A reduced curcuminoid analog as a novel inducers of fetal hemoglobin

Nattawara Chaneiam[a,b], Chatchawan Changtam[c], Thongperm Mungkongdee[b], Umaporn Suthatvoravut[d], Pranee Winichagoon[b], Jim Vadolas[e], Apichart Suksamrarn[c], Suthat Fucharoen[b] and Saovaros Svasti[b,f,*]

[a]*Institute of Nutrition, Mahidol University, Nakhonpathom 73170, Thailand*
[b]*Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakhonpathom 73170, Thailand*
[c]*Department of Chemistry and Center for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand*
[d]*Department of Pediatric, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand*
[e]*CAGT Research Group, The Murdoch Childrens Research Institute, Royal Children's Hospital, Victoria 3052, Australia*
[f]*Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

Running title: Curcuminoid analog as HbF inducer

***Corresponding author:** Saovaros Svasti, Thalassaemia Research Center, Institute of Molecular Biosciences, Mahidol University, Salaya Campus, 25/25 Phuttamonthon 4 Rd, Phuttamonthon, Nakornpathom 73170, Thailand. Phone: +66 2 889 2557, Fax: +66 2 889 2559, E-mail: stssv@mahidol.ac.th

Word count: abstract 147 words, Total 3,655 words.
Number of tables: 1
Number of figures: 3

Disclosures:
Conflict of interest: none
Redundant publication: no substantial overlap with previous papers

Authors' contributions:
NC contributed to the study design, molecular study, specimen collection and drafted manuscript. CC and AS purify and synthesis the compounds. TM and SF contributed to specimen collection. US, PW and JV, contributed to concept of study. SS was the principal investigator and responsible for study design, concept of the study, analysis of data and drafting manuscript. Final approval of the version to be published was made by all authors.

Abbreviations: Hb, hemoglobin**;** BDMC, bisdemethoxycurcumin; THBDMC, tetrahydrobisdemethoxycurcumin; HHBDMC, hexahydrobisdemethoxycurcumin; OHBDMC, octahydrobisdemethoxycurcumin

Abatract

Thalassemia is an inherited disorder of hemoglobin molecules that is characterised by an imbalance of α- and β-globin chain synthesis. Accumulation of unbound α-globin chains in erythroid cells is the major cause of pathology in β-thalassemia. Stimulation of γ-globin production can ameliorate disease severity as it combines with the α-globin to form fetal hemoglobin. We examined γ-globin inducing effect of curcuminoids extracted from *Curcuma longa* L. and their metabolite reduced forms in erythroid leukemia K562 and human primary erythroid precursor cells. The results showed that curcuminoid compounds, especially bisdemethoxycurcumin (BDMC) are potential γ-globin enhancers. We also demonstrated that its reduced analog, hexahydrobisdemethoxycurcumin (HHBDMC), is most effective and leads to induction of γ-globin mRNA and HbF in primary erythroid precursor cells for 3.6±0.4 and 2.0±0.4 folds respectively. This suggested that HHBDMC is the potential agent to be developed as a new therapeutic drug for β-thalassemia and related β-hemoglobinopathies.

Keywords: Hemoglobin F; Thalassemia; Curcumin; Reduced curcuminoid analog

Introduction

β-Thalassemia is an inherited disorder of hemoglobin synthesis characterized by the absence or reduction of β-globin chains in erythroid cells [1,2] . The continued synthesis of α-globin chains, in normal amount, leads to excess α-globins, which do not bind with β-globins and accumulate within erythroid precursors and mature red blood cells. These insoluble aggregates of α-globin cause membrane damage, leading to short red-cell survival. The resulting anemia causes an intense proliferative drive of ineffective ineffective erythropoiesis in bone marrow, which leads to skeletal deformities and a variety of growth and metabolic abnormalities. Furthermore, hyperplasia of the bone marrow leads to increased iron absorption and iron loading resulting in progressive iron deposition in the tissue, organ failure and finally death, if the iron is not removed [1,2]. The degree of imbalance in α- and β-globin chain synthesis also determines the thalassemia phenotype and the severity level in these patients [3].

Pharmacological stimulation of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) production is a potential therapeutic approach for β-thalassemia [1,4] . The rationale for this approach is based on the observation that γ-globins can combine with α-globins and compensate for the reduced amount of β-globin synthesis in β-thalassemia. A number of clinical trials have investigated several potential HbF inducers. The current drugs for Hb F induction, such as 5-azacytidine, hydroxyurea, and butyric acid, have low efficacy and specificity, and some agents are potential carcinogens and associated with high toxicity [5,6]. Therefore, the identification of new pharmacological HbF inducing agents with greater efficacy and less toxicity, is urgently needed for treatment of β-thalassemia patients. A number of plant extracts and plant constituents have been reported to induce HbF expression [7]. *Curcuma longa* L. is a

medicinal plant used widely as Asian traditional herbal remedy and dietary spice. Its curcuminoid constituents are known to have the biological activities as antioxidant, anti-inflammation and anticarcinogen [8,9]. This study will explore the γ-globin expression and HbF inducing effect of curcuminoids from *C. longa* extract. Their curcuminoid constituents, curcumin, demethoxycurcumin and bisdemethoxycurcumin (BDMC) and the metabolite reduced analogs of these curcuminoids were also assessed, aiming to find a new drug for treatment of hemoglobinopathies, especially β-thalassemic disease.

Materials and methods

Curcuminoids and reduced curcuminoid analogs

The three natural curcuminoids, curcumin, demethoxycurcumin and BDMC, were obtained from the rhizomes of *C. longa* as described previously [10]. Tetrahydrobisdemethoxycurcumin (THBDMC), hexahydrobisdemethoxycurcumin (HHBDMC) and octahydrobisdemethoxycurcumin (OHBDMC) were prepared by catalytic hydrogenation of BDMC in the same manner described for the preparation of reduced analogs of curcumin [10,11]. The mixture of crude products was chromatographed to afford THBDMC (60 mg, 60%), HHBDMC (22 mg, 22%) and OHBDMC (10 mg, 10%). The synthesized analogs were characterized by spectroscopic techniques including IR, NMR and mass spectrometry. The ^{1}H NMR data of THBDMC were consistent with those reported previously [12] whereas those of THBDMC and OHBDMC were consistent with their spectroscopic data (see supplementary data).

Stock compound solutions

The curcuminoids and analogs were dissolved in 100% dimethyl sulfoxide (DMSO) into a final concentration of 10 mM as the stock solution and kept at 4 °C in the dark. Hemin (Sigma-Aldrich, St. Louis, MO) solution was freshly prepared by dissolving in DMSO to a final concentration of 5 mM and filtered through a 0.45 µm filter paper. Cisplatin (Pfizer (Perth), Bentley, Australia), 3.3 mM stock solution, was prepared by dissolving in distilled water and filtered through a 0.45 µm filter paper before storage at 4 °C.

Cell lines and treatment

A stable reporter cell line, K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cells, harboring green fluorescent protein gene in-frame replacement of the $^{G}\gamma$- and $^{A}\gamma$-globin coding sequence in the intact human β-globin locus was used for screening of HbF inducer [13]. These cells and K562 cells were cultured in RPMI1640 supplement with 20% fetal bovine serum (FBS, GIBCO-Invitrogen, Carlsbad, CA). The cells were treated with or without the test compounds. EGFP expression and HbF staining were analyzed by FACS after 5 days of treatment. The untreated cells were used as the negative control, and cells treated with 50 µM hemin and 20 µM cisplatin as positive controls.

Human erythroid primary cells culture

Infomred consent forms for ethical clearance permitted by the Institutional Review Board of Mahidol University, Thailand were signed by all healthy volunteers before 20 ml peripheral blood samples were collected. CD34 positive cells were isolated by immunomagnetic

positive cell selection using anti-CD34 immunomagnetic bead (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were cultured by two-phase methods as previous described [14]. Briefly, the cells were initially cultured in phase I culture medium composed of Iscoves modified Dulbecco medium (IMDM, GIBCO-Invitrogen) and supplemented with 30% FBS, 0.01% bovine serum albumin (BSA, GIBCO-Invitrogen), 1% L-glutamin, 10 μg/ml cyclosporin A (Novartis, East Hanover, NJ), 25 ng/ml human interleukin-3 (IL-3, Promokine, Germany), 50 ng/ml recombinant human stem cell factor (rhSCF, Promokine), and 0.1 U/ml recombinant human erythropoietin (rhEPO, EPREX, Belgium). On day 7, cells were subcultured in phase II culture media contained IMDM and supplemented with 30% FBS, 1% BSA, 1 μM hydrocortisone (Sigma-Aldrich), 0.1 mM β-mercaptoethanol (GIBCO-Invitrogen), 0.1 ng/ml IL-3, and 5 U/ml rhEPO. The cells were treated with curcuminoids on day 7 and harvested for analysis of globin gene expression and HbF production on day 14.

Cell viability measurement

The viability of cells was measured by using an annexin V/PI apoptosis kit (BD Bioscience, San Jose, CA). Positive cell staining was detected by FACSCalibur flow cytometer and analysis is performed using BD Cell Quest software (BD Bioscience). The percentage of cell viability was calculated based on the number of non-positive cells.

Analysis of EGFP expression and HbF production

The induction of EGFP expression in curcuminoids treated K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cells was measured by FACSCalibur flow cytometer (BD Bioscience) and Cell Quest software (BD Bioscience). The levels of HbF production were determined by staining with fluorescein

isothiocyanate (FITC)-conjugated monoclonal antibody against HbF (BD Bioscience) and analyzed by FACSCalibur flow cytometer. The mean fluorescent intensity of EGFP expression or HbF production was measured and used for calculation of the fold change responsed to various inducing agents between the induced and non-induced cells.

Globin mRNA analysis

Total RNA was extracted by TriReagent® (Molecular Research Center, Cincinnati, OH) according to the manufacturer instructions. One microgram of total RNA was reverse-transcribed to cDNA using using Oligo-dT primer and Super-Script^{TM} II Reverse Transcriptase (Invitrogen, USA). Multiplex quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) for quantification of α-, β- and γ-mRNA was performed using TaqMan^{TM} One-Step RT-PCR Master Mix kit (Bio-Rad Laboratories, Hercules, CA) previously described [15]. The globin gene expression between treated and untreated samples was calculated as $2^{-\Delta\Delta Ct}$ relatively to the reference gene, β-actin.

Statistic analyses

Data are shown as mean ± SD of three independent measurements and statistically analyzed using Student's t-test. Values of significance were considered at $P < 0.05$.

Results

Induction of γ-globin gene expression using K562::$\Delta^{G}\gamma$-$^{A}\gamma$EGFP reporter cells treated with C. longa extracts and its reduced analogs

C. longa crude extract was firstly screened to investigate its activity as γ-globin and HbF enhancer by using the K562::$\Delta^{G}\gamma$-$^{A}\gamma$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 [13]. The result showed that 30 µg/ml *C. longa* crude extract enhanced EGFP expression by 1.4±0.5 fold with 89% cell viability when compared to untreated cells (Fig. 1A). This result prompted us to continue to screen individual curcuminoid compounds; curcumin, demethoxycurcumin and bisdemethoxycurcumin (BDMC). Interestingly, all curcuminoid compounds enhanced γ-gene promoter activity in a dose-dependent manner, as shown by induced EGFP expression. BDMC was found to give the highest EGFP expression at 30 µM with a 3.5±0.4 fold increase, compared to the untreated control (Fig. 1B, Table 1).

Previous studies of curcumin metabolites in rodents and humans have revealed that curcumin undergoes rapid metabolism and formation of reduced metabolites. Data from the individual curcuminoid compound initiated our interest to study BDMC and its metabolite reduced analogs, tetrahydrobisdemethoxycurcumin (THBDMC), hexahydrobisdemethoxycurcumin (HHBDMC) and octahydrobisdemethoxycurcumin (OHBDMC). The result showed that the parent compound and its reduced analogs enhanced EGFP expression in K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cells in a dose-dependent manner (Fig. 2A). The most effective compound was HHBDMC at 40 µM, which gave a significant fold change, 4.2±0.1, when compared with the untreated control, whereas 30 µM BDMC, 40 µM

THBDMC and 50 µM OHBDMC moderately enhanced EGFP expression by 3.5±0.4, 3.7±0.2 and 2.2±0.3 fold, respectively (Fig. 2A, Table 1). Moreover, all curcuminoids compounds were found to have less toxicity, with more than 80% cell viability, when compared to the positive control such as 50 µM hemin (60.8±9.8%) and 20 µM cisplatin (76.3±6.3%).

Induction of HbF production and γ-globin gene expression in K562 cells

To confirm the γ-globin inducing effect of curcuminoids, the native erythroid leukemia (K562) cells were exposed to BDMC, THBDMC, HHBDMC and OHBDMC at the concentration ranging from 10 to 50 µM. After 5 days, cells were harvested to analyze the HbF production, γ-globin expression and cell viability. The results shown in the native erythroid leukemia cell lines were consistent with those of the K562::$\Delta^{G}\gamma$-$^{A}\gamma$EGFP reporter cells, which demonstrated that 40 µM HHBDMC was most effective, and HbF production was induced up to 2.1±0.2 folds when compared with that of the untreated K562 cells (Fig. 2B, Table 1). While 20 µM cisplatin and 50 µM hemin induced HbF production by 1.9±0.2 and 2.1±0.3 fold, respectively when compared to the untreated cells (Fig. 2B). In addition, cell viability was more than 80% suggesting that all natural compounds at the highest dose (up to 40 µM) was still less toxic than the positive control, 66.8±2.5 % with 50 µM hemin and 78.8±3.5 % with 20 µM cisplatin.

Alteration of α- and γ-globin gene expression in K562 cells was also found to be dose-dependent with the test curcuminoid compounds. The most prominent result was that of the 30 µM HHBDMC, which significantly increased γ-globin mRNA level to 3.6±0.4 folds when compared to the untreated cells, whereas the other natural compounds increased γ-globin expression at a lower level (Fig. 2C). The induction of γ-globin mRNA levels was

consistent with the increased HbF levels, and EGFP expression following OHBDMC THBDMC, BDMC and HHBDMC treatment, which are in the order of increasing activity. This study also showed that the positive controls, 20 μM cisplatin and 50 μM hemin, significantly increased γ-globin gene expression when compared to the untreated cells by 3.9±0.4 and 4.0±0.5 fold respectively (Fig. 2C).

Induction of HbF production and γ-globin gene expression in the primary human erythroid cell cultures

The effect of BDMC and its reduced form were assessed for the induction of human γ-globin gene and HbF production in erythroid progenitor cells culture derived from healthy volunteers. Based on the results in K562 cells, we decided to investigate only BDMC, THBDMC and HHBDMC. The human erythroid progenitor cells were treated with 10 to 30 μM of the test compounds at day 7 and harvested for globin mRNA expression study as well as HbF production on day 14. A dose dependent induction of HbF production was observed in erythroid precursor cells treated with BDMC and its reduced metabolites (Fig. 3). The compound concentrations that resulted in the highest HbF inducing potency in erythroid precursor cells were 30, 20 and 20 μM, respectively for BDMC, THBDMC and HHBDMC. At 20 μM HHBDMC significantly increased HbF level by 2.0±0.4 fold when compared to the untreated cells. The increased level of HbF was the same as that of the positive control, 20 μM cisplatin (1.8±0.4) and 50 μM hemin (2.4±0.1). The compounds had less cell toxicity as measured by cell viability at 30 μM BDMC (79.6±2.8%), 20 μM THBDMC (83.5±2.3%) and 20 μM HHBDMC (83.6±3.0%) compare to 20 μM cisplatin (66.7±2.7%) and 50 μM hemin (77.5±4.4%).

We also investigated the level of globin mRNA expression in human erythroid cells following exposure with the test curcuminoid compounds. The result showed that all natural compounds increased all three globin mRNA levels, but the effect was more pronounce in γ-globin mRNA level, especially 20 μM HHBDMC, which significantly increased γ-globin gene expression by 3.2±0.2 fold, which is similar to 20 μM cisplatin (3.1±0.2 fold) and 50 μM hemin (3.4±0.1 fold) treated cells (Fig. 3B). While its parent compound, BDMC, and the other reduced analogs, THBDMC, were less effective at γ-globin gene induction.

Discussion

The identification of bioactive compound isolated from *C. longa* is a promising strategy to develop a new pharmaceutical from medicinal plant. Our data suggested that the phytochemicals profiles of curcuminoids can induce γ-globin expression and, more importantly, increase HbF production in human erythroid cell culture. Among the three curcuminoids, curcumin, demethoxycurcumin and BDMC, the last curcuminoid was the most effective compound that enhanced γ-globin mRNA expression and HbF synthesis. We found that not only BDMC but also its reduced analogs enhanced EGFP expression when these compounds were tested in all three types of erythroid cells, K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP reporter cell line, native K562 and primary human erythroid cell culture. HHBDMC was the most effective reduced analog of BDMC that induced γ-globin expression and HbF production.

Since BDMC was more active than demethoxycurcumin, it was thus concluded that introduction of one methoxyl group on the aromatic ring to give demethoxycurcumin structure resulted in a decreased activity. BDMC was therefore chosen as a structure lead to further structural modifications to achieve the reduced analogs, such as THBDMC and

HHBDMC (Table 1). Additions of hydroxyl group to increase the solubility property are required for high HbF enhancing ability.

Toxicity studies, either in animal or in human showed that there is no toxicity at a dosage of 2.000 or 8.000 curcumin/kg/day curcumin, respectively. In addition, many recent studies have shown that curcumin has very poor bioavailability due to poor absorption and rapid metabolism. The maximum concentration can be detected within 1-2 hours after 4,000 mg/kg oral uptake of curcumin. For dose escalation in 34 human subjects, only in the subjects who have administered 10,000 or 12,000 mg as single oral, the low level of curcumin in serum but not in plasma can be detected, and no toxicity appeared in the high dose [16-18]. The study also showed that not only the parent compounds but also their metabolite reduced analogs have the neuroprotective activity. That is shown by inhibited nitric oxide (NO) production and the decreased mRNA expression level of inducible NO synthase from Lipopolysaccharide exposed in HAPI microglial cells [19].

A number of plant extracts and plant constituents have been reported to induce HbF expression such as resveratrol from grape [20], angelicin from *Aegle marmelos* [21], labdane diterpenes from *Curcuma comosa* [22] , citropten and bergapten from *Citrus bergamia* [23]. This is the first study that demonstrated γ-globin expression and HbF inducing properties of *C. longa* extract, curcuminoids and reduced analogs in human erythroid cells. Recent studies suggested that curcuminoids are a potent epigenetic modification agent by interaction with histone deacetylases (HDAC), histone acetyltransferases (HAT) and DNA methlytranferase I (DNMT1) [24,25]. The studies in human cancer cells showed that the mode of action of curcumin was through direct interaction with target proteins as well as epigenetic modulation of target genes in a time- and concentration-dependent manner. Curcumin covalently binds with DNMT1 and blocks the catalytic activity, leading to exertion of its global DNA hypomethylation in a leukemia cell line [26-28].

In this study, we report for the first time that curcuminoids can induce γ-globin expression and, more importantly, increase HbF production in human erythroid cell culture. We demonstrate that HHBDMC was most effective in two independent assay systems. Based on our findings, the analysis of the effects of HHBDMC and related compounds in erythroid cells derived from thalassemia patients is warranted. Mechanistically, curcuminoids have been shown to be potent epigenetic modifying agent by interacting with HDACs, HAT and DNMT1. Further studies are needed to clarify the mechanism of γ-globin inducer of these compounds in order to develop targeted therapeutic strategies for hemoglobin disorders such as β-thalassemia and sickle cell disease.

Acknowledgements

This study was supported in part by Office of the Higher Education Commission and Mahidol University under the National Research University Initiative, The Thailand Research Fund (TRF) and National Science and Technology Development Agency, Thailand. NC was supported by the Strategic Scholarship Fellowship Frontier Research Networks, Office of the Higher Education Commission and Mahidol University**.** C.C. and A.S. acknowledge supports from the Strategic Basic Research Grant of TRF and the Center of Excellence for Innovation in Chemistry, Office of the Higher Education Commission.

References

1. Cao A, Galanello R (2010) β-thalassemia. Genet Med 12(2):61-76

2. Rund D, Rachmilewitz E (2005) β-thalassemia. N Engl J Med 353(11):1135-1146

3. Thein SL (2008) Genetic modifiers of the β-haemoglobinopathies. Br J Haematol 141(3):357-366

4. Perrine SP, Castaneda SA, Chui DH, Faller DV, Berenson RJ, Siritanaratku N, Fucharoen S (2010) Fetal globin gene inducers: novel agents and new potential. Ann N Y Acad Sci 1202:158-164

5. El-Beshlawy A, Hamdy M, El Ghamrawy M (2009) Fetal globin induction in β-thalassemia. Hemoglobin 33 Suppl 1:S197-203

6. Testa U (2009) Fetal hemoglobin chemical inducers for treatment of hemoglobinopathies. Ann Hematol 88(6):505-528

7. Bianchi N, Zuccato C, Lampronti I, Borgatti M, Gambari R (2009) Fetal Hemoglobin Inducers from the Natural World: A Novel Approach for Identification of Drugs for the Treatment of β-Thalassemia and Sickle-Cell Anemia. Evid Based Complement Alternat Med 6(2):141-151

8. Hatcher H, Planalp R, Cho J, Torti FM, Torti SV (2008) Curcumin: from ancient medicine to current clinical trials. Cell Mol Life Sci 65(11):1631-1652

9. Sharma RA, Gescher AJ, Steward WP (2005) Curcumin: the story so far. Eur J Cancer 41(13):1955-1968

10. Changtam C, de Koning HP, Ibrahim H, Sajid MS, Gould MK, Suksamrarn A (2010) Curcuminoid analogs with potent activity against Trypanosoma and Leishmania species. Eur J Med Chem 45(3):941-956.

11. Changtam C, Hongmanee P, Suksamrarn A (2010) Isoxazole analogs of curcuminoids with highly potent multidrug-resistant antimycobacterial activity. Eur J Med Chem 45(10):4446-4457

12. Portes E, Gardrat C, Castellan A (2007) A comparative study on the antioxidant properties of tetrahydrocurcuminoids and curcuminoids. Tetrahedron 63:9092-9099

13. Vadolas J, Wardan H, Orford M, Williamson R, Ioannou PA (2004) Cellular genomic reporter assays for screening and evaluation of inducers of fetal hemoglobin. Hum Mol Genet 13(2):223-233

14. Perrine SP, Mankidy R, Boosalis MS, Bieker JJ, Faller DV (2009) Erythroid Kruppel-like factor (EKLF) is recruited to the g-globin gene promoter as a co-activator and is required for g-globin gene induction by short-chain fatty acid derivatives. Eur J Haematol 82(6):466-476

15. Tubsuwan A, Munkongdee T, Jearawiriyapaisarn N, Boonchoy C, Winichagoon P, Fucharoen S, Svasti S (2011) Molecular analysis of globin gene expression in different thalassaemia disorders: individual variation of β^E pre-mRNA splicing determine disease severity. Br J Haematol 154(5):635-643

16. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4(6):807-818

17. Lao CD, Ruffin MTt, Normolle D, Heath DD, Murray SI, Bailey JM, Boggs ME, Crowell J, Rock CL, Brenner DE (2006) Dose escalation of a curcuminoid formulation. BMC Complement Altern Med 6:10

18. Sharma RA, McLelland HR, Hill KA, Ireson CR, Euden SA, Manson MM, Pirmohamed M, Marnett LJ, Gescher AJ, Steward WP (2001) Pharmacodynamic and pharmacokinetic study of oral Curcuma extract in patients with colorectal cancer. Clin Cancer Res 7(7):1894-1900

19. Tocharus J, Jamsuwan S, Tocharus C, Changtam C, Suksamrarn A (2012) Curcuminoid analogs inhibit nitric oxide production from LPS-activated microglial cells. J Nat Med 66(2):400-405

20. Fibach E, Prus E, Bianchi N, Zuccato C, Breveglieri G, Salvatori F, Finotti A, Lipucci di Paola M, Brognara E, Lampronti I, Borgatti M, Gambari R (2012) Resveratrol:

Antioxidant activity and induction of fetal hemoglobin in erythroid cells from normal donors and β-thalassemia patients. Int J Mol Med 29(6):974-982

21. Lampronti I, Bianchi N, Borgatti M, Fibach E, Prus E, Gambari R (2003) Accumulation of γ-globin mRNA in human erythroid cells treated with angelicin. Eur J Haematol 71(3):189-195
22. Chokchaisiri R, Chaneiam N, Svasti S, Fucharoen S, Vadolas J, Suksamrarn A (2010) Labdane diterpenes from the aerial parts of *Curcuma comosa* enhance fetal hemoglobin production in an erythroid cell line. J Nat Prod 73(4):724-728
23. Guerrini A, Lampronti I, Bianchi N, Zuccato C, Breveglieri G, Salvatori F, Mancini I, Rossi D, Potenza R, Chiavilli F, Sacchetti G, Gambari R, Borgatti M (2009) Bergamot (*Citrus bergamia* Risso) fruit extracts as γ-globin gene expression inducers: phytochemical and functional perspectives. J Agric Food Chem 57(10):4103-4111
24. Fu S, Kurzrock R (2010) Development of curcumin as an epigenetic agent. Cancer 116(20):4670-4676
25. Huang J, Plass C, Gerhauser C (2011) Cancer Chemoprevention by Targeting the Epigenome. Curr Drug Targets 12(13):1924-1956
26. Jha AK, Nikbakht M, Parashar G, Shrivastava A, Capalash N, Kaur J (2010) Reversal of Hypermethylation and Reactivation of the RARβ2 Gene by Natural Compounds in Cervical Cancer Cell Lines. Folia Biol (Praha) 56(5):195-200
27. Liu Z, Xie Z, Jones W, Pavlovicz RE, Liu S, Yu J, Li PK, Lin J, Fuchs JR, Marcucci G, Li C, Chan KK (2009) Curcumin is a potent DNA hypomethylation agent. Bioorg Med Chem Lett 19(3):706-709
28. Meeran SM, Ahmed A, Tollefsbol TO (2010) Epigenetic targets of bioactive dietary components for cancer prevention and therapy. Clin Epigenetics 1(3-4):101-116

Figure legend

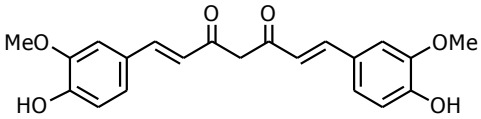
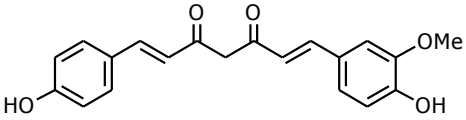
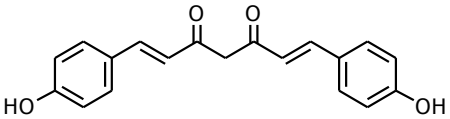
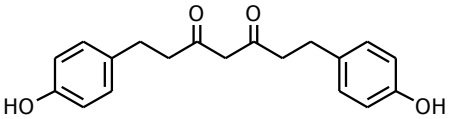
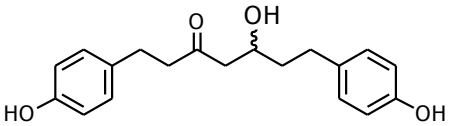
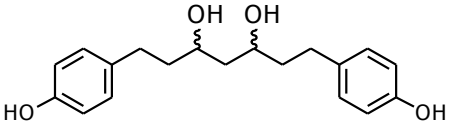
Fig. 1. Analysis of EGFP expression induction by *Curcuma longa* crude extract and individual curcuminoids in K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell. (A) Histogram plot of EGFP analysis by flow cytometry. Induction of EGFP expression in the K562::ΔGγ-Aγ EGFP reporter assay cell line by *Curcuma longa* crude extract was observed. (B) Induction of EGFP expression by three natural curcuminoids. 20 μM Cisplatin and 50μM hemin were used as positive control. ** $P < 0.01$, * $P < 0.05$ when compared to untreated cultures

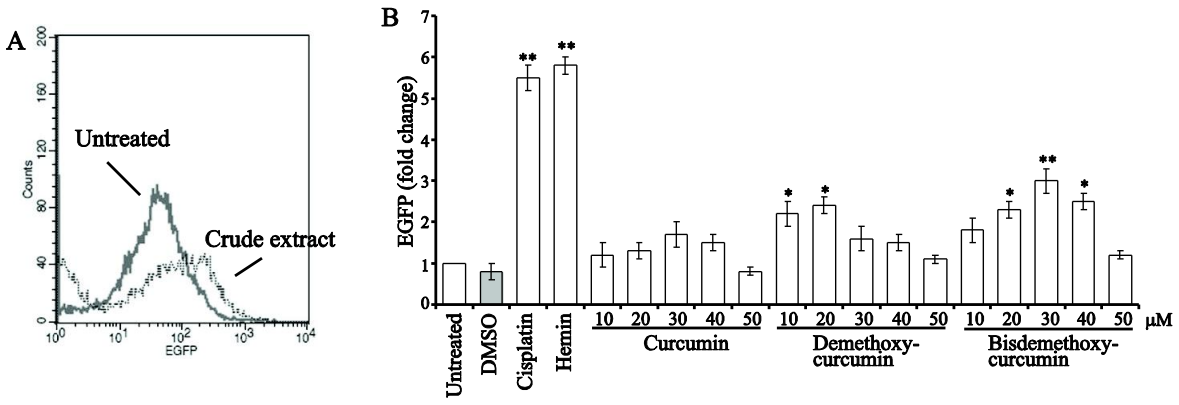
Fig. 2. Analysis of enhanced EGFP and γ-globin expression by BDMC and its reduced analogs. The K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP and K562 cells were treated with the compounds for 5 days. (A) Analysis for EGFP expression in K562::ΔGγ-Aγ EGFP cells and (B) HbF level in K562 cells by flow cytometry. The results showed fold changes of the increasing mean fluorescent intensity of EGFP and HbF levels, with dose-dependent manner, in treated cells compared with untreated cells. (C) Measurement of α- and γ-globin mRNAs in K562 cells by RT-qPCR. Cells were treated with the compounds at concentration yielding the highest HbF induction activity. The results showed fold changes of α- and γ-globin mRNA expression in treated cells compared with the untreated cells using β-actin as normalizer. 20 μM Cisplatin and 50μM hemin were used as positive control. ** $P < 0.01$, * $P < 0.05$ when compared to untreated cells.

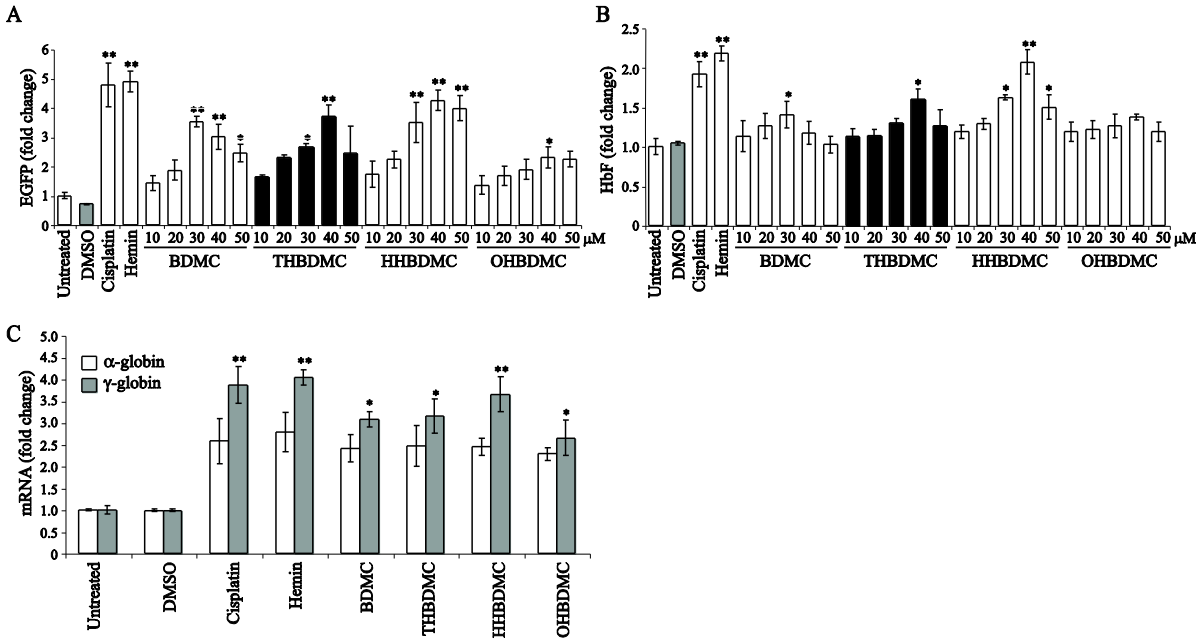
Fig. 3. Analysis of HbF and γ-globin mRNA in primary erythroid precursor cells treated with BDMC and its reduced analogs. The $CD34^{+}$ cells were isolated from healthy volunteers. The erythroid precursor cells were treated on day 7 of culture and harvested on day 14 for determination of HbF and γ-globin mRNA levels. (A) Analysis of HbF production

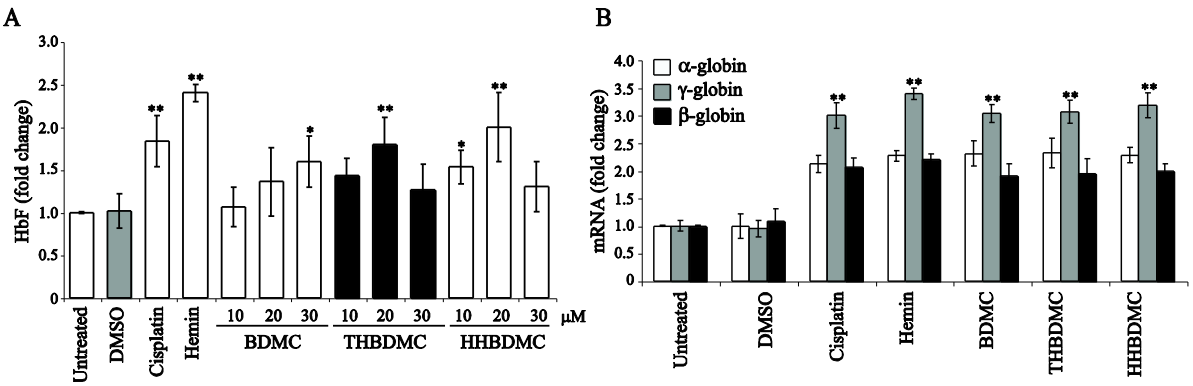
by immunostaining with FITC-conjugated anti-HbF and flow cytometry. The results showed fold changes in HbF levels in treated cells compared with the untreated cells. (B) Measurement of α-, β- and γ-globin mRNAs by RT-qPCR. The cells were treated with the compounds at concentration that resulted in highest HbF induction activity. The results showed fold changes of α-, β- and γ-globin mRNA expression in treated cells compared with the untreated cells using β-actin as normalizer. 20 μM Cisplatin and 50μM hemin were used as positive control. ** $P < 0.01$, * $P < 0.05$ when compared to untreated cells.

Table 1
The effect of curcuminoids, tetrahydro- hexahydro- and octahydrobisdemethoxycurcumin on K562::$\Delta^{G}\gamma$-$^{A}\gamma$ EGFP and K562 cells.

Compound	Dose effective (μM)	EGFP[a] (fold change)	HbF [b] (fold change)	% Cell viability
 Curcumin	30	1.8±0.9	1.2±0.4	80.5±4.4
 Demethoxycurcumin	20	2.4±0.6	1.2±0.3	75.6±4.3
 Bisdemethoxycurcumin (BDMC)	30	3.5±0.4	1.4±0.5	86.3±2.4
 Tetrahydrobisdemethoxycurcumin (THBDMC)	40	3.7±0.2	1.6±0.6	85.4±4.4
 Hexahydrobisdemethoxycurcumin (HHBDMC)	40	4.25±0.1	2.1±0.5	80.0±6.5
 Octahydrobisdemethoxycurcumin (OHBDMC)	40	2.24±0.3	1.3±0.5	85.8±3.8


A
Untreated
Crude extract
Counts
EGFP
B
EGFP (fold change)
Untreated
DMSO
Cisplatin
Hemin
10 20 30 40 50
Curcumin
Demethoxy-curcumin
Bisdemethoxy-curcumin
μM


A
EGFP (fold change)
Untreated
DMSO
Cisplatin
Hemin
10 20 30 40 50 μM
BDMC
THBDMC
HHBDMC
OHBDMC
B
HbF (fold change)
C
α-globin
γ-globin
mRNA (fold change)


A
HbF (fold change)
3.0
2.5
2.0
1.5
1.0
0.5
0
Untreated
DMSO
Cisplatin
Hemin
10 20 30 10 20 30 10 20 30 μM
BDMC
THBDMC
HHBDMC
B
α-globin
γ-globin
β-globin
mRNA (fold change)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Untreated
DMSO
Cisplatin
Hemin
BDMC
THBDMC
HHBDMC

Author/s:
Chaneiam, N; Changtam, C; Mungkongdee, T; Suthatvoravut, U; Winichagoon, P; Vadolas, J; Suksamrarn, A; Fucharoen, S; Svasti, S

Title:
A reduced curcuminoid analog as a novel inducer of fetal hemoglobin

Date:
2013-03-01

Citation:
Chaneiam, N., Changtam, C., Mungkongdee, T., Suthatvoravut, U., Winichagoon, P., Vadolas, J., Suksamrarn, A., Fucharoen, S. & Svasti, S. (2013). A reduced curcuminoid analog as a novel inducer of fetal hemoglobin. ANNALS OF HEMATOLOGY, 92 (3), pp.379-386. https://doi.org/10.1007/s00277-012-1604-1.