Lysine-specific histone demethylase 1 inhibition enhances robust fetal hemoglobin induction in human β^0-thalassemia/hemoglobin E erythroid cells

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Abstract

Induction of fetal hemoglobin (HbF) ameliorates the clinical severity of β-thalassemias. Histone methyltransferase LSD1 enzyme removes methyl groups from the activating chromatin mark histone 3 lysine 4 at silenced genes, including the γ-globin genes. LSD1 inhibitor RN-1 induces HbF levels in cultured human erythroid cells. Here, the HbF-inducing activity of RN-1 was investigated in erythroid progenitor cells derived from β^0-thalassemia/hemoglobin E (HbE) patients. The significant and reproducible increases in γ-globin transcript and HbF expression upon RN-1 treatment were demonstrated in erythroid cells with divergent HbF baseline levels, the average of HbF induction was 17.7±0.8%. RN-1 at low concentration did not affect viability and proliferation of erythroid cells, but decreases in cell number were observed in cells treated with RN-1 at high concentration. Delayed terminal erythroid differentiation was revealed in β^0-thalassemia/HbE erythroid cells treated with RN-1 as similar to other compounds that target LSD1 activity. Downregulation of repressors of γ-globin expression; NCOR1 and SOX6, was observed in RN-1 treatment. These findings provide proof of the concept that LSD1 epigenetic enzyme is a potential therapeutic target for β^0-thalassemia/HbE patients.

Introduction

β-Thalassemia/hemoglobin E patients constitute one-half of the clinically severe β-thalassemias worldwide. Hemoglobin E (HbE; HBB:c.79G>A) is the most common hemoglobin variant in Southeast Asia, making β-thalassemia/HbE a significant health problem in the region. Reduction in the expression of the β-globin gene results in excess unmatched α-globin chains that precipitate and damage erythroid cell membranes, causing hemolysis and ineffective erythropoiesis.[1] Increased fetal hemoglobin (HbF; $\alpha_2\gamma_2$) can ameliorate phenotypic severity of β-thalassemia/HbE patients by reducing the burden of imbalanced α- to non-α-globin chains.[2] HbF levels in patients with β^0-thalassemia/HbE range from 2 to 76%, and those who have increased HbF levels usually have milder clinical symptoms.[2] Many attempts were made to induce HbF expression to reach the therapeutic level in adult β-thalassemia patients, however, the results have been heterogeneous.[3]

Lysine-specific histone demethylase 1 (LSD1) has been identified as a therapeutic target for HbF induction through RNA interference strategies and pharmacological inhibitors.[4] LSD1 is an epigenetic modifying enzyme that removes methyl groups from histone 3 lysine 4 (H3K4) and lysine 9 (H3K9), the activating and repressive chromatin mark, respectively.[5,6] Inhibition of LSD1 activity has been demonstrated to induce HbF expression in adult erythroid cells by increasing di-methylated histone 3 lysine 4 (Me2H3K4) at γ-globin promoter.[4] RN-1, a potent and selective LSD1 inhibitor, increased γ-globin and HbF synthesis in a sickle cell disease mouse model and led to improvement of many aspects of disease pathology including red blood cell survival.[7,8] RN-1 also recapitulated the fetal pattern of hemoglobin induction in baboons (*papio anubis*).[9] Induction of HbF with RN-1 appears to be similar to that of histone demethylase DNMT1 inhibitor decitabine, the most potent inducer of HbF thus far identified. Here, the therapeutic potential of RN-1 was investigated in erythroid progenitor cells derived from β^0-thalassemia/HbE patients with divergent HbF baseline levels.

Materials and methods

In vitro erythroid cell culture and drug treatment

This study was performed under the Institutional Review Board approval from Mahidol University, Thailand (EOC No.2013/136.2512). All the participants signed the informed consent before sample collection. Mononuclear cells were separated from peripheral blood samples by density gradient centrifugation [density = 1.077 g/mL; Lymphoprep™ (AXIS-SHIELD PoC

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AS, Oslo, Norway)]. CD34+ hematopoietic progenitors were selected using the anti-CD34 magnetic microbeads positive selection kit [MACS Technology (Miltenyi Biotec, CA, USA)] according to the manufacturer's protocols. Following separation, CD34+ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, New York, USA) supplemented with 20% fetal bovine serum (Merck, Darmstadt, Germany), 100 U/mL penicillin-streptomycin (Gibco) and 0.3 mg/mL human holotransferrin (PromoCell, Heidelberg, Germany) as a basal media. The cells were incubated at 37°C in 5% CO_2 for 14 days. This study used a three-phase liquid culture system; in the first phase (day 0-4), the basal media was supplemented with 10 ng/mL human interleukin-3 (IL3; Cell Signaling Technologies, Danvers, USA), 50 ng/mL human stem cell factor (SCF; Cell Signaling Technologies) and 2 U/mL erythropoietin (EPO)(CILAG GmbH, Zug, Switzerland), whereas in the second phase of culture (day 4-8), the basal media was supplemented with 10 ng/mL SCF and 2 U/mL EPO. In the third phase (day 8-14), the basal media was supplemented with 4 U/mL EPO. Various concentrations of RN-1 (LSD1 inhibitor)(Calbiochem, San Diego, USA) were added on day 8 of culture and maintained until day 14.

Cell viability, proliferation and differentiation

Cell number and viability of erythroid cells were analyzed by trypan blue staining and counted with a hemocytometer. Cell differentiation analysis was performed by immunostaining using phycoerythrin (PE)-conjugated antibody against the human transferrin receptor (CD71; BD Biosciences, Franklin Lakes, USA) and allophycocyanin (APC)-conjugated antibody against human glycophorin A (CD235a; BD Bioscience). Fluorescence signals were detected using the FACSCalibur flow cytometer. Cells were gated into 4 sub-populations: $CD71^{pos}/CD235a^{neg}$, $CD71^{high}/CD235a^{pos}$, $CD71^{medium}/CD235a^{pos}$ and $CD71^{low}/CD235a^{pos}$ according to the fluorescent intensity of each markers. After staining, the fluorescent intensity was detected and analyzed by the FACSCalibur flow cytometer and CellQuest™ software (BD Biosciences).

Quantitative real-time polymerase chain reaction and high-performance liquid chromatography analysis

Total RNA was isolated from erythroid cells on days 11 and 14 of culture using TRIzol Reagent (Thermo Fisher Scientific, CA, USA) and treated with DNase I (Thermo Fisher Scientific) following the manufacturer's instructions. cDNA was generated using the RevertAid first strand

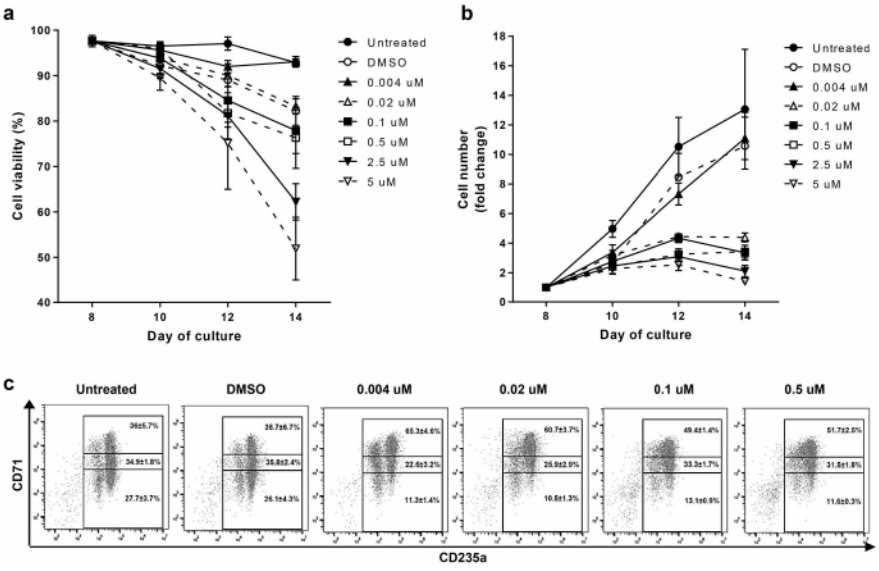

Figure 1. Effects of RN-1 treatment on cell viability, proliferation, and differentiation of erythroid cells derived from β⁰-thalassemia/hemoglobin E (HbE) patients. CD34+ cells were differentiated to erythroid cells in the presence of RN-1 at the indicated concentrations or vehicle control (DMSO) in days 8-14 of culture. Untreated culture condition (UNT) and DMSO treatment were used as the negative control. (a) Cell viability and (b) cell proliferation assessed by trypan blue staining (mean±SEM, n=4). (c) Representative flow cytometry dot plots for erythroid cell differentiation analysis of cells day 14 of culture. Cells were gated into 4 sub-populations: $CD71^{pos}/CD235a^{neg}$, $CD71^{high}/CD235a^{pos}$, $CD71^{medium}/CD235a^{pos}$ and $CD71^{low}/CD235a^{pos}$ according to the fluorescent intensity of each markers.

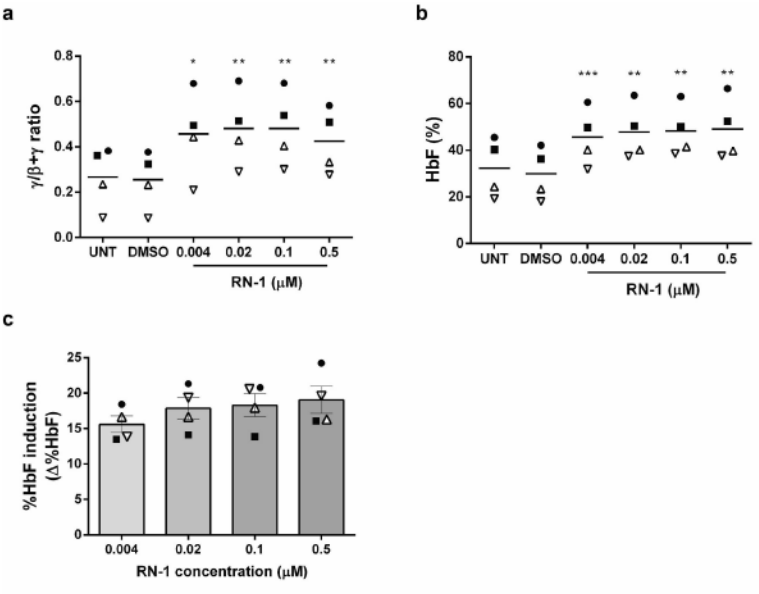

Figure 2. RN-1 increases γ-globin mRNA and fetal hemoglobin (HbF) expression in erythroid cells derived from four individual β⁰-thalassemia/hemoglobin E (HbE) patients with divergent HbF baseline levels. (a) Globin mRNA expression (γ/β+γ ratio) measured by quantitative real-time PCR in cells day 14 of culture. (b) Percentage of HbF in total hemoglobin determined by HPLC. (c) % HbF induction (Δ%HbF) was calculated by subtracting the RN-1 treated samples from vehicle control (DMSO). *P<0.05; **P<0.01; ***P<0.001. UNT (untreated control); DMSO (vehicle control).

cDNA synthesis kit (Thermo Fisher Scientific) with oligo-dT18 primer as per the manufacturer's protocol. Quantitative RT-PCR assay was performed using gene specific primers and SYBR® select master mix for CFX (Thermo Fisher Scientific) in the CFX Connect™ Real-Time PCR machine (Bio-Rad Laboratories, Hercules, USA). The relative fold changes were calculated using the ΔΔC(T) method by normalizing against ribosomal protein S18 (RPS18) expression. The levels of hemoglobin types were determined in cells on day 14 of culture by high-performance liquid chromatography (HPLC) using the VARIANT™ II β-thalassemia Short Program (Bio-Rad Laboratories).

Statistical analysis

Statistical analyses were performed by GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). The data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made using paired *t*-tests. A P-value of <0.05 was considered statistically significant.

Results

In this study, the *in vitro* three-phase liquid culture system was employed to promote erythroid differentiation of $CD34^+$ hematopoietic stem/progenitor cells isolated from peripheral blood of four individual β^0-thalassemia/HbE patients of different HbF baseline levels. Initially, the dose-response effect of RN-1, when added during days 8-14 of culture, was determined on cell proliferation and cell differentiation. There was no significant change in the viability and proliferation of cells treated with RN-1 at a concentration of 0.004 uM (Figure 1a and b, respectively), but the number of cells was reduced in RN-1 treatment at 0.02 uM and higher. Furthermore, RN-1 at 2.5 uM and 5 uM were obviously cytotoxic to the cells as shown by viable cells less than 70% on day 14 of culture. A slightly delay of terminal erythroid differentiation in cells treated with RN-1 at concentrations of 0.004 uM and higher was observed as evidenced by lower percentages of cells expressing low levels of CD71 ($CD71^{low}$/$CD235a^{pos}$ population = 11.3±1.4% in 0.004 uM RN-1 vs. 21.1±4.3% in DMSO control; Figure 1c).

The effect of RN-1 on HbF production was investigated in both mRNA and protein levels in cells on day 14 of culture using quantitative RT-PCR and HPLC, respectively. The significant increase of γ-globin mRNA (γ/β+γ ratio) was revealed in all concentrations of RN-1 treatment, consistent with the elevation of HbF (Figure 2a and b). The significant changes in β-globin and α-globin mRNAs were not observed (data not shown). HbF was induced in average of 15.6±1.2, 17.9±1.6, 18.3±1.6 and 19.1±1.9% upon RN-1 treated at concentrations 0.004, 0.02, 0.1 and 0.5 uM, respectively. Figure 1c shows the effects of RN-1 on HbF induction presented as increases in HbF percentage from the baseline level in DMSO-treated cells from the same donor (Δ%HbF). The induction levels of HbF were not significantly different among the different RN-1 treatment concentrations in individual β^0-thalassemia/HbE erythroblasts with divergent HbF baseline levels, the average of Δ%HbF was 17.7±0.8%. Furthermore, the effect of RN-1 treatment at concentrations of 0.004 and 0.02 uM on the transcriptional regulation of HbF regulators was determined in cells on days 11 and 14 of culture (Figure 3). The quantitative RT-PCR analysis revealed that two

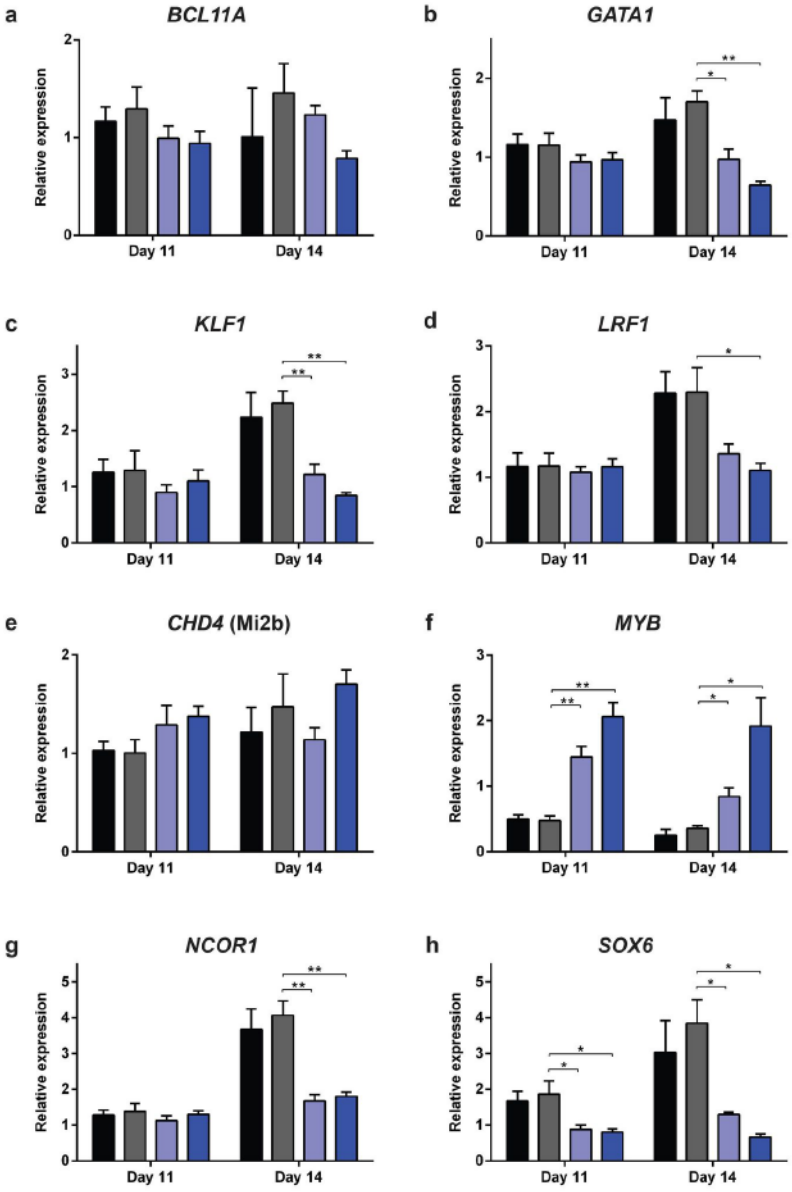

Figure 3. Effect of RN-1 on mRNA expression of fetal hemoglobine (HbF) regulators in β^0-thalassemia/hemoglobine E (HbE) erythroid cells. Relative mRNA abundance of (a) BCL11A, (b) GATA1, (c) KLF1, (d) LRF1, (e) Mi2b, (f) MYB, (g) NCOR1, and (h) SOX6 normalized to ribosomal protein S18 (RPS18) determined by quantitative real-time PCR in cells days 11 and 14 of culture (3 and 6 days after RN-1 treatment). Data are represented as mean±SEM of relative fold change to DMSO vehicle control (n=4). *P<0.05; **P<0.01. Black bar, untreated control; gray bar, DMSO control; light blue bar, 0.004 uM RN-1 treatment; dark blue bar, 0.02 uM RN-1 treatment.

repressors of γ-globin expression; NCOR1 and SOX6, were significantly downregulated by RN-1 treatment (Figure 3g and h). Moreover, the expression of GATA1, KLF1 and LRF1 were modestly but significantly downregulated after treatment with RN-1 (Figure 3b-3d), whereas MYB was significantly upregulated (Figure 3f). Of note, BCL11A and Mi2b were unaffected by RN-1 treatment. (Figure 3a and e).

Discussion and conclusions

Several epigenetic modifying enzymes involved in γ-globin repression are attractive therapeutic targets for the induction of HbF in β-thalassemia patients. LSD1 removes the methyl groups from mono- and dimethyl-histone H3 lysine 4 (MeH3K4 and Me2H3K4, respectively), which are epigenetic signatures that mark transcriptionally active genes.[5] In addition, LSD1 participates as cofactors to form a tetrameric core DRED (direct repeat erythroid-definitive) repressor complex, which directly binds to the promoters of the embryonic and fetal globin genes to repress their expression in adult erythroid progenitor cells.[10] The present study exhibited the induction of γ-globin and HbF synthesis after disruption of LSD1 activity by RN-1 treatment in *in vitro* human β^0-thalassemia/HbE erythroid cells, these cells have very high HbF baseline levels. The results demonstrated that RN-1 treatment at 0.004 uM significantly increased the expression of γ-globin mRNA and HbF in β^0-thalassemia/HbE erythroid cells without significant toxicity, consistent with the previous studies in sickle cell mouse model and anemic as well as non-anemic baboon.[7-9] The HbF induction achieved by RN-1 treatment was between 14-22% above baseline levels, which is comparable to previous reports that inhibit LSD1 activity via use of either RNA interference or tranylcypromine (TCP; the FDA-approved antidepressant LSD1 inhibitor) in human adult erythroid cells derived from mobilized circulating $CD34^+$ progenitors.[4] Noteworthy, erythroid cells derived from individual β^0-thalassemia/HbE patients with different HbF baseline levels all exhibited a similar degree of HbF induction in response to RN-1 treatment. These data demonstrated that RN-1 potently induced HbF expression in β^0-thalassemia/HbE erythroid cells regardless of HbF baseline levels. Furthermore, the combination treatment of RN-1 at effective but non-toxic concentration with other compounds targeted to different regulatory pathways may magnitude the effect of HbF induction.

Transcript levels of several key γ-globin repressors and co-repressors in β^0-thalassemia/HbE erythroid cells were significantly changed by RN-1 treatment. NCOR1 and SOX6 transcripts were decreased whereas MYB transcript was increased. NCOR1 has been identified as an adapter protein scaffolding between DNA-binding and epigenetic enzyme components (*e.g.*, DNMT1 and LSD1) in the DRED complex.[11] SOX6 is a chromatin-associated protein that binds and induces a marked bending of DNA.[12] SOX6 acts as a repressor by directly binding to the εγ promoter in definitive erythropoiesis.[13] In adult human erythroid progenitors, SOX6 and BCL11A along with GATA1 co-occupy the human β-globin cluster and cooperate in silencing γ-globin transcription.[14] A recent study has shown that downregulation of SOX6 induces γ-globin production in erythroid cells derived from β-thalassemia major patients.[15] MYB is an essential hematopoietic transcription regulator, highly expressed in immature proliferating cells of all hematopoietic lineages.[16] In human embryonic/fetal K562 cell line, increased levels of MYB expression results in inhibited γ-globin expression, while reduced expression of MYB in the human erythroid progenitor cells promotes HbF synthesis.[17,18] Furthermore, the GATA1, KLF1, and LRF1 were also downregulated by RN-1 treatment in β^0-thalassemia/HbE erythroid cells, whereas BCL11A and Mi2b were unaffected. Altogether, the findings indicate that the mechanisms of action of RN-1 in induction of HbF synthesis partly involve transcriptional regulation of key HbF repressors and/or co-repressors. Currently, there is no evidence of RN-1 treatment directly regulating the expression of erythroid genes, thus these phenomena may indirectly be affected by the suppressing of LSD1 activity. Although numerous studies suggest that, under such circumstances, variation of protein levels is primarily determined by their respective mRNA levels, no linear relationship between transcript concentration and individual protein abundance can be assumed. This study reported the dysregulation of certain erythroid regulators upon the RN-1 treatment at the transcriptional level, hence additional experiments are warranted to investigate the significant change in protein synthesis and its sequential effects.

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