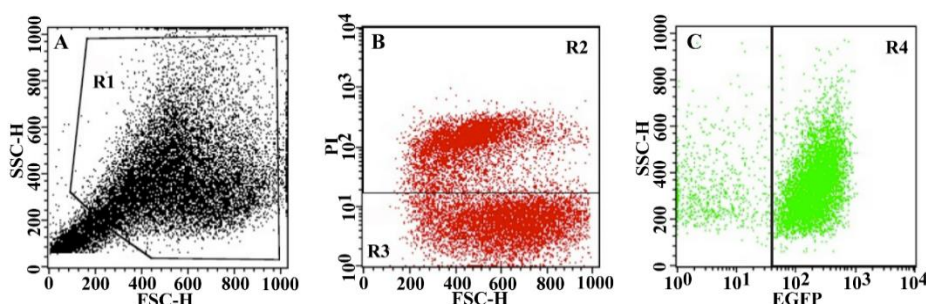


Figure 1 EGFP expression and cell viability analysis by Flow cytometry. (A) R1 region presents the K562:: $\Delta^G\gamma$ - γ EGFP cells population as determined by forward scatter (FSC) and side scatter (SSC). (B) The dead cells (PI-positive, R2 region) and living cells (PI-negative, R3 region) were identified by PI staining. (C) The PI-negative cells were further analyzed for EGFP MFI of EGFP positive cells (R4 region).

Statistical analysis

The results of test compounds were compared to untreated cells by paired-T test to analyze statistical significance in the fold change of EGFP expression and the percentage of cell viability. P-value ≤ 0.05 was considered as statistical significance.

Results

The compounds were screened for its HbF inducer activity by using the K562:: $\Delta^G\gamma$ - γ EGFP reporter cells. This cell line has previously been shown to respond to known HbF inducers as shown by the increased EGFP expression linked to γ -globin promoter activity (Vadolas et al., 2004). The K562:: $\Delta^G\gamma$ - γ EGFP cells were initially treated with positive compounds, cisplatin and hemin, and vehicle compound, DMSO. The increased HbF production was observed in cisplatin and hemin treated cells, while there was showed no enhanced HbF production in cells treated with DMSO (Fig. 2). The cells were treated with 52 compounds at 10-30 μ M for 5 days without changing media in order to screen for the effective compounds. After treatment, the MFI of EGFP expression and cell viability examined with PI were measured using flow cytometry.

From screening, we classified compounds into 5 groups based on criteria of induction of EGFP gene expression over 1.5 fold and toxicity, with more than 80% cell viability. The results showed that 2 compounds, compound code 355 and 358 can enhance EGFP expression. The highest of EGFP expression induced by compound 355 and 358 (Fig. 3) were observed at 20 μ M and 30 μ M, respectively. Both of them give the percentage of cell viability over 80% (Fig. 2 and Table 2). The remaining compounds can be categorized into 5 groups based on criteria of induction of EGFP gene expression and toxicity as showed in (Fig. 4).

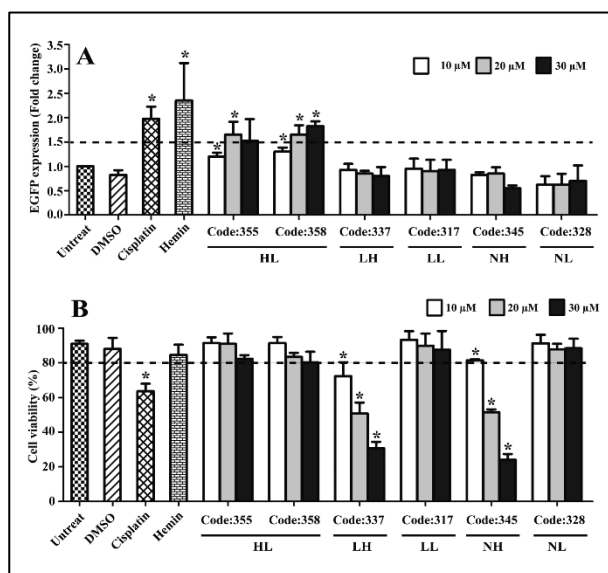
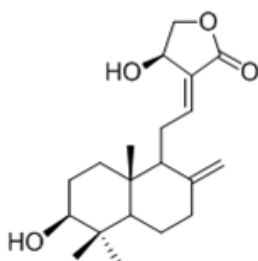


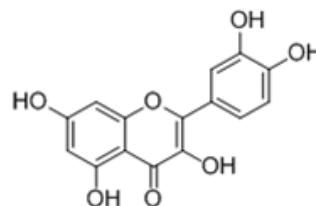
Figure 2 Analysis of enhanced EGFP and cytotoxicity by compound in high induction, low toxicity group and representative compound from other groups. The K562:: $\Delta^G\gamma$ - γ EGFP cells were treated with the compounds for 5 days. * $P < 0.05$ when compared to untreated cells.

Table 2 The effect of compound in high induction, low toxicity group on K562:: $\Delta^G\gamma$ - γ EGFP cells.

Compound	Concentration (μ M)	EGFP (Fold change)	Cell viability (%)
355	10	1.2 $\pm$ 0.1	91.4 $\pm$ 3.3
	20	1.7 $\pm$ 0.3	90.9 $\pm$ 6.0
	30	1.5 $\pm$ 0.4	82.3 $\pm$ 2.1
358	10	1.3 $\pm$ 0.1	91.4 $\pm$ 3.4
	20	1.7 $\pm$ 0.2	83.4 $\pm$ 2.3
	30	1.8 $\pm$ 0.1	80.3 $\pm$ 6.1



Compound 355



Compound 358

Figure 3 Structure of compound 355 and 358.

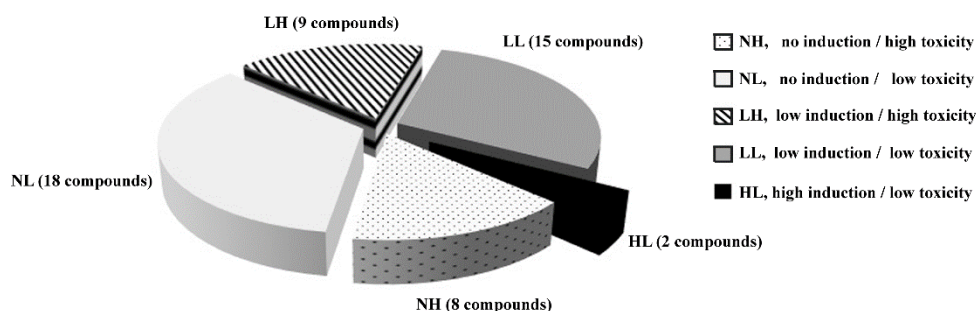


Figure 4 Classification of 52 compounds based on criteria of induction of γ -globin gene expression and toxicity.

Discussion and Conclusions

In an effort to find the novel HbF inducer compounds, we treated K562:: $\Delta^G\gamma$ - $\Delta^A\gamma$ EGFP cell lines with 52 compounds extracted from Thai medicinal plants then measured EGFP expression and cytotoxicity. We found that 2 out of 52 compounds, code 355 and 358, could induce EGFP expression. When compared the effect of compound code 355 and 358 with control agents, cisplatin and hemin, the results showed that compound 355 and 358 can enhanced EGFP expression at lower level compared to positive control while have higher percentage of cell viability than cisplatin. These indicate that compound 355 and 358 have potential to induce γ -globin gene expression and not toxic to cells. A number of plant extracts and plant constituents have been reported to induce HbF expression such as resveratrol from grape, angelicin from *Aegle marmelos* (Bianchi et al., 2009), labdane diterpenes from *Curcuma comosa* (Changtam et al., 2010), citropten, bergapten from *Citrus bergamia* (Guerrini et al., 2009) and curcuminoid from *Curcuma longa* (Chaneiam et al., 2013). However, these compounds had less HbF inducer activity compared to chemical drug compounds. Modification of the 2 candidate lead compounds may results in a higher γ -globin gene expression inducing activity with lower toxicity. A slight different in structure of curcuminoid compounds can give a large different effect on γ -globin gene expression (Chaneiam et al., 2013). Thus, there might be some synthetic analogs of the 2 compounds that can give more potential than their parent compounds.

In conclusion, 52 compounds extracted from Thai medicinal plant had been screened for HbF inducing property in K562:: $\Delta^G\gamma$ - $\Delta^A\gamma$ EGFP cell lines. The results showed that 2 compounds from 52 compounds can enhance EGFP expression more than 1.5 fold changes when compared to untreated cells. The effect of compound 355 and 358 on HbF expression induction should be further determined in primary human erythroid progenitor cells.

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