รายงานวิจัยฉบับสมบูรณ์

การกระตุ้นการสร้างฮีโมโกลบินเอฟโดยโพมาลิโดไมด์เพื่อการรักษาโรคเบต้าธาลัสซีเมีย

Repurposing of Pomalidomide to Induce Fetal Hemoglobin Expression for β-Thalassemia Treatment

โดย ดร.นที เจียรวิริยะไพศาล และคณะ

สิงหาคม 2563

สัญญาเลขที่ MRG5680092

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
สำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

Abstract

Project Code : MRG5680092

Project Title : Repurposing of Pomalidomide to Induce Fetal Hemoglobin Expression for β-Thalassemia Treatment

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Clinical studies have shown that induction of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) expression in adult erythroid cells can ameliorate the severity of β-thalassemia and sickle cell disease. Pomalidomide, an FDA-approved immunomodulatory drug with anti-tumor activity for the treatment of multiple myeloma, has been shown to induce HbF expression in erythroid progenitor cells from normal individuals and sickle cell patients through unknown mechanisms, but at least in part by downregulating factors involved in γ-globin repression. Here we repurposed pomalidomide as a potential HbF inducer for treatment of β-thalassemia where HbF becomes expressed at very high levels. We found that treatment of β^0-thalassemia/HbE erythroid progenitor cells with pomalidomide led to significant increases in HbF expression without any significant cytotoxicity. To achieve higher levels of HbF induction, we combined pomalidomide with hydroxyurea, decitabine, and RN-1. The combination of pomalidomide and decitabine showed the greatest additive effects on HbF induction. High levels of HbF induction achieved by these treatments were shown to be partly associated with downregulation of transcription factors and corepressors involved in γ-globin repression. These findings demonstrate that if shown to be safe, pomalidomide alone or in combination with decitabine could be repurposed as a potential therapeutic HbF inducer to treat β-thalassemia.

Keywords : fetal hemoglobin induction, β-thalassemia, pomalidomide, drug repurposing

บทคัดย่อ

รหัสโครงการ : MRG5680092

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(ขอพักการได้รับทุนเพื่อ: มิถุนายน 2557 -พฤษภาคม 2560)

การศึกษาและการวิจัยทางคลินิกแสดงให้เห็นว่าการกระตุ้นการสร้างฮีโมโกลบินเอฟ (HbF, $\alpha_2\gamma_2$) ในเม็ดเลือดแดงสามารถที่จะลดความรุนแรงของโรคเบต้าธาลัสซีเมียและโรคโลหิตจางแบบซิกเคิลเซลล์ (Sickle cell anemia) ได้ โพมาลิโดไมด์เป็นยาปรับระบบภูมิคุ้มกัน (immunomodulatory drug) ที่ได้รับการรับรองจากองค์การอาหารและยาสหรัฐอเมริกาสำหรับการรักษาโรคมัลติเพิล มัยอิโลม่า (Multiple Myeloma) ซึ่งมีการรายงานว่ามีความสามารถในการกระตุ้นการสร้างฮีโมโกลบินเอฟได้ในเม็ดเลือดแดงตัวอ่อนของคนปกติและคนไข้โรคโลหิตจางแบบซิกเคิลเซลล์โดยยังไม่ทราบกลไกที่แน่ชัด แต่อาจจะกระตุ้นการสร้างฮีโมโกลบินเอฟผ่านการกดการแสดงออกของโปรตีนที่ยับยั้งการสร้างแกมม่าโกลบิน การศึกษานี้ได้นำยาโพมาลิโดไมด์ซึ่งเป็นยารักษาโรคมัลติเพิล มัยอิโลม่ามาศึกษาประสิทธิภาพในการกระตุ้นการสร้างฮีโมโกลบินเอฟเพื่อใช้ในการรักษาโรคเบต้าธาลัสซีเมีย พบว่าโพมาลิโดไมด์สามารถกระตุ้นการสร้างฮีโมโกลบินเอฟได้สูงขึ้นอย่างมีนัยสำคัญในเม็ดเลือดแดงตัวอ่อนจากคนไข้โรคเบต้าธาลัสซีเมีย/ฮีโมโกลบินอี โดยไม่มีความเป็นพิษต่อเซลล์ นอกจากนั้นยังมีการเพิ่มประสิทธิภาพการกระตุ้นการสร้างฮีโมโกลบินเอฟโดยการใช้โพมาลิโดไมด์ร่วมกับสารกระตุ้นการสร้างฮีโมโกลบินเอฟอื่นๆ ได้แก่ ไฮดรอกซียูเรีย (hydroxyurea) ดีไซทาบีน (decitabine) และอาร์เอ็นวัน (RN-1) โดยพบว่าโพมาลิโดไมด์ร่วมกับดีไซทาบีนสามารถกระตุ้นการสร้างฮีโมโกลบินเอฟได้สูงสุด และพบว่าการกระตุ้นการสร้างฮีโมโกลบินเอฟโดยโพมาลิโดไมด์มีผลบางส่วนมาจากการกดการแสดงออกของโปรตีนที่ยับยั้งการแสดงออกของแกมม่าโกลบิน ดังนั้นการศึกษานี้แสดงให้เห็นว่ามีความเป็นไปได้ที่จะนำโพมาลิไมด์มาใช้ในการกระตุ้นการสร้างฮีโมโกลบินเอฟเพื่อใช้ในการรักษาโรคเบต้าธาลัสซีเมียโดยอาจจะใช้เดี่ยวหรือร่วมกับดีไซทาบีน

คำหลัก : การกระตุ้นการสร้างฮีโมโกลบินเอฟ โรคเบต้าธาลัสซีเมีย โพมาลิโดไมด์ การนำยาที่มีการใช้ในการรักษาโรคมาใช้รักษาโรคอื่น

Executive Summary

β-Thalassemia and sickle cell disease (SCD) are the two most common monogenic inherited disorders in man, leading to lifelong, severe medical consequences and early death. While both can be cured by hematopoietic stem cell transplantation, the demographics of both diseases indicates that this is not an accessible therapeutic option for more than 95% of affected individuals. The best currently approved medication for treatment is hydroxyurea, a drug discovered more than 30 years ago, which is ineffective in approximately 50% of treated patients, and has no effect on diminished longevity. In this report we discovered a potentially new treatment for β-thalassemia. We found that treatment of β-thalassemic erythroid progenitor cells with pomalidomide leads to massive upregulation of γ-globin mRNA and fetal hemoglobin, which can fully replace the mutant or missing adult β-globin chains in β-thalassemia. We believe that pomalidomide or structural refinements thereof may lead to future fully effective and widely accessible therapies for these disorders.

Final Report

Objective

The ultimate goal of this work to investigate the therapeutic potential of pomalidomide as a HbF inducer in erythroid progenitor cells from β^0-thalassemia/HbE patients

Results

Pomalidomide induces HbF expression in erythroid progenitor cells from β^0-thalassemia/HbE patients in a time- and dose-dependent manner

We investigated the effects of the recently-identified HbF inducer, pomalidomide, on the induction of HbF expression in erythroid progenitor cells from β^0-thalassemia/HbE patients using a 3-phase liquid culture system that supports terminal maturation of erythroid cells. In phase I (days 0-4) and phase II (days 4-8) of the culture, $CD34^+$ hematopoietic stem/progenitor cells commit to erythroid lineage progenitors and expand to generate >80% erythroid progenitor cells as characterized by the expression levels of two cell surface markers, transferrin receptor (CD71) and glycophorin A (GPA/CD235a): $CD71^{high}/GPA^{low}$ and $CD71^{high}/GPA^{high}$ at day 8 of culture (Figure 1). Erythroid progenitor cells further differentiate during phase III (days 8-14) to generate up to 50% $CD71^{low}/GPA^{high}$ erythroid cells by day 14. We observed a delay in erythroid differentiation in erythroblasts from β^0-thalassemia/HbE patients when compared to normal controls (Figure 1).

We first assessed the optimal period of pomalidomide treatment in β^0-thalassemia/HbE erythroid progenitors. Cells were treated with pomalidomide at a concentration of 1.0 μM for 3 different treatment durations: days 4-8, days 8-14, or days 4-14. Using high performance liquid chromatography (HPLC) to determine HbF expression at day 14, we found that the highest increase in HbF percentage from baseline level in DMSO control-treated cells (Δ%HbF) was achieved when pomalidomide was exposed to the cell cultures from days 4-14 (Figure 2A) without any significant effects on cell proliferation (Figure 4A) or viability (Figure 4B). There was a small increase in HbF expression in cells treated with pomalidomide from days 8-14, suggesting that the response to pomalidomide treatment was more pronounced when cells exposed to pomalidomide during early stages of erythroid development. Based on

the optimal period of pomalidomide treatment, we then performed a dose titration study of pomalidomide in β^0-thalassemia/HbE erythroid progenitors with concentrations of 0.5 and 4.0 μM. We found that pomalidomide increased HbF synthesis in a dose-dependent manner (Figure 2B). The strongest induction of HbF was achieved at 4.0 μM with a 27.0 ± 4.0% HbF increase. Therefore, 4.0 μM pomalidomide treatment during days 4-14 of erythroid culture was chosen for subsequent investigations.

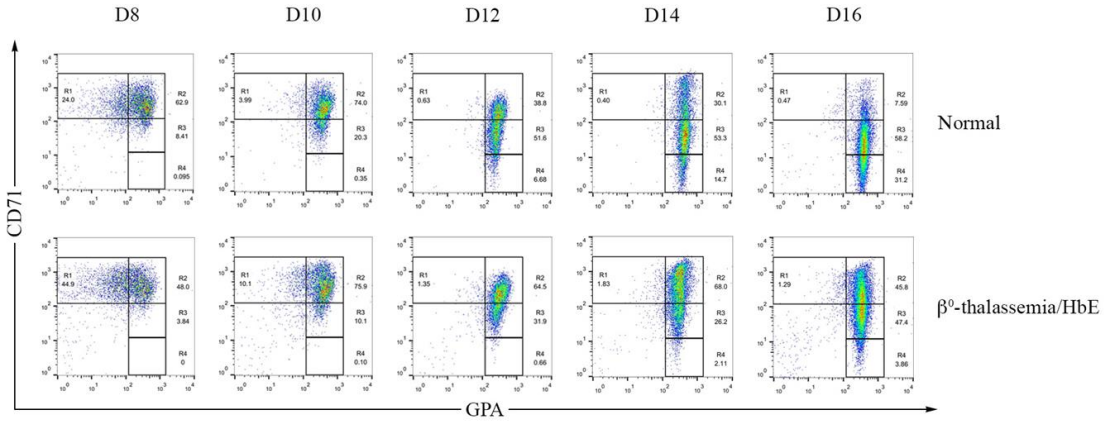

Figure 1. Delayed erythroid differentiation is observed in β^0-thalassemia/HbE comparing to normal erythroblast culture. Representative flow cytometry dot plots for erythroid differentiation analysis during a 3-phase *ex vivo* erythroid cell culture from normal individual and β^0-thalassemia/HbE patient. Erythroid cells were gated into R1 to R4 populations according to the expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a).

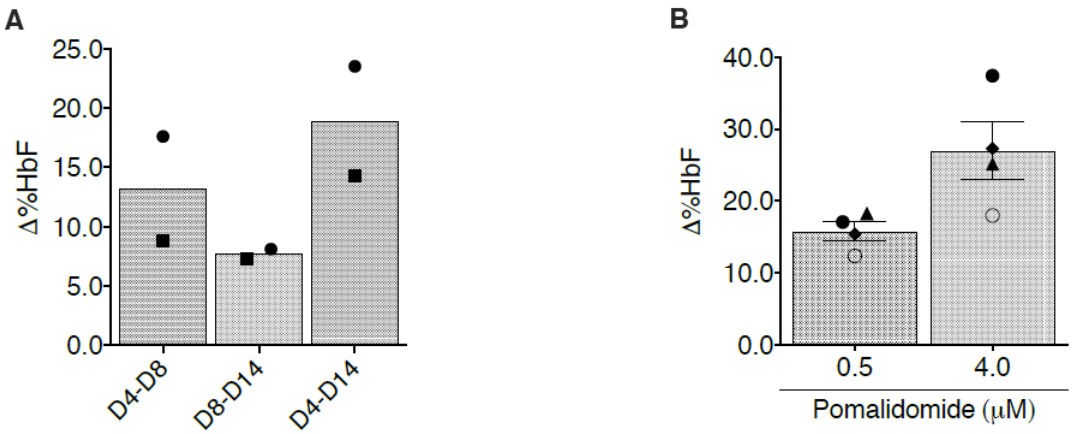

Figure 2. Time- and dose-dependent inducing effects of pomalidomide on HbF induction in erythroid cells from β^0-thalassemia/HbE patients. (A) β^0-Thalassemia/HbE erythroblasts were treated with 1.0 μM pomalidomide for 3 different treatment durations: days 4-8, days 8-14, days 4-14. The percentage of HbF relative to total Hb (%HbF + %HbE) determined by HPLC at day 14 of erythroid differentiation. The increase in HbF percentage after treatment from the baseline level (DMSO control) was expressed as Δ%HbF (%HbF [compound treatment] - %HbF [DMSO control]). (n = 2). (B) β^0-Thalassemia/HbE erythroblasts were treated with different concentrations of pomalidomide during days 4-14 of culture. The increase of the HbF percentage in pomalidomide-treated cells from the baseline level in DMSO control was expressed as Δ%HbF. (n = 4).

Pomalidomide plus decitabine potently and additively induces HbF

To probe the therapeutic potential of pomalidomide for treatment of β-thalassemia, we investigated the efficacy of pomalidomide in erythroid progenitor cells from 12 different cases of compound heterozygous β^0-thalassemia/HbE (Table 1). We also compared the efficacy of pomalidomide to other pharmacological HbF inducers that have been previously reported, including hydroxyurea, decitabine and RN-1. Based on our previous studies, hydroxyurea, decitabine and RN-1 showed maximum HbF-inducing activities without significant cytotoxic effects at concentrations of 1.0, 0.1 and 0.02 μM, respectively, when added during days 8-14 of β^0-thalassemia/HbE erythroid culture (data not shown). Comparison results using the optimal conditions of each compound in β^0-thalassemia/HbE erythroid culture revealed that pomalidomide was much more effective in inducing HbF expression than hydroxyurea, decitabine or RN-1 (Figure 3A-

B). The greatest increase in HbF percentage from baseline level (DMSO control) was observed in pomalidomide-treated cells, achieving 24.5 ± 1.2% as determined by HPLC (Figure 3A-B). Using quantitative RT-PCR to assess the quantity of γ-globin mRNA at day 12 of culture, we found that pomalidomide significantly increased γ-globin (*HBG*) mRNA expression, achieving a 2.3 ± 0.3-fold increase compared with DMSO-treated cells, with a concomitant decrease of β-globin (*HBB*) mRNA expression (Figure 3C). There was no significant change in α-globin (*HBA*) mRNA expression. These results strongly support that pomalidomide is a more effective HbF inducer than hydroxyurea, decitabine or RN-1.

To enhance the level of HbF induction, we investigated the effects of combinatorial treatment of pomalidomide with other pharmacological HbF inducers. The combination of pomalidomide and decitabine had an additive effect on HbF induction as shown by increased HbF expression level when compared to treatment with any single compound (Figure 3A-B). Hydroxyurea did not generate any additional increase in HbF when combined with pomalidomide. The combination of pomalidomide and RN-1 did increase the percentage of HbF (Figure 3A-B) and at the same time decreased *HBA*, *HBB*, and *HBG* mRNA expression (Figure 3C) compared to single treatments, suggesting that this combination negatively affected total globin mRNA expression. Taken together, these results suggest that pomalidomide and decitabine act through independent mechanisms to additively induce high level HbF expression, implying cooperative therapeutic potential for the treatment of β-thalassemia.

Table 1 Hematological parameters of β^0-thalassemia/HbE patients participated in this study.

Sample name	*HBB* alleles	Hb* (g/dL)	HbF %*	HbE %*	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW-CV (%)
BE1	IVSI-1 (G>T):CD26(G>A)	7.2	49.0	51.0	25	78.4	23	28.8	24.4
BE2	Codon41/42(-TTCT) :CD26(G>A)	5.7	34.8	65.2	18	62.8	20	31.5	29.6
BE3	Codon17(A>T):CD26(G>A)	6.2	47.3	54.1	20	58.3	18.3	31.5	28.4
BE4	Initiation codon (T>G) :CD26(G>A)	6.7	19.4	21.9	23	68.8	20.5	29.8	ND
BE5	Codon17(A>T):CD26(G>A)	7.1	57.7	36.7	22	57.7	18.8	32.6	ND
BE6	Codon41/42(-TTCT) :CD26(G>A)	6.3	26.6	51.6	21	60.6	18.3	30.1	ND
BE7	Codon17(A>T):CD26(G>A)	7.5	31	42.6	23	56.8	18.4	32.5	32.2
BE8	Codon41/42(-TTCT) :CD26(G>A)	8.9	57.3	42.7	27	75.4	25	33.3	ND
BE9	Codon41/42(-TTCT) :CD26(G>A)	6.6	44.4	55.6	20	56	18	32.4	ND
BE10	IVSI-1(G>T):CD26(G>A)	8.3	62.6	37.4	25.6	72.8	24	32.7	22.3
BE11	Codon17(A>T):CD26(G>A)	7.1	27.6	72.4	22	66.2	21	31.8	UD
BE12	Codon17(A>T):CD26(G>A)	6.7	36.8	63.2	20	63	21	33.7	ND

Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; ND, not determined

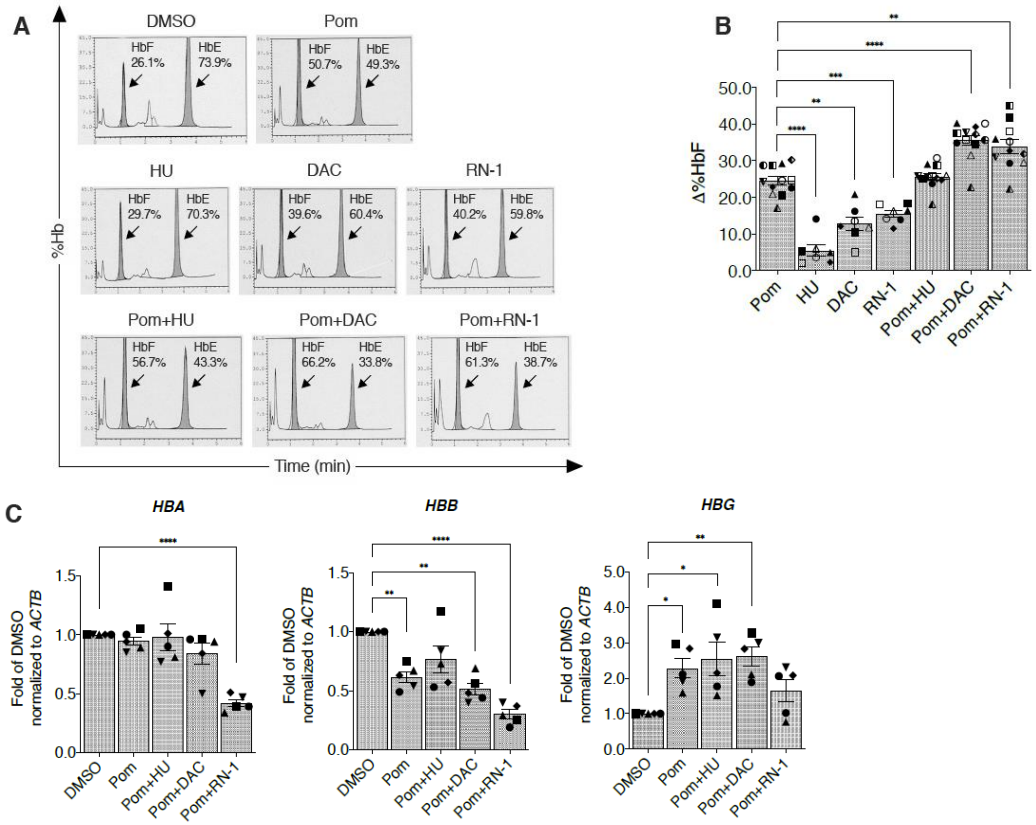

Figure 3. High level of HbF induction in β^0-thalassemia/HbE erythroid cells by pomalidomide alone or in combination with other HbF inducers. β^0-Thalassemia/HbE erythroblasts were treated with 4.0 μM pomalidomide only (Pom, from day 4 to 14), 1.0 μM hydroxyurea only (HU, from day 8 to 14), 0.1 μM decitabine only (DAC, from day 8 to 14), 0.02 μM RN-1 (from day 8 to 14), the combination of pomalidomide with hydroxyurea, the combination of pomalidomide with decitabine, or the combination of pomalidomide with RN-1. (A) Representative HPLC chromatograms showing hemoglobin composition at day 14 of erythroid differentiation. (B) The percentage of HbF relative to total Hb (%HbF + %HbE) determined by HPLC at day 14 of erythroid differentiation. The increase in HbF percentage after treatment from the baseline level (DMSO control) was expressed as Δ%HbF (%HbF [compound treatment] - %HbF [DMSO control]). (mean ± standard error of the mean [SEM], n = 7 for HU, DAC and RN-1, n = 12 for Pom, Pom+HU and Pom+DAC, n = 11 for Pom+RN-1). (C) Quantitative RT-PCR analysis showing relative *HBA*, *HBB* and *HBG* mRNA expression levels normalized to β-actin (*ACTB*) at day 12 of erythroid differentiation. Data are presented as the mean of relative fold change of DMSO ± SEM. (n = 5) $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$; $^{****}P < 0.0001$.

Pomalidomide does not affect erythroid proliferation, viability, or terminal differentiation

To assess the cytotoxicity of pomalidomide or its combinations with other HbF inducers, erythroid cell proliferation, viability, differentiation, and morphology were investigated. We found that pomalidomide did not significantly affect erythroid cell proliferation (Figure 4A) or viability (Figure 4B) under our culture conditions. However, pomalidomide plus decitabine showed a reduction of cell proliferation at day 12 of the culture without affecting cell viability.

Erythroid cell proliferation and viability were reduced in cells exposed to pomalidomide plus RN-1 (Figure 4A-B), suggesting toxicity of this combination. Analysis of erythroid differentiation at day 12 of culture by flow cytometry revealed that the erythroid differentiation pattern of cells treated with pomalidomide or its combinations with hydroxyurea or decitabine was similar to that of DMSO-treated cells (Figure 4C-D), suggesting that these treatments did not cause defects in the erythroid terminal differentiation. However, significantly delayed erythroid differentiation was observed in RN-1 combined with pomalidomide as evidenced by an increased proportion in the flow-evaluated R1 population ($CD71^{high}/GPA^{low}$) at day 12 of culture (Figure 4C-D). Similarly, modified Giemsa-stained cytospins showed an increased number of early-stage erythroblasts in cells exposed to the combination of pomalidomide and RN-1 when compared to DMSO-treated cells (Figure 4E). The morphology of erythroid cells treated with pomalidomide or its combinations with hydroxyurea or decitabine was similar to that of DMSO control cells, confirming the absence of any toxic effects of these treatments.

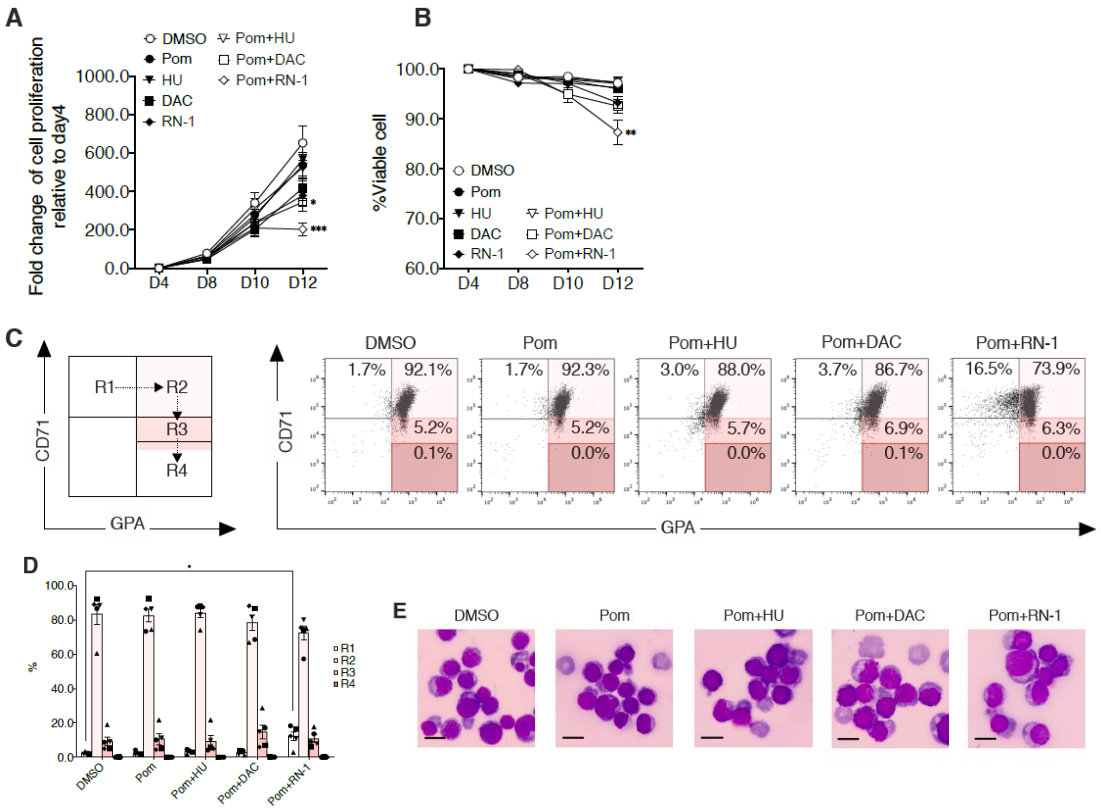

Figure 4. Effect of pomalidomide and its combinations on cell proliferation, viability, and differentiation of cultured erythroid cells from β^0-thalassemia/HbE patients. β^0-Thalassemia/HbE erythroblasts were treated with 4.0 μM pomalidomide only (Pom, from day 4 to 14), 1.0 μM hydroxyurea only (HU, from day 8 to 14), 0.1 μM decitabine only (DAC, from day 8 to 14), 0.02 μM RN-1 (from day 8 to 14), the combination of pomalidomide with hydroxyurea, the combination of pomalidomide with decitabine, or the combination of pomalidomide with RN-1. (A) Erythroid proliferation and (B) viability during differentiation assessed by trypan blue staining. The fold change of cell proliferation represents the ratio of cell number at the indicated time points versus day 4. (mean ± SEM, n = 7 for HU, DAC and RN-1, n = 12 for Pom, Pom+HU, Pom+DAC and Pom+RN-1). $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$, relative to DMSO control. (C) Representative flow cytometry dot plots for erythroid differentiation analysis on day 12 of culture. Erythroid cells were gated into R1 to R4 populations according to the expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a). (D) The histogram represents the quantitation of erythroid subpopulations analyzed by flow cytometry. (mean ± SEM, n = 5). $^{*}P < 0.05$, relative to DMSO control. (E) Representative modified Giemsa-stained cytospins at day 12 of culture showing erythroid morphology after DMSO and compound treatments. Scale bar = 10 μm.

Effects of pomalidomide on HbF regulators in β-thalassemia erythroid cell culture

It has been shown that fetal to adult hemoglobin switching is highly regulated by transcriptional repressors and their corepressors. Recently, pomalidomide has been shown to induce fetal hemoglobin expression in erythroid progenitor cells from normal individuals and SCD patients partly through decreasing BCL11A and SOX6, two major repressors of γ-globin expression. To investigate the effects of pomalidomide and its combinations on transcriptional regulation in β-thalassemic erythroid progenitor cells, we determined mRNA expression levels of 14 known HbF-modifying transcription factors including *BCL11A*, *SOX6*, *KLF1*, *LRF*, *GATA1*, *GATA2*, *FOXO3*, *NRF2*, *ID2*, *LSD1*, *DNMT1*, *CHD4*, *HBS1L*, and *MYB* on day 12 of erythroid culture. The quantitative RT-PCR analyses revealed that the key γ-globin repressor *BCL11A* was slightly reduced by treatments of pomalidomide and the combination of pomalidomide and hydroxyurea. *BCL11A* was significantly downregulated (by 1.8- and 5.6-fold) after treatment with the combinations of pomalidomide with decitabine or pomalidomide with RN-1, respectively (Figure 5). Moreover, the expression of *SOX6*, *LRF*, *GATA1*, and *HBS1L* was modestly but significantly downregulated by pomalidomide, whereas the others were unaffected. In addition to the treatment of pomalidomide alone, combined pomalidomide and decitabine treatment, which showed an additive effect on HbF induction, decreased the expression of *KLF1*, *LSD1*, and *CHD4*, among which the two latter factors are epigenetic modifying enzymes. The combination of pomalidomide and RN-1 significantly and remarkably affected the expression of several key regulators including *SOX6*, *KLF1*, *GATA1*, *FOXO3*, *NRF2*, *ID2*, *LSD1*, *DNMT1*, *CHD4*, *HBS1L*, and *MYB* (Figure 5), consistent with the result that combined pomalidomide and RN-1 significantly reduced cell proliferation and viability and delayed erythroid differentiation (Figure 4). However, *GATA2*, a potential activator of γ-globin expression, was unaffected by any of these treatments. Taken together, these results indicated that the mechanisms of action of pomalidomide and its combinations in induction of HbF expression partly involve transcriptional regulation of key HbF repressors and/or corepressors.

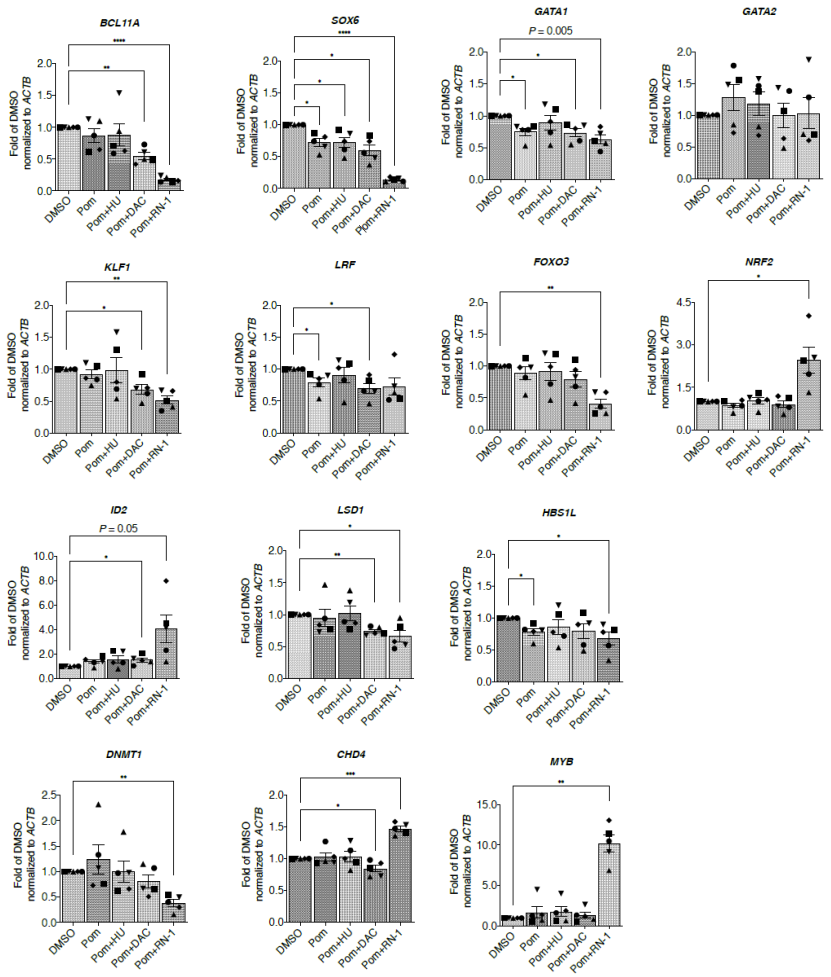

Figure 5. Treatment of pomalidomide and its combinations alter the expression of HbF regulators in β^0-thalassemia/HbE erythroid cells. Relative mRNA abundance of known HbF regulators normalized to β-actin (*ACTB*) determined by quantitative RT-PCR at day 12 of erythroid culture. Gene names are shown at the top of each histogram. Data are presented as the mean of relative fold change of DMSO ± SEM. (n = 5) $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$; $^{****}P < 0.0001$.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ
   งานวิจัยนี้มีผลงานวิจัยตีพิมพ์จำนวน 2 เรื่อง ได้แก่

1.1 Nualkaew T, Khamphikham P, Pongpaksupasin P, Kaewsakulthong W, Songdej D, Paiboonsukwong K, Sripichai O, Engel JD, Hongeng S, Fucharoen S, Jearawiriyapaisarn N (2020) UNC0638 induces high levels of fetal hemoglobin expression in beta-thalassemia/HbE erythroid progenitor cells. Ann Hematol 99 (9):2027-2036. doi:10.1007/s00277-020-04136-w

1.2 Khamphikham P, Nualkaew T, Pongpaksupasin P, Kaewsakulthong W, Songdej D, Paiboonsukwong K, Engel JD, Hongeng S, Fucharoen S, Sripichai O, Jearawiriyapaisarn N (2020) High-level induction of fetal haemoglobin by pomalidomide in beta-thalassaemia/HbE erythroid progenitor cells. Br J Haematol 189 (6):e240-e245. doi:10.1111/bjh.16670

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ: นักวิจัยมีการนำข้อมูลที่ได้จากงานวิจัยนี้มาใช้ในการวางแผนการทดสอบประสิทธิภาพของ pomalidomide ในหนูทดลองที่เป็นโรค β-thalassemia เพื่อนำไปสู่การพัฒนาและการศึกษาผลของยา pomalidomide ในทางคลินิก (clinical trials) สำหรับการรักษาโรค β-thalassemia ในคนไข้ต่อไป

ภาคผนวก

ผลงานวิจัยตีพิมพ์เรื่องที่ 1

doses).[11] This adds weight to the argument of starting chelation with deferasirox early in the course of MDS when lower doses may suffice and improve compliance.

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High-level induction of fetal haemoglobin by pomalidomide in β-thalassaemia/HbE erythroid progenitor cells

Studies have shown that increased expression of fetal haemoglobin (HbF; $\alpha_2\gamma_2$) can ameliorate red blood cell deficiencies in patients with β-thalassaemia and sickle cell disease (SCD).[1–3] Pharmacological induction of HbF expression in β-thalassaemia has been investigated using several classes of small molecules,[4] including 5-azacytidine,[5] decitabine,[6] hydroxyurea,[7] LSD1 inhibitors (tranylcypromine and RN-1),[8,9] and short chain fatty acid derivatives.[10,11] Among these molecules, hydroxyurea is the only U.S. Food and Drug Administration (FDA) currently approved drug for the treatment of SCD and/or β-thalassaemia. However, hydroxyurea has shown modest and variable responses with potential myelosuppression in β-thalassaemia patients. Therefore, more robust and safer HbF therapeutics are highly desired.

Pomalidomide, an FDA-approved immunomodulatory drug for the treatment of multiple myeloma,[12,13] stimulates γ-globin mRNA and HbF expression in erythroid progenitor cells by downregulating factors involved in γ-globin repression, including *BCL11A, SOX6, GATA1, KLF1* and *LSD1*.[14–16] In addition, treatment of a humanized mouse model of SCD with pomalidomide induced comparable HbF expression to hydroxyurea, but without myelosuppressive effects.[15]

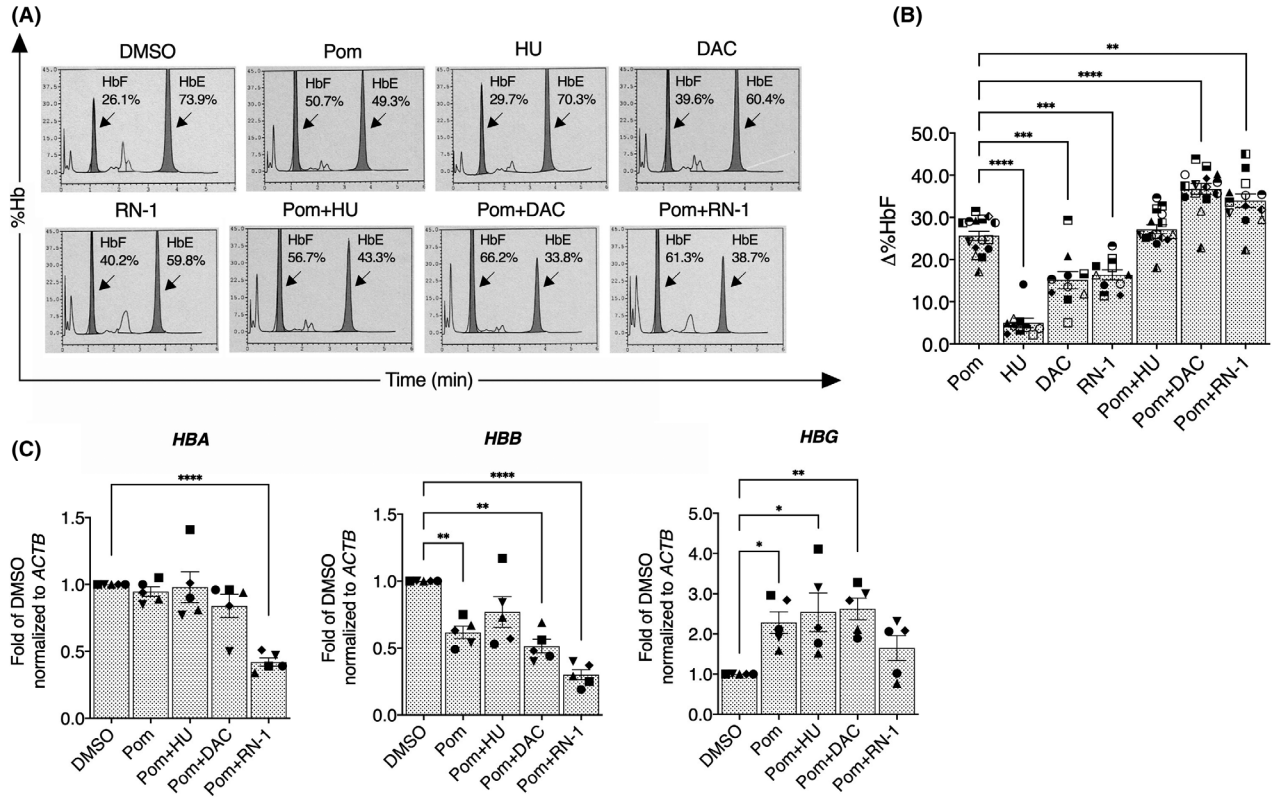

Fig 1. Robust HbF induction in β^0-thalassaemia/HbE erythroid cells by pomalidomide alone or in combination with other HbF inducers. β^0-Thalassaemia/HbE erythroblasts were treated with 4·0 μmol/l pomalidomide only (Pom, from day 4–14), 1·0 μmol/l hydroxyurea only (HU, from day 8–14), 0·1 μmol/l decitabine only (DAC, from day 8–14), 0·02 μmol/l RN-1 only (from day 8–14), or in combination of Pom with HU, with DAC, or with RN-1. (A) Representative high-performance liquid chromatograms (HPLC) showing haemoglobin composition at day 14 of erythroid differentiation. (B) The percentage of HbF relative to total Hb (%HbF + %HbE) determined by HPLC at day 14 of erythroid differentiation. The increase in HbF percentage after treatment from the baseline level in dimethyl sulfoxide (DMSO) control was expressed as Δ%HbF (% HbF [compound treatment] − %HbF [DMSO control]). Mean ± standard error of the mean [SEM], n = 10 for HU, DAC and RN-1; n = 15 for Pom, Pom + HU and Pom + DAC; and n = 13 for Pom + RN-1. (C) Quantitative reverse transcription polymerase chain reaction analysis showing relative *HBA*, *HBB* and *HBG* mRNA expression levels normalised to β-actin (*ACTB*) at day 12 of erythroid differentiation. Data are presented as the mean (± SEM) of relative fold change of DMSO (n = 5) *P < 0·05; **P < 0·005; ***P < 0·0005; ****P < 0·0001.

Here, we investigated the therapeutic potential of pomalidomide and its combined effects with other HbF inducers, including hydroxyurea, decitabine and RN-1, in erythroid progenitor cells from compound heterozygous β^0-thalassaemia/HbE (*HBB*:c.79G>A) patients (Table SI) using a three-phase liquid culture system that supports terminal maturation of erythroid cells (Data S1 and Fig S1). Comparison of results using optimal conditions for each compound (Figs S2 and S3) revealed that pomalidomide was much more effective in inducing HbF expression than hydroxyurea, decitabine or RN-1 (Fig 1A,B). The greatest increase in HbF percentage from the baseline level was observed in pomalidomide-treated cells, achieving 25·6 ± 1·1% as determined by high-performance liquid chromatography (HPLC) (Fig 1A,B). β^0-thalassaemia/HbE precursors from patients of different β^0-thalassemic mutations (Table SI) showed similarly increased levels of HbF induction in response to pomalidomide treatment. This result suggested that deficient progenitors, regardless of specific β^0-thalassemic mutation or baseline HbF level, are all susceptible to strong induction with pomalidomide (Fig 1A,B and Tables SI and SII). The percentage of cells expressing HbF (F cells) increased from 49·8 ± 4·7% for dimethyl sulfoxide (DMSO) controls to 60·6 ± 2·5% after pomalidomide treatment (Fig S4). By quantitative reverse transcription polymerase chain reaction (RT-PCR), we found that pomalidomide significantly increased γ-globin (*HBG*) mRNA expression, achieving a 2·3 ± 0·3-fold increase over control cells, with coincidentally diminished β-globin (*HBB*) expression, without significant change in α-globin (*HBA*) expression (Fig 1C).

To enhance the level of HbF induction, we investigated the effects of combined treatment of pomalidomide either with or without other pharmacological HbF inducers. The combination of pomalidomide and decitabine had an additive effect on induction, as shown by the differential HbF level (Δ% HbF = 36·7 ± 1·3) when compared to treatment with any single agent (Fig 1A,B). Hydroxyurea did not generate any additional increase in HbF when combined with pomalidomide. The

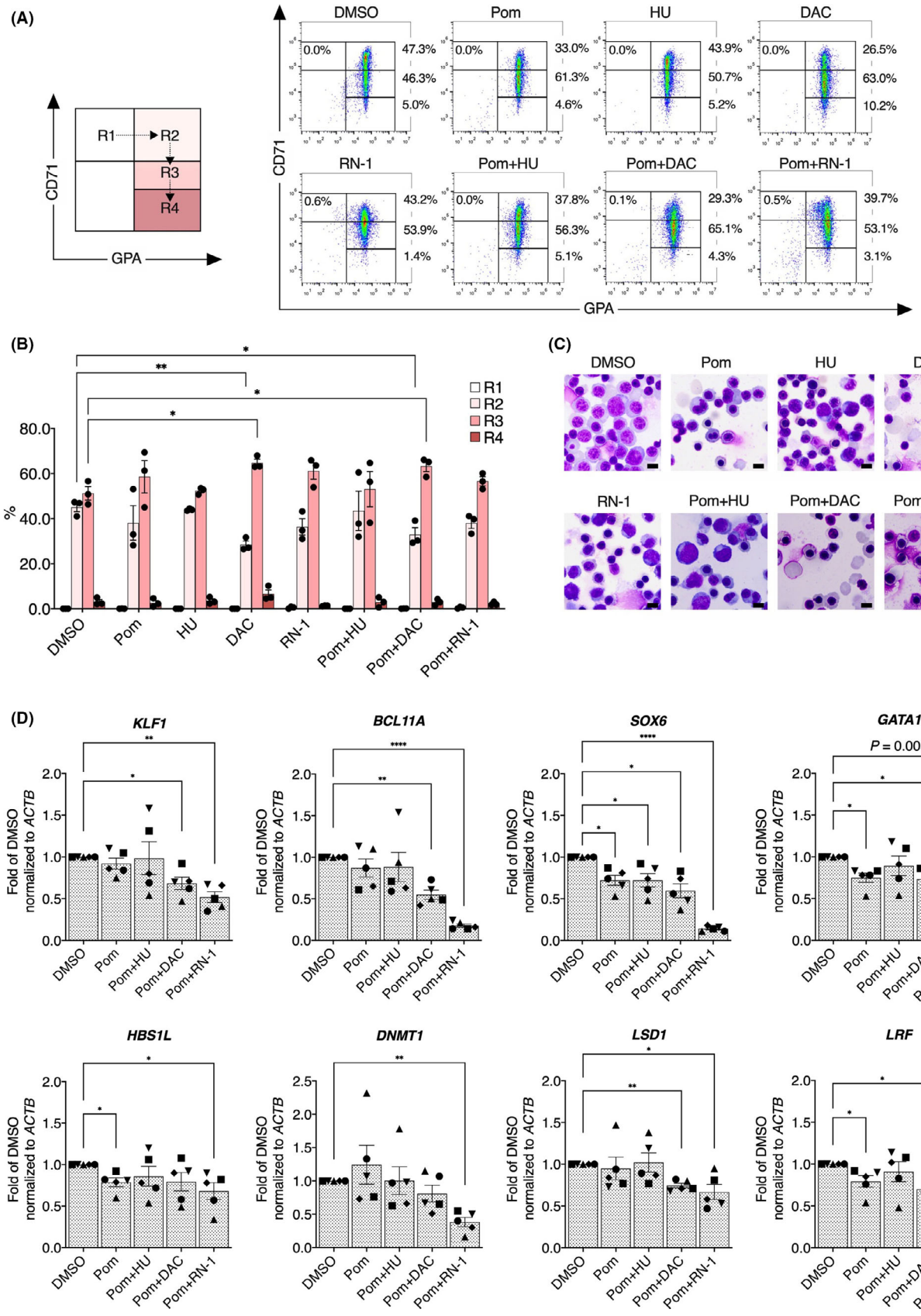

Fig 2. Effect of pomalidomide and its combinations on erythroid differentiation and mRNA expression of HbF regulators in cultured erythroid cells from β^0-thalassaemia/HbE patients. β^0-Thalassaemia/HbE erythroblasts were treated with 4·0 μmol/l pomalidomide only (Pom, from day 4 –14), 1·0 μmol/l hydroxyurea only (HU, from day 8–14), 0·1 μmol/l decitabine only (DAC, from day 8– 14), 0·02 μmol/l RN-1 only (from day 8–14), the combination of Pom with HU, with DAC, or with RN-1. (A) Representative flow cytometry dot plots for erythroid differentiation analysis on day 12 of culture. Erythroid cells were gated into R1 to R4 populations according to the expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a). (B) The histogram represents the quantitation of erythroid subpopulations analysed by flow cytometry. (mean ± SEM, $n = 3$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$, relative to dimethyl sulfoxide (DMSO) control. (C) Representative modified Giemsa-stained cytospins at day 12 of culture showing erythroid morphology after DMSO or compound treatments. Scale bar = 10 μm. (D) Relative mRNA abundance of known HbF regulators normalized to β-actin (*ACTB*) determined by quantitative reverse transcription polymerase chain reaction at day 12 of erythroid cell culture. Gene names are shown at the top of each histogram. Data are presented as the mean (± SEM) of relative fold change of DMSO . ($n = 5$) $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$; $^{****}P < 0{\cdot}0001$.

combination of pomalidomide and RN-1 did increase the percentage of HbF (Fig 1A,B) and at the same time reduced *HBA*, *HBB* and *HBG* mRNA expression (Fig 1C), suggesting that this combination negatively affected total globin mRNA expression. Taken together, these results suggest that pomalidomide and decitabine act through independent pathways to induce, additively, high-level HbF expression, implying a cooperative therapeutic potential for the treatment of β-thalassaemia.

We next determined the cytotoxicity of treatments and found that pomalidomide did not significantly affect erythroid cell proliferation (Fig S5A) or viability (Fig S5B). However, pomalidomide plus decitabine showed a reduction in cell proliferation on day 12 of culture without affecting cell viability. Erythroid cell proliferation and viability were significantly reduced in cells exposed to pomalidomide plus RN-1 (Fig S5A,B), suggesting toxicity of the latter combination. Analysis of erythroid differentiation of cells treated with hydroxyurea or pomalidomide plus hydroxyurea was similar to that of DMSO-treated cells (Fig 2A,B), suggesting that these treatments did not affect erythroid terminal differentiation. We noted a trend towards increased differentiation of cells treated with pomalidomide, RN-1 and pomalidomide plus RN-1, compared with the controls. Interestingly, significantly accelerated erythroid differentiation was observed in decitabine alone and pomalidomide plus decitabine, as evidenced by elevated transferrin receptor $(CD71)^{medium}$/(glycophorin A $(GPA)^{high}$ population and decreased $CD71^{high}$/GPA^{high} cells (Fig 2A,B). Similarly, modified Giemsa-stained cytospins showed an increased number of late-stage erythroblasts in cells exposed to decitabine alone and pomalidomide plus decitabine when compared to control cells, indicating a shift towards normal erythroid cell maturation (Fig 2C and Fig S1). These results suggested that the differentiation of β^0-thalassaemia/HbE progenitor cells significantly improved after treatment with either decitabine alone or pomalidomide plus decitabine.

To investigate the effects of pomalidomide plus or minus these effectors on transcriptional regulation in β-thalassaemic erythroid progenitor cells, quantitative RT-PCR analyses revealed that one key γ-globin repressor mRNA, *BCL11A,* was only slightly reduced after treatment with pomalidomide or pomalidomide plus hydroxyurea. *BCL11A* was significantly downregulated (by 1·8- and 5·6-fold) after treatment with pomalidomide plus decitabine or pomalidomide plus RN-1 respectively (Fig 2D). Moreover, the expression of *SOX6*, *GATA1*, *HBS1L* and *LRF* were modestly but significantly downregulated by pomalidomide, whereas other erythroid regulators were unaffected (Fig 2D and Fig S6). In addition, combined pomalidomide and decitabine treatment, which showed additive effects on HbF induction, reduced the expression of *KLF1*, *LSD1* and *CHD4*. The combination of pomalidomide plus RN-1 significantly affected the expression of several key regulators, including *KLF1*, *SOX6*, *GATA1*, *HBS1L*, *DNMT1*, *LSD1*, *ID2*, *CHD4*, *FOXO3*, *NRF2* and *MYB* (Fig 2D and Fig S6), consistent with the fact that this same combination significantly reduced cell proliferation and viability (Fig S5). Taken together, these results indicate that the mechanisms of action of pomalidomide and several co-effectors in induction of HbF expression partly involve transcriptional regulation of key HbF repressors and/or co-repressors.

In summary, the present data show that pomalidomide is a potent HbF inducer and is more potent than hydroxyurea. The combination of pomalidomide and decitabine provide additive effects in inducing HbF expression in erythroid cells from β^0-thalassaemia/HbE patients. Despite these promising results, it must be emphasized that the potential risks associated with the use of pomalidomide include developmental defects (if taken during pregnancy), thrombosis and pancytopenia,[17] which are similar to the toxicities of the parental drugs, lenalidomide and thalidomide. Development of pomalidomide structural refinements or analogues with similar biological effects may lead to future, fully effective, reduced adverse effects and possible clinical application.

Acknowledgements

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

PK, TN, OS and NJ designed the research; PK, TN, PP and WK performed the experiments; PK, TN and NJ analysed data; DS, KP, SH and SF provided samples and resources; PK, JDE, and NJ wrote the manuscript; JDE, SH, SF, OS and NJ conceptualized the idea and supervised the project. All the authors read and approved the final manuscript.

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Keywords: fetal haemoglobin induction, β-thalassaemia/HbE, pomalidomide, hydroxyurea, decitabine

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplemental methods.

Fig S1. Delayed erythroid differentiation is observed in β^0-thalassaemia/HbE comparing to normal erythroblast culture.

Fig S2. Time- and dose-dependent inducing effects of pomalidomide on HbF induction in erythroid cells from β^0-thalassaemia/HbE patients.

Fig S3. HbF-inducing effects of hydroxyurea (HU), decitabine (DAC), and RN-1 in erythroid cells from β^0-thalassaemia/HbE patients.

Fig S4. Pomalidomide and its combinations increase erythroid cells expressing HbF (F-cells).

Fig S5. Effect of pomalidomide and its combinations on cell proliferation, viability of cultured erythroid cells from β^0-thalassaemia/HbE patients.

Fig S6. Treatment of pomalidomide and its combinations alter the expression of HbF regulators in β^0-thalassaemia/HbE erythroid cells.

Table SI Haematological parameters of β^0-thalassaemia/HbE patients participated in this study.

Table SII Induction of HbF in erythroid progenitor cells from β^0-thalassaemia/HbE after treatments with pomalidomide, hydroxyurea, decitabine, RN-1 and their combinations.

Table SIII Primers used in this study.

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Clinical and biological correlates of the expression of select Polycomb complex genes in Brazilian children with acute promyelocytic leukaemia

De novo acute promyelocytic leukaemia (APL) is an aggressive subtype that accounts for 5–10% of all childhood acute myeloid leukaemia (AML). In some Latin American and European populations, APL is more frequent than in other geographic populations. Approximately 97% of patients with APL present t(15;17)(q22;q21.1)/promyelocytic leukaemia (PML)-retinoic acid receptor alpha (RARA) fusion protein and nearly all of the affected patients respond to therapy with all-*trans* retinoic acid (ATRA) combined with arsenic trioxide (ATO).[1] Additionally, studies in transgenic mice revealed that this transcript is necessary, but not sufficient, for APL development, suggesting that additional genetic or epigenetic changes are also required for the APL establishment.[2]

The FMS-like tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) has been frequently reported in patients with APL presenting a more aggressive disease course, with lower overall and disease-free survival rates. These mutations are known to co-operate with other initiating events to advance disease progression, but they do not initiate leukaemia independently.[3] Regarding paediatric APL, genomic studies reported that APL cells have fewer genetic alterations than those from other AML subtypes.[4] These data indicate that childhood APL development may require more than just genetic alterations to manifest the disease phenotype.

In this context, there are several reports on the epigenetic alterations implicated in AML development, and they are mainly presented in APL. For instance, patients with APL are characterised by a specific DNA methylation pattern, which may be due to relatively late events in APL leukaemogenesis, contributing to APL maintenance rather than leukaemia initiation.[5]

Besides, PML/RARA induces a multitude of alterations in chromatin architecture, including the recruitment of crucial epigenetic-modifying factors, such as histone deacetylase complexes and DNA methyltransferases. Moreover, pieces of evidence have revealed that the Polycomb repressor complex (PRC) could contribute to the alterations observed in the typical APL epigenetic landscape.[6]

Polycomb group (PcG) proteins are histone modifiers in two multiprotein complexes: Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). Radulovic et al.[6] highlighted the emerging implications of these genes in haematopoietic neoplasms, including myeloid neoplasia. Therefore, changes in the expression profile of individual PcG genes might yield novel information about APL pathogenesis.

We evaluated the PcG gene levels, namely, enhancer of zeste homologue 2 (*EZH2*), Yin and Yang 1 protein (*YY1*), BMI1 proto-oncogene, Polycomb ring finger (*BMI1*) and suppressor of zeste 12 protein homologue (*SUZ12*), in a cohort of 25 Brazilian children with APL, with and without a *FLT3*-ITD mutation. In addition, we compared them to those found in patients with other AML subtypes, with and without the *FLT3* mutation, to verify whether this mutation status could be associated with the APL epigenetic landscape.

Amongst the 25 patients with APL, eight had additional chromosome abnormalities that mostly involved chromosomes 6, 8, 20 and 21. In the AML group, 39 patients had their karyotype evaluated and the abnormalities detected were as follows: 15 (38·5%) presented with lysine methyltransferase 2A (*KMT2A*) gene abnormalities; eight (20·5%) presented with non-recurrent chromosome abnormalities; four (10·3%) presented with RUNX1 translocation partner 1 (*RUNX1*)/*RUNXT1* fusion genes; four (10·3%) presented with core binding factor subunit beta (*CBFB*)/myosin heavy chain 11 (*MYH11*) fusion genes; four (10·3%) presented with normal karyotypes; three (7·7%) presented with abnormalities in chromosomes 5 and 7; and one (2·4%) presented with no mitosis. In relation to *FLT3* status, 21 (30·9%) patients had *FLT3*-ITD mutations, 13 (61·9%) were patients with APL and eight (38·1%) were patients with AML [AML-M5 (five patients), AML-M2 (two) and AML-M6 (one)] (Clinical data are in Table S1).

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ORIGINAL ARTICLE

UNC0638 induces high levels of fetal hemoglobin expression in β-thalassemia/HbE erythroid progenitor cells

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Abstract

Increased expression of fetal hemoglobin (HbF) improves the clinical severity of β-thalassemia patients. EHMT1/2 histone methyltransferases are epigenetic modifying enzymes that are responsible for catalyzing addition of the repressive histone mark H3K9me2 at silenced genes, including the γ-globin genes. UNC0638, a chemical inhibitor of EHMT1/2, has been shown to induce HbF expression in human erythroid progenitor cell cultures. Here, we report the HbF-inducing activity of UNC0638 in erythroid progenitor cells from β-thalassemia/HbE patients. UNC0638 treatment led to significant increases in γ-globin mRNA, HbF expression, and HbF-containing cells in the absence of significant cytotoxicity. Moreover, UNC0638 showed additive effects on HbF induction in combination with the immunomodulatory drug pomalidomide and the DNMT1 inhibitor decitabine. These studies provide a scientific proof of concept that a small molecule targeting EHMT1/2 epigenetic enzymes, used alone or in combination with pomalidomide or decitabine, is a potential therapeutic approach for HbF induction. Further development of structural analogs of UNC0638 with similar biological effects but improved pharmacokinetic properties may lead to promising therapies and possible clinical application for the treatment of β-thalassemia.

Keywords Fetal hemoglobin induction · β-Thalassemia/HbE · UNC0638 · Pomalidomide · Decitabine

Introduction

β-Thalassemia is one of the most common genetic blood disorders and is designated as a global health burden by the World Health Organization [1]. It is caused by multiple mutations in the β-globin locus, resulting in the complete absence or a reduction in the expression of the β-globin gene and reduced adult hemoglobin (HbA; $\alpha_2\beta_2$) production in erythroid cells. This results in excess unmatched α-globin chains that precipitate and damage erythroid cell membranes, causing ineffective erythropoiesis, hemolysis, anemia, and extramedullary erythropoiesis [2, 3]. Coinheritance of the β-thalassemia allele and the structural variant hemoglobin E (HbE, *HBB*:c.79G > A), resulting in β-thalassemia/HbE disease, is one of the most common severe β-thalassemias worldwide [4, 5]. Current treatments for β-thalassemia are primarily based on supportive therapies including regular, lifelong blood transfusions combined with iron chelators. Hematopoietic stem cell transplantation remains the only curative treatment; however, it is accessible to only a small fraction of patients. More recently, gene therapy for β-thalassemia

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appears to be more promising [6], but it is unlikely to be widely applied, again due to clinical accessibility.

Patients with β-thalassemia/HbE show remarkable variability in clinical severity, ranging from nearly asymptomatic to severe, transfusion-dependent thalassemia [7–9]. It is known that increased levels of HbF expression is an important modifying factor that can ameliorate the clinical severity of β-thalassemia/HbE patients due to improved α/β-globin chain imbalance [7, 9].

Hydroxyurea was the first US Food and Drug Administration (FDA)-approved HbF inducer for sickle cell disease (SCD) and/or β-thalassemia [10]. However, the response to hydroxyurea treatments is highly variable in SCD patients [10] and limited in β-thalassemia patients [11–14]. Moreover, hydroxyurea poses undesirable side effects including myelosuppression and possible long-term carcinogenesis [15]. L-glutamine has more recently been approved by the FDA for treatment of SCD by reducing oxidative stress [16], exhibiting quite modest clinical benefit. More effective and less toxic HbF-inducing agents are thus warranted.

γ-Globin gene repression requires several epigenetic modifying enzymes, including DNA methyl transferase 1 (DNMT1), histone deacetylases (HDAC), lysine-specific demethylase 1 (LSD1), euchromatin histone lysine methyltransferases 1/2 (EHMT1/2), and protein arginine *N*-methyltransferase 5 (PRMT5), which have each been investigated as potential therapeutic targets for HbF induction [17–19]. A number of pharmacological agents such as the DNMT1 inhibitor decitabine [20], HDAC inhibitors (e.g., HQK-1001 [21]), LSD1 inhibitors (e.g., tranylcypromine [22] and RN-1 [23]), and EHMT1/2 inhibitors (e.g., UNC0638 [24, 25]) have been shown to induce HbF expression. Moreover, the immunomodulatory drug pomalidomide has been reported to be a potent HbF inducer partly by downregulation of the key γ-globin repressors, BCL11A and SOX6 [26–28].

It has been demonstrated that γ-globin gene repression is associated with accumulation of the repressive chromatin mark histone H3 dimethyl-lysine 9 (H3K9me2) at the γ-globin loci [29]. Addition of H3K9me2 is mediated by the EHMT1 (GLP) and EHMT2 (G9a) histone methyltransferases [30]. Selective inhibition of EHMT1/2 by the small chemical molecule UNC0638 has been shown to induce γ-globin mRNA and HbF expression in erythroid progenitor cells from normal individuals [24, 25, 31]. The stimulation of HbF expression by UNC0638 treatment was associated with diminished accumulation of H3K9me2 near the γ-globin loci and with increased loop formation between the locus control region (LCR) and the γ-globin promoters through recruitment of the LDB1 complex to the γ-globin promoters [24, 25].

In this study, the HbF-inducing activity of UNC0638, either alone or in combination with other pharmacological HbF inducers, was investigated in erythroid progenitor cells isolated from β^0-thalassemia/HbE patients. These data confirm earlier studies examining *HBG* induction in tissue culture cells and demonstrate that UNC0638, alone or in combination with pomalidomide or decitabine, potently induces elevated HbF expression, suggesting that inhibition of EHMT1/2 holds therapeutic potential for β-thalassemia treatment.

Materials and methods

Ex vivo differentiation of human CD34$^+$ cells

Studies of human erythroid progenitor cell culture were approved by Institutional Review Boards of Mahidol University; written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. CD34$^+$ hematopoietic stem/progenitor cells (HSPC) were isolated from the peripheral blood of β^0-thalassemia/HbE patients. Hematological parameters and the percentages of HbF in the peripheral blood of these patients are shown in Supplementary Table S1. Briefly, peripheral blood mononuclear cells were isolated using Lymphoprep (Axis-Shield, Oslo, Norway), and CD34$^+$ cells were purified using the CD34 microbead kit with cell separation columns according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). CD34$^+$ cells were differentiated toward the erythroid lineage using a 3-phase liquid culture system employing three different erythroid differentiation culture media. The composition of the basal medium is Iscove's modified Dulbecco medium (Biochrom GmbH, Berlin, Germany) supplemented with 20% fetal bovine serum (Merck, Temecula, CA, USA), 300 μg/mL holo-transferrin (ProSpec, Rehovot, Israel), and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). CD34$^+$ cells were cultured in the basal medium supplemented with either 10 ng/mL human interleukin-3 (Miltenyi Biotec), 50 ng/mL human stem cell factor (SCF; Miltenyi Biotec), and 2 U/mL erythropoietin (EPO; Janssen-Cilag, Bangkok, Thailand) during phase I (days 0–4) or 10 ng/mL SCF and 2 U/mL EPO during phase II (days 4–8) or 4 U/mL EPO during phase III (days 8–14). Cell concentration was maintained at 1–2 × 10^6 cells/mL during phase III of culture. Cells were incubated at 37 °C, 5% CO_2 in a 100% humidified atmosphere.

Chemical treatment of primary human erythroid progenitor cells

All compounds were purchased from Sigma-Aldrich. UNC0638 (U4885), pomalidomide (P0018), and decitabine (A3656) were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich). Compounds were freshly diluted and added to cells at the designated concentrations and durations. Culture medium containing 0.1% v/v of DMSO served as a concentration-matched vehicle control.

Hemoglobin analysis by HPLC

The proportion of HbF (%HbF) was determined by the Bio-Rad Variant II Hemoglobin Testing System (Bio-Rad) with β-Thalassemia Short Program. At least one million differentiating erythroid cells were subjected to HPLC analysis. The Lyphochek Hemoglobin A2 control (Bio-Rad) was used for normalization. The percentage of HbF was reported relative to total Hb (HbF + HbE) in β^0-thalassemia/HbE erythroid cells. The increase in HbF percentage after treatment from the baseline level (DMSO control) was expressed as Δ%HbF (%HbF [compound treatment] − %HbF [DMSO control]).

Cell proliferation, viability, and morphology

Cell number and viability of erythroid cells were analyzed by trypan blue staining and counted with a hemocytometer. Erythroid cell morphology was examined by modified Giemsa staining (Sigma-Aldrich) of cytospins.

Flow cytometry analysis

To assess erythroid differentiation, erythroid cells at day 12 of culture were stained with antibodies against erythroid surface markers, including a phycoerythrin (PE)-conjugated anti-human CD71 (clone CY1G4; Biolegend, San Diego, CA, USA) and an allophycocyanin (APC)-conjugated anti-human CD235a (clone GA-R2; BD Biosciences, San Jose, CA, USA) and analyzed on BD Accuri C6 Plus cytometer (BD Biosciences). To assess HbF-containing cells (F-cells), erythroid cells at day 14 of culture were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then stained with either a fluorescein isothiocyanate (FITC)-conjugated anti-human fetal hemoglobin antibody (clone 2D12; BD Biosciences) or a FITC-conjugated mouse IgG1, κ isotype control (clone MOPC-21, BD Biosciences). The stained cells were analyzed on the Accuri C6 Plus cytometer. Data analysis was performed using FlowJo version 10.3.0 (FlowJo LLC, Ashland, OR, USA) software.

RNA isolation and gene expression analysis

Total RNA was isolated from erythroid cells at day 12 of culture using TRIzol Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were treated with DNase I (ThermoFisher Scientific, Waltham, MA, USA) and subsequently subjected to cDNA synthesis using RevertAid First-Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's instruction. Quantitative real-time PCR was carried out in CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) using FastStart Essential DNA Green Master (Roche, Mannheim, Germany) according to the manufacturer's instruction. Relative expression was calculated using the ΔΔCT method by normalizing to β-actin (*ACTB*) expression. Primer sequences are provided in Supplementary Table S2.

Statistical analysis

Data are presented as mean ± standard deviation (SD). All statistical analyses were performed using unpaired Student's *t* test by GraphPad Prism version 8.2.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was assumed at a *P* value less than 0.05 ($P < 0.05$).

Results

UNC0638-mediated induction of HbF in β^0-thalassemia/HbE erythroid cell culture

To determine the therapeutic potential of UNC0638 as a HbF inducer, we employed a 3-phase liquid culture system to promote erythroid differentiation of primary human $CD34^+$ hematopoietic stem/progenitor cells (HSPC) isolated from peripheral blood of β^0-thalassemia/HbE patients. During phase I (days 0–4) and phase II (days 4–8) of culture, $CD34^+$ cells differentiate into erythroid lineage progenitors. By day 8 of differentiation, the majority of cells are basophilic erythroblasts as evidenced by high expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a) (Supplementary Fig. S1). During phase III (days 8–14) of differentiation, cells undergo terminal erythroid maturation.

We initially determined the time-dependent effect of UNC0638 on HbF induction in β^0-thalassemia/HbE erythroid progenitor cells. UNC0638 at a concentration of 1.0 μM (previously determined [24]) was added to cells during days 4–14 or days 8–14 of culture. Because of the normally highly variable HbF baseline levels in β^0-thalassemia/HbE erythroid cells, the effects of UNC0638 on HbF induction are presented as increases in HbF percentage from the baseline level in DMSO-treated cells from the same donor (Δ%HbF). We found that addition of UNC0638 during days 4–14 induced a higher increase in HbF percentage (Δ%HbF = 36.5 ± 3.4) compared with when the addition was performed during days 8–14 (Δ%HbF = 9.3 ± 1.6) (Fig. 1a). This suggested that in order to achieve the maximal effect on HbF induction, UNC0638 should be added during early stages of erythroid differentiation. The addition of 1.0 μM UNC0638 to cells during days 4–14 did not affect cell viability (Fig. 1b); however, it was associated with a significant reduction of erythroid proliferation (Fig. 1c). We next assessed the effects of varying the concentrations of UNC0638, when added during days 4–14, on the induction of HbF in β^0-thalassemia/HbE erythroid progenitor cells. We found that UNC0638 induced HbF production in a dose-

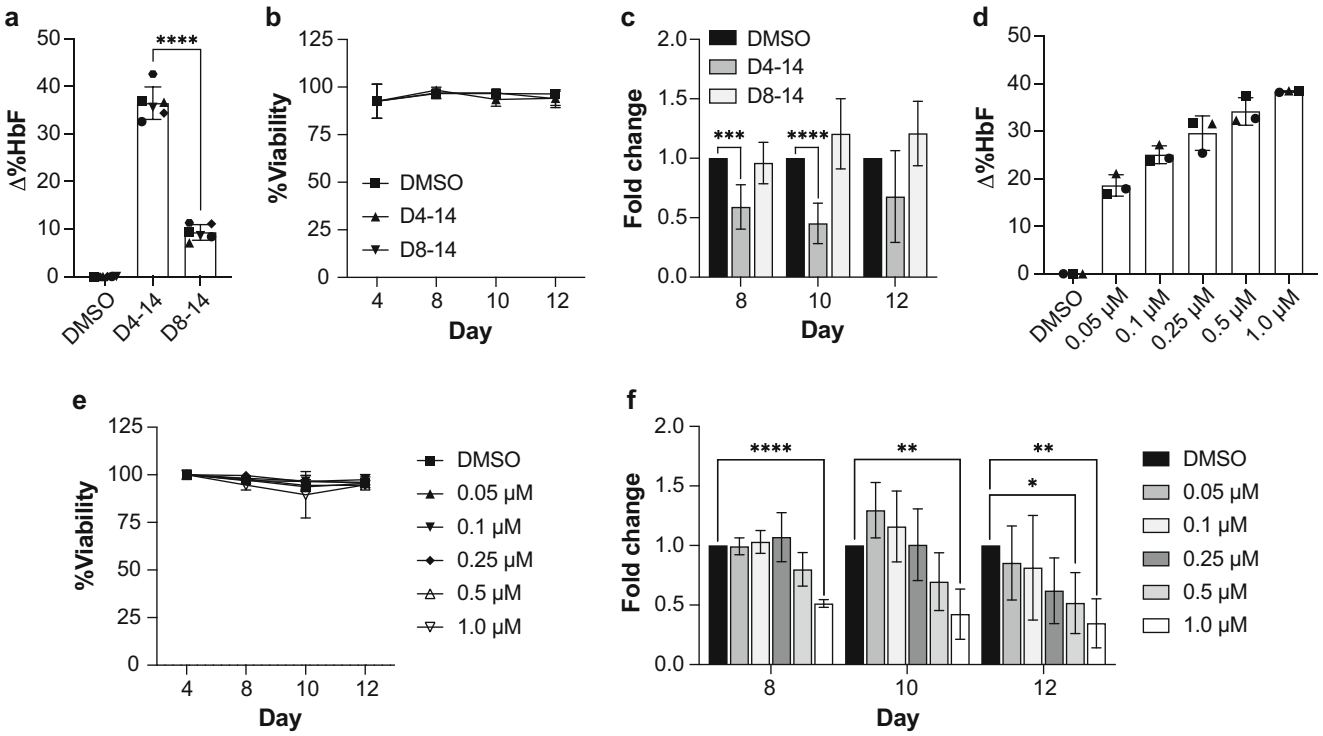

Fig. 1 UNC0638 induces HbF expression in a time- and dose-dependent manner in erythroid progenitor cells from β^0-thalassemia/HbE patients. **a**–**c** β^0-Thalassemia/HbE erythroid progenitor cells recovered from the peripheral blood of random patients ($n = 6$) were cultured in the presence of 1.0 μM UNC0638 for the indicated length of time. **a** The increase in HbF percentage analyzed by HPLC at day 14 was expressed as Δ%HbF (%HbF [compound treatment] − %HbF [DMSO control]). **b** Cell viability and **c** proliferation during erythroid differentiation. The cell proliferation was expressed as a fold change relative to DMSO control. **d**–**f** β^0-Thalassemia/HbE erythroid progenitor cells ($n = 3$) were cultured in the presence of UNC0638 at the indicated concentrations during 4–14 days of culture. **d** The increase in HbF percentage analyzed by HPLC at day 14 (Δ%HbF). **e** Cell viability and **f** proliferation during erythroid differentiation. Data are presented as mean ± standard deviation (SD). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

dependent fashion without affecting erythroid cell viability at any tested dose (Fig. 1d, e). However, a significant reduction in cell proliferation during erythroid differentiation was quite evident at 1.0 and 0.5 μM of UNC0638 (Fig. 1f). We thus chose to treat cells with 0.25 μM UNC0638 during days 4–14 of erythroid culture in the subsequent investigations.

We next determined the therapeutic potential of UNC0638 in erythroid progenitor cells derived from 6 individual compound heterozygous β^0-thalassemia/HbE patients carrying different β^0-thalassemia mutations, including 4 cases of $\beta^{codon17(A>T)}/\beta^E$ and 2 cases of $\beta^{codon41/42(-TCTT)}/\beta^E$ (Supplementary Table S1). Based on the optimal concentration and duration of treatment, we found that UNC0638 increased HbF significantly and reproducibly in β^0-thalassemia/HbE erythroid progenitor cells. Although the %HbF baseline levels in individual cases varied from 15 to 50%, all of them responded to virtually the same extent upon UNC0638 addition (Fig. 2a, b and Supplementary Table S3). The increase of HbF (Δ%HbF) achieved by UNC0638 treatments was 25.5 ± 4.2% above the DMSO control baseline levels (Fig. 2b). These results demonstrated that β^0-thalassemia/HbE erythroid progenitor cells bearing a variety of different β^0-thalassemia mutations with divergent HbF baseline levels were all susceptible to significantly elevated HbF induction upon UNC0638 treatment. Moreover, the significant increase in HbF levels analyzed by HPLC paralleled the increase in the percentage of cells expressing HbF (F cells) analyzed by flow cytometry. The average percentage of F-cells was elevated from 50.2 ± 7.3% in DMSO-treated cells to 76.0 ± 10.4% in UNC0638-treated cells (Fig. 2c). HbF expression induced by UNC0638 correlated with the increase in γ-globin mRNA analyzed by quantitative RT-PCR. The results revealed that UNC0638 significantly induced γ-globin (*HBG*) mRNA accumulation, resulting in a 2.1 ± 0.6-fold increase compared with DMSO-treated cells (Fig. 2d). The increase in γ-globin mRNA was coordinated with reduced β-globin (*HBB*) transcription without a significant change in α-globin (*HBA*) expression. Erythroid cell viability and proliferation were not significantly altered after UNC0638 treatments (Fig. 2e, f), suggesting that there are no significant cytotoxic effects of UNC0638 under these culture conditions.

The erythroid differentiation pattern and morphology were also analyzed by flow cytometry and modified Giemsa-stained cytospins, respectively, in the absence or presence of UNC0638. The results showed that erythroid

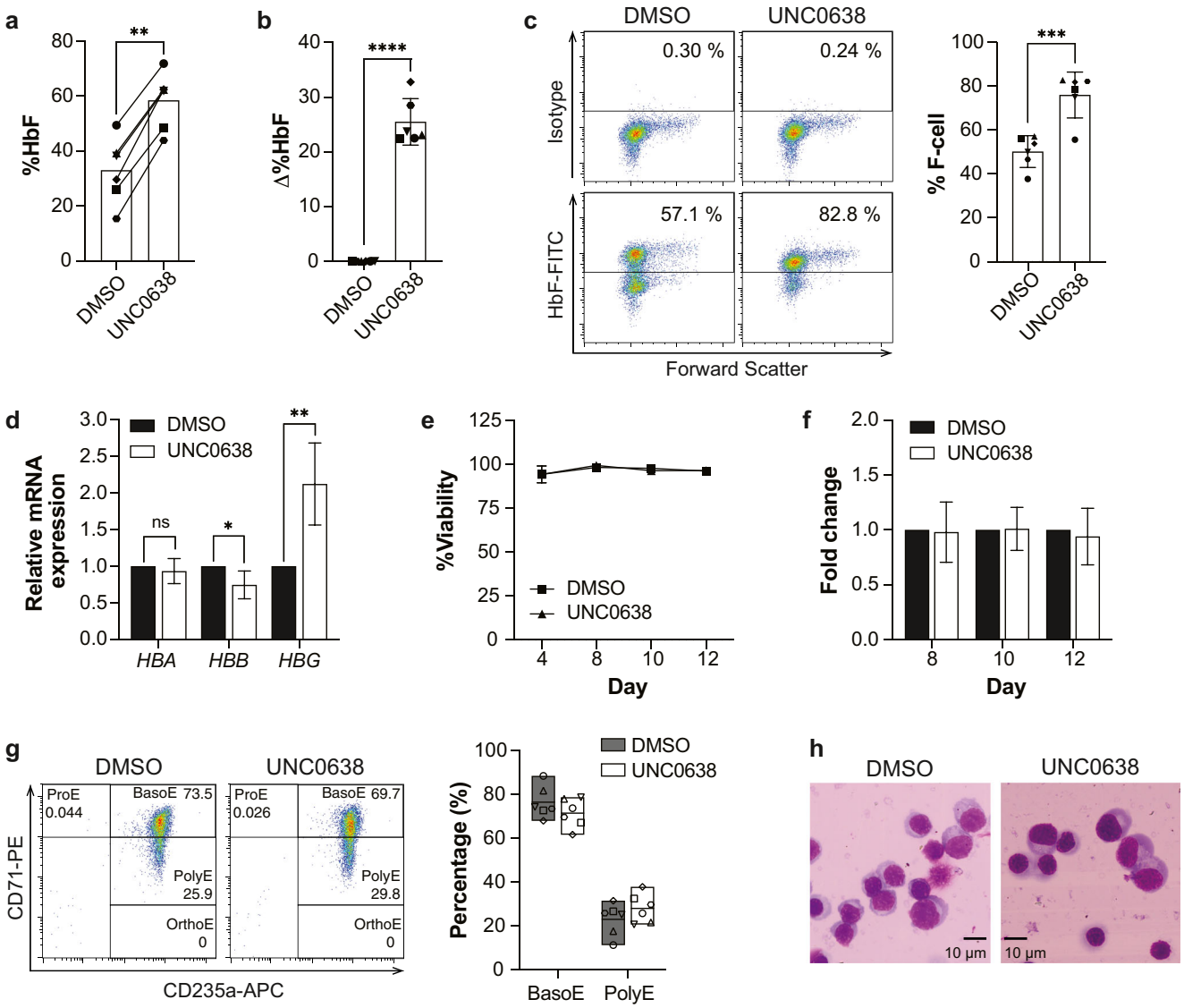

Fig. 2 UNC0638 potently increases γ-globin gene expression and HbF production in erythroid progenitor cells from β^0-thalassemia/HbE patients. β^0-Thalassemia/HbE erythroid progenitor cells were treated with 0.25 μM UNC0638 during days 4–14 of culture. **a** The percentage of HbF analyzed by HPLC at day 14 of culture. **b** The increase in HbF percentage (Δ%HbF) in UNC0638-treated cells from the baseline level in DMSO-treated cells. **c** Representative flow cytometry dot plots and quantitative analysis of the percentage of F-cells. **d** Quantitative RT-PCR showing the relative fold change of *HBA*, *HBB*, and *HBG* mRNA expression levels normalized to β-actin (*ACTB*) after 12 days of culture. **e** Cell viability and **f** proliferation during erythroid differentiation. The fold change of erythroid proliferation represents the cell number in UNC0638-treated samples versus DMSO controls. **g** Representative flow cytometry dot plots and quantitative analysis of erythroid subpopulations assessed by the expression levels of CD71 and CD235a surface markers at day 12 of culture. BasoE, basophilic erythroblasts; PolyE, polychromatophilic erythroblasts. (h) Representative images of modified Giemsa-stained cytospins at day 12 of culture. Scale bar = 10 μm. Data are presented as mean ± SD ($n = 6$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

cells in the presence or absence of UNC0638 exhibited a comparable differentiation pattern and morphology by 12 days of culture (Fig. 2g, h). However, we noted a trend toward accelerated erythroid differentiation for cells treated with UNC0638 as evidenced by the higher number of polychromatophilic erythroblasts. These results suggest that UNC0638 is a potent inducer of γ-globin mRNA expression and HbF production, in the absence of significant cytotoxicity, in β^0-thalassemia/HbE erythroid progenitor cells.

UNC0638 treatment reveals additive effects with pomalidomide and decitabine

To investigate the possibility for even more robust HbF induction for therapeutic purposes, the combined use of UNC0638 with two other potentially therapeutic HbF agents, pomalidomide and decitabine, was evaluated in β^0-thalassemia/HbE erythroid progenitor cells. Based on preliminary data, the maximum HbF-inducing activities for pomalidomide and decitabine were observed when 4.0 μM pomalidomide

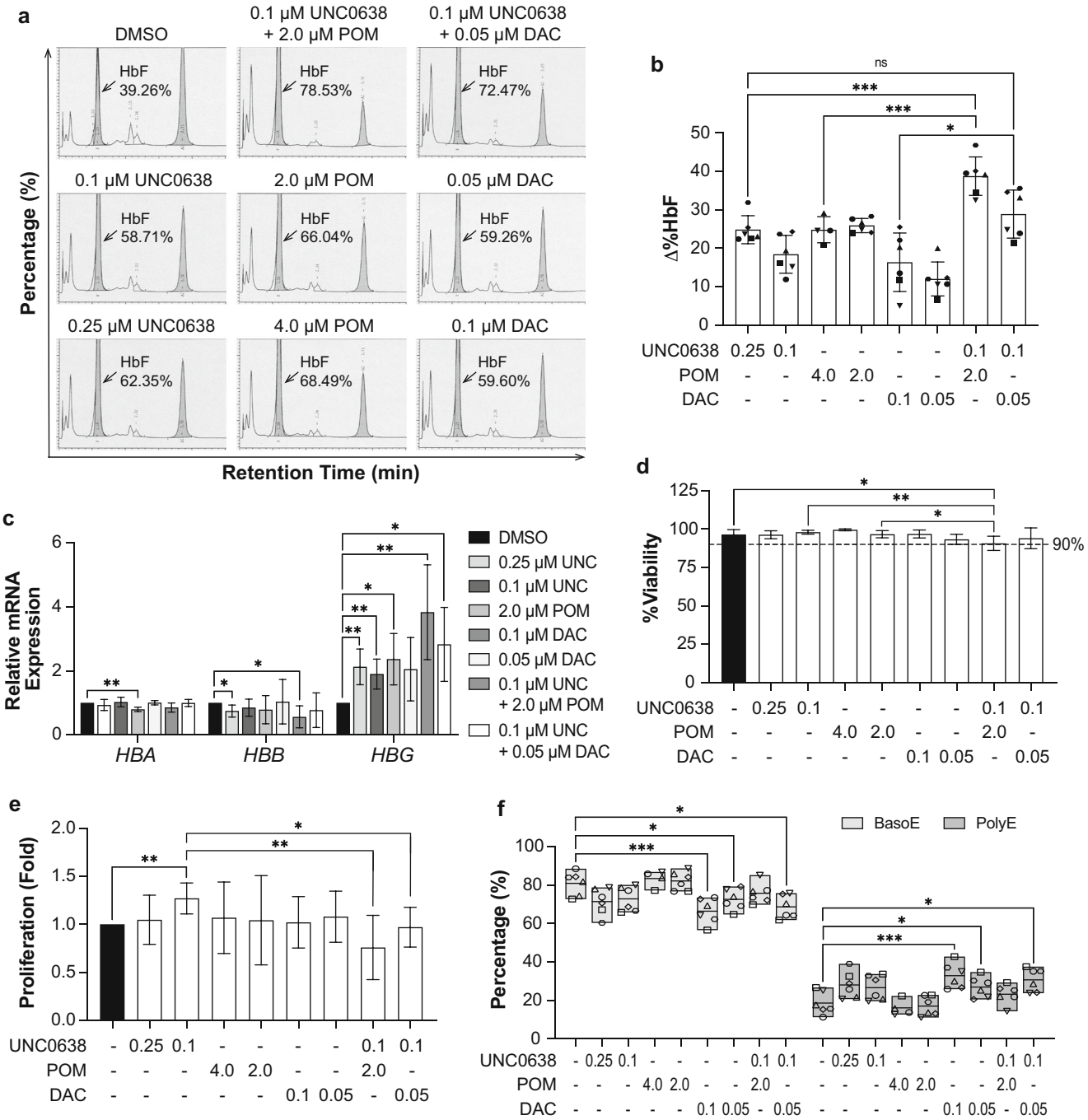

Fig. 3 UNC0638 plus pomalidomide or decitabine additively induces HbF production in erythroid progenitor cells from β^0-thalassemia/HbE patients. β^0-Thalassemia/HbE erythroid progenitor cells were treated with UNC0638 alone (UNC, from days 4–14), pomalidomide alone (POM, from days 4–14), decitabine alone (DAC, from days 8–14), UNC0638 + POM, or UNC0638 + DAC at the indicated concentrations. **a** Representative HPLC chromatograms depicting hemoglobin composition at day 14 of culture. **b** The increase in HbF percentage (Δ%HbF) in compound-treated cells from the baseline level in DMSO-treated cells. (mean ± SD, $n = 4$ for 4.0 μM POM and $n = 6$ for other treatments). **c** Quantitative RT-PCR showing relative fold change of *HBA*, *HBB*, and *HBG* mRNA expression levels normalized to β-actin (*ACTB*) at day 12 of culture. (mean ± SD, $n = 4$). **d** Cell viability and **e** proliferation of erythroid cells at day 10 of culture. The fold change of erythroid proliferation represents the cell number in compound-treated samples versus DMSO controls. **f** The histogram represents the quantitative analysis of erythroid subpopulations assessed by the expression levels of CD71 and CD235a surface markers at day 12 of culture. BasoE, basophilic erythroblasts; PolyE, polychromatophilic erythroblasts. (mean ± SD, $n = 4$ for 4.0 μM POM and $n = 6$ for other treatments). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

and 0.1 μM decitabine were added to erythroid progenitor cells during days 4–14 and 8–14 of culture, respectively. Under these conditions, 4.0 μM pomalidomide and 0.25 μM UNC0638 induced comparable levels of HbF while 0.1 μM

decitabine showed less effective HbF-inducing activity (Fig. 3a, b). We used half of these doses for all compounds in the combination treatments to minimize cytotoxicity. We found that the combination of 0.1 μM UNC0638 with either 2.0 μM pomalidomide or 0.05 μM decitabine additively increased HbF levels in all samples. In particular, the strongest response was observed when 0.1 μM UNC0638 was combined with 2.0 μM pomalidomide, resulting in a 38.8 ± 5.0% HbF increase (Fig. 3a, b and Supplementary Table S3). This increase was significantly higher than that observed with any single compound at both full and partial doses. We found that 0.1 μM UNC0638 plus 2.0 μM pomalidomide significantly induced a 3.8-fold increase in γ-globin mRNA above baseline in DMSO control with a coincident decrease in β-globin mRNA level (Fig. 3c) in the absence of α-globin mRNA change. To a lesser extent, we observed a similar result for the combination of 0.1 μM UNC0638 and 0.05 μM decitabine. The Δ%HbF increased from 18.5 ± 4.9% for 0.1 μM UNC0638 and 12.0 ± 4.4% for 0.05 μM decitabine to 28.9 ± 6.3% in combination.

Erythroid cell viability and proliferation in the combination treatment of 0.1 μM UNC0638 and 2.0 μM pomalidomide was significantly but modestly reduced when compared to that of single-molecule treatments (Fig. 3d, e), suggesting minimal cytotoxic effects of this combinatorial regimen. Additionally, the combination of 0.1 μM UNC0638 and 0.05 μM decitabine slightly decreased proliferation but not viability of erythroid cells. We next determined erythroid differentiation after 12 days of culture by flow cytometry. We found that treatment with UNC0638 or in combination with pomalidomide did not change the erythroid differentiation pattern compared with DMSO treatment (Fig. 3f). Interestingly, cells treated with decitabine alone or decitabine plus UNC0638 exhibited a significant increase in erythroid differentiation as shown by an increase in polychromatophilic erythroblasts with a diminished number of basophilic erythroblasts (Fig. 3f). This result suggested that decitabine may accelerate the differentiation of β^0-thalassemia/HbE erythroid progenitor cells.

Taken together, these results strongly suggest that UNC0638 is a potent HbF inducer without cytotoxicity under these tested conditions. UNC0638 exhibits additive effects with pomalidomide and decitabine, implying that it induces γ-globin mRNA and HbF expression through a mechanism of action that differs from either pomalidomide or decitabine. Further analyses of UNC0638 alone or in combination with pomalidomide or decitabine may lead to improved treatments for β-thalassemia.

Discussion

The induction of γ-globin and increase in HbF expression has been shown to ameliorate the pathophysiology and severity of β-thalassemia patients by reducing excess unmatched α-globin chains in red blood cells [9, 32, 33]. A current FDA-approved HbF inducer, hydroxyurea, is not effective in more than 50% of β-thalassemia patients [14], and therefore, more effective HbF inducers are sorely needed. Several epigenetic modifying enzymes involved in γ-globin repression, including DNMT1, LSD1, and EHMT1/2 histone methyltransferases, are attractive therapeutic targets for induction of HbF [19]. Inhibition of EHMT1 (GLP) and EHMT2 (G9a) histone methyltransferases by the small chemical compound UNC0638 has shown to potently induce γ-globin and HbF expression in erythroid progenitor cells from normal adult donors by decreasing the repressive histone H3K9Me2 mark at the γ-globin promoters and by facilitating loop formation between the LCR and the γ-globin promoters [24, 25, 31]. Moreover, UNC0638 treatment does not affect the expression of key erythroid transcription factors (GATA1, KLF1, and NFE2), or key γ-globin repressors (BCL11A and MYB) [25], suggesting that it does not induce HbF expression via an effect on delayed erythroid differentiation or downregulation of γ-globin repressors.

In this report, we further evaluated the effects of UNC0638 on HbF induction in β^0-thalassemia/HbE erythroid progenitor cells; as is well appreciated, these cells have very high HbF baseline levels. We found that UNC0638 robustly increased γ-globin mRNA, HbF, and F-cells in β^0-thalassemia/HbE erythroid progenitor cell cultures. The HbF induction achieved by UNC0638 treatment was 25.5 ± 4.2% above baseline levels, which is comparable to previous reports in normal erythroid progenitor cells [24, 25]. Moreover, UNC0638 exhibited HbF-inducing activity similar to pomalidomide, while exhibiting even stronger induction than decitabine under these culture conditions. Interestingly, erythroid precursor cells from different β^0-thalassemia/HbE patients with varying HbF baseline levels all exhibited a similar degree of HbF induction in response to UNC0638 treatment. These data demonstrated that UNC0638 potently induced HbF expression in β^0-thalassemia/HbE erythroid progenitor cells regardless of specific β^0-thalassemia mutations or HbF baseline levels.

In agreement with a previous study [24], the level of HbF induction was more pronounced when UNC0638 was added at an early stage (day 4) than when added in a late stage (day 8) of erythroid differentiation. Since the pattern of globin gene expression is highly regulated and still reversible during early erythroid differentiation stages, UNC0638 was shown to act more effectively during this period. Thus, the time of addition of UNC0638 in erythroid cell culture was critical for inhibiting EHMT1/2 and reactivating HbF expression.

Combinatorial therapy by multiple HbF inducers is a promising therapeutic strategy to achieve clinical improvement in patients. Given that the major limitation of combination therapy is the increase in toxicity, we therefore used half the amount determined to be the most effective concentration of each compound in combination to achieve greater HbF

induction and avoid adverse effects in comparison to single-drug treatment. Pomalidomide, an FDA-approved immunomodulatory drug for the treatment of multiple myeloma, has been shown to induce γ-globin and HbF expression, at least partially through the downregulation of BCL11A and SOX6 [26–28, 34]. Decitabine, a DNMT1 inhibitor, has been shown to induce γ-globin and HbF expression in patients with SCD [35, 36] and β-thalassemia [20]. Here, we found that the combination of UNC0638 with pomalidomide or with decitabine increased HbF expression more than any of the three individually. Although, we observed a statistically significant reduction in viability and proliferation of cells treated with UNC0638 plus pomalidomide, these reductions were modest when compared to treatment with any single agent (full or half doses) or DMSO alone. Further studies examining precise dose titration of these compounds in a combination regimen would reduce the negative effect on cell viability and proliferation while potentially maintaining the high level of HbF induction. Interestingly, the differential HbF generated by the combination of UNC0638 and pomalidomide was greater than 30% of total hemoglobin, which is the level that has been demonstrated to achieve a significant clinical improvement of patients with SCD [37–40] and β-thalassemia [14]. These results suggest that the combination of compounds that have different mechanisms of action has the potential to additively increase HbF expression.

Although UNC0638 has demonstrated a strong HbF-inducing activity in ex vivo erythroid cell culture systems, its in vivo pharmacokinetic properties are poor due to a lack of drug-like properties [41]. Further development of EHMT1/2 inhibitors with higher potency and improved in vivo pharmacokinetic properties should reveal novel HbF inducers that would be suitable for clinical applications.

In summary, the present study confirms that inhibition of EHMT1/2 histone methyltransferases by a small molecule, UNC0638, exhibits strongly elevated HbF induction in β-thalassemia/HbE erythroid progenitor cells. Furthermore, UNC0638 was shown to have additive effects on HbF induction when combined with either pomalidomide or decitabine. Therefore, searching for novel potent and selective EHMT1/2 inhibitors with improved drug-like properties may lead to clinical application in the treatment of β-thalassemia.

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Authors' contributions Contribution: T.N., P.K., and N.J. designed the research; T.N., P.K., P.P., and W.K. performed the experiments; T.N., P.K., and N.J. analyzed the data; D.S., K.P., S.H., and S.F. provided the samples and resources; T.N., J.D.E., and N.J. wrote the manuscript; O.S., J.D.E., S.H., S.F., and N.J. conceptualized the idea and supervised the project; and all the authors read and approved the final manuscript.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics approval The study was approved by Institutional Review Boards of Mahidol University and was conducted in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all participants before being included in the study.

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<u>รายงานสรุปการนำผลงานวิจัยไปใช้ประโยชน์</u>

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ชื่อโครงการ การกระตุ้นการสร้างฮีโมโกลบินเอฟโดยโพมาลิโดไมด์เพื่อการรักษาโรคเบต้าธาลัสซีเมีย

Repurposing of Pomalidomide to Induce Fetal Hemoglobin Expression for β-Thalassemia Treatment

หัวหน้าโครงการ ดร.นที เจียรวิริยะไพศาล **หน่วยงาน** ศูนย์วิจัยธาลัสซีเมีย, สถาบันชีววิทยาศาสตร์โมเลกุล, มหาวิทยาลัยมหิดล

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สถานะผลงาน □ **ปกปิด** ☑ **ไม่ปกปิด**

ความสำคัญ / ความเป็นมา

โรค β-thalassemia เป็นโรคทางพันธุกรรมที่พบบ่อยในประเทศไทย เกิดจากการกลายพันธุ์ของยีน β-globin ทำให้มีการสร้างสาย β-globin ลดลงหรือสร้างไม่ได้เลย ทำให้การสร้างฮีโมโกลบิน (HbA, $\alpha_2\beta_2$) ได้ลดลง และเกิดความไม่สมดุลของสายโกลบินซึ่งส่งผลให้กระบวนการสร้างเม็ดเลือดแดงไม่มีประสิทธิภาพ เม็ดเลือดแดงมีรูปร่างผิดปกติ ขาดความยืดหยุ่น มีอายุสั้นและแตกทำลายง่าย ผู้ป่วยธาลัสซีเมียจึงมีภาวะซีดเรื้อรังและมีอาการอื่น ๆ ตามมา การรักษาผู้ป่วยโรค β-thalassemia โดยการกระตุ้นการสร้าง HbF ($\alpha_2\gamma_2$) ที่ทดแทนการขาดหายไปของ HbA ($\alpha_2\beta_2$) เป็นวิธีการรักษาที่มีประสิทธิภาพและผู้ป่วยส่วนใหญ่สามารถเข้าถึงการรักษาได้ซึ่งแตกต่างจากการรักษาแบบหายขาดด้วยการปลูกถ่ายเซลล์เม็ดเลือดต้นกำเนิด (Hematopoietic stem cell transplantation) ที่มีราคาค่อนข้างสูงและมากกว่า 95% ของผู้ป่วยไม่สามารถเข้าถึงการรักษาได้ โดยปัจจุบัน hydroxyurea เป็นยาเพียงชนิดเดียวที่ได้รับการรับรองจาก U.S. Food and Drug Administration (FDA) เพื่อใช้ในการกระตุ้นการสร้าง HbF เพื่อการรักษาโรค sickle cell disease และมีการใช้เป็นทางเลือกสำหรับการรักษาโรค β-thalassemia แต่พบว่ามากกว่า 50% ของผู้ป่วย sickle cell disease และ β-thalassemia ไม่ตอบสนองต่อการรักษาด้วย hydroxyurea และยังมี side effect ได้แก่ myelosuppression ดังนั้นจึงมีความจำเป็นที่จะต้องมีการศึกษาค้นหายากระตุ้นการสร้าง HbF ชนิดใหม่ที่มีประสิทธิภาพสูงขึ้นและมีความเป็นพิษลดลง

จากการรายงานในวารสารทางวิชาการพบว่า Pomalidomide ซึ่งเป็นยาในกลุ่ม immunomodulatory drug (IMiD) ที่ได้รับการรับรองจาก FDA สำหรับการรักษาโรค multiple myeloma มีความสามารถในการกระตุ้นการสร้าง HbF ได้ในปริมาณที่สูงในเซลล์เม็ดเลือดแดงตัวอ่อนที่แยกมาจากคนปกติ คนที่เป็นโรค sickle cell disease โดยยังไม่ทราบกลไกที่แน่ชัด แต่อาจจะกระตุ้นการสร้าง HbF ผ่านการกดการแสดงออกของโปรตีนที่ยับยั้งการสร้าง γ-globin ดังนั้นการศึกษานี้จึงได้นำยา pomalidomide ซึ่งเป็นยารักษาโรค multiple myeloma มาศึกษาประสิทธิภาพในการกระตุ้นการสร้าง HbF เพื่อใช้ในการรักษาโรค β-thalassemia

วัตถุประสงค์ของโครงการ

เพื่อศึกษาประสิทธิภาพของ pomalidomide ในการกระตุ้นการสร้าง HbF ในเม็ดเลือดแดงตัวอ่อนที่แยกมาจากคนไข้ β^0-thalassemia/HbE เพื่อนำไปเป็นต้นแบบหรือพัฒนาต่อเป็นยาที่สามารถใช้ในการรักษาโรค β-thalassemia

ผลการวิจัย

1. Pomalidomide สามารถกระตุ้นการสร้าง HbF ได้สูงขึ้นอย่างมีนัยสำคัญในเม็ดเลือดแดงตัวอ่อนจากคนไข้โรค β^0-thalassemia/HbE โดยไม่มีความเป็นพิษต่อเซลล์ และมีประสิทธิภาพดีกว่า hydroxyurea, decitabine และ RN-1 ซึ่งเป็นสารที่เคยมีรายงานมาว่าสามารถกระตุ้นการสร้าง HbF ได้
2. การใช้ pomalidomide ร่วมกับ decitabine สามารถกระตุ้นการสร้าง HbF ได้สูงสุด และมากกว่าเมื่อใช้ร่วมกับ hydroxyurea หรือ RN-1
3. การกระตุ้นการสร้าง HbF โดย pomalidomide มีผลบางส่วนมาจากการกดการแสดงออกของโปรตีนที่ยับยั้งการแสดงออกของ γ-globin
4. การศึกษานี้แสดงให้เห็นว่ามีความเป็นไปได้ที่จะนำ pomalidomide มาใช้ในการกระตุ้นการสร้าง HbF เพื่อใช้ในการรักษาโรค β-thalassemia โดยอาจจะใช้เดี่ยวหรือร่วมกับ decitabine

คำสืบค้น (Keywords)

Fetal hemoglobin induction, β-thalassemia, pomalidomide, drug repurposing

การกระตุ้นการสร้างฮีโมโกลบินเอฟ โรคเบต้าธาลัสซีเมีย โพมาลิโดไมด์ การนำยาที่มีการใช้ในการรักษาโรคมาใช้รักษาโรคอื่น

การนำผลงานวิจัยไปใช้ประโยชน์ *(ดูคำจำกัดความ และตัวอย่างด้านหลังแบบฟอร์ม)*

☐ **ด้านนโยบาย** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)*.....

มีการนำไปใช้อย่างไร

.....

☐ **ด้านสาธารณะ** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* .....

มีการนำไปใช้อย่างไร

.....

☐ **ด้านชุมชนและพื้นที่** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* .....

มีการนำไปใช้อย่างไร

.....

☐ **ด้านพาณิชย์** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* .....

มีการนำไปใช้อย่างไร

.....

☑ **ด้านวิชาการ** **โดยใคร** *(กรุณาให้ข้อมูลเจาะจง)* นักวิจัย

มีการนำไปใช้อย่างไร *(กรุณาให้ข้อมูลเจาะจง)*

มีการนำข้อมูลที่ได้จากงานวิจัยนี้มาใช้ในการวางแผนการทดสอบประสิทธิภาพของ pomalidomide ในหนูทดลองที่เป็นโรค β-thalassemia เพื่อนำไปสู่การพัฒนาและการศึกษาผลของยา pomalidomide ในทางคลินิก (clinical trials) สำหรับการรักษาโรค β-thalassemia ในคนไข้ต่อไป

☐ **ยังไม่มีการนำไปใช้** (โปรดกรอกในกรอบถัดไป)

การเผยแพร่/ประชาสัมพันธ์ *(กรุณาให้รายละเอียด พร้อมแนบหลักฐาน)*

1. สิ่งพิมพ์ หรือสื่อทั่วไป

   ☐ **หนังสือพิมพ์** ☐ **วารสาร** ☐ **โทรทัศน์** ☐ **วิทยุ** ☐ **เว็บไซต์** ☐ **คู่มือ/แผ่นพับ** ☐ **จัดประชุม/อบรม** ☐ **อื่น ๆ**

   .....

2. สิ่งพิมพ์ทางวิชาการ *(วารสาร, การประชุม ให้ระบุรายละเอียดแบบการเขียนเอกสารอ้างอิง เพื่อการค้นหาซึ่งควรประกอบด้วย ชื่อผู้แต่ง ชื่อเรื่อง แหล่งพิมพ์ ปี พ.ศ. (ค.ศ.) ฉบับที่ หน้า)*

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ORIGINAL ARTICLE

UNC0638 induces high levels of fetal hemoglobin expression in β-thalassemia/HbE erythroid progenitor cells

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Abstract

Increased expression of fetal hemoglobin (HbF) improves the clinical severity of β-thalassemia patients. EHMT1/2 histone methyltransferases are epigenetic modifying enzymes that are responsible for catalyzing addition of the repressive histone mark H3K9me2 at silenced genes, including the γ-globin genes. UNC0638, a chemical inhibitor of EHMT1/2, has been shown to induce HbF expression in human erythroid progenitor cell cultures. Here, we report the HbF-inducing activity of UNC0638 in erythroid progenitor cells from β-thalassemia/HbE patients. UNC0638 treatment led to significant increases in γ-globin mRNA, HbF expression, and HbF-containing cells in the absence of significant cytotoxicity. Moreover, UNC0638 showed additive effects on HbF induction in combination with the immunomodulatory drug pomalidomide and the DNMT1 inhibitor decitabine. These studies provide a scientific proof of concept that a small molecule targeting EHMT1/2 epigenetic enzymes, used alone or in combination with pomalidomide or decitabine, is a potential therapeutic approach for HbF induction. Further development of structural analogs of UNC0638 with similar biological effects but improved pharmacokinetic properties may lead to promising therapies and possible clinical application for the treatment of β-thalassemia.

Keywords Fetal hemoglobin induction · β-Thalassemia/HbE · UNC0638 · Pomalidomide · Decitabine

Introduction

β-Thalassemia is one of the most common genetic blood disorders and is designated as a global health burden by the World Health Organization [1]. It is caused by multiple mutations in the β-globin locus, resulting in the complete absence or a reduction in the expression of the β-globin gene and reduced adult hemoglobin (HbA; $\alpha_2\beta_2$) production in erythroid cells. This results in excess unmatched α-globin chains that precipitate and damage erythroid cell membranes, causing ineffective erythropoiesis, hemolysis, anemia, and extramedullary erythropoiesis [2, 3]. Coinheritance of the β-thalassemia allele and the structural variant hemoglobin E (HbE, *HBB*:c.79G > A), resulting in β-thalassemia/HbE disease, is one of the most common severe β-thalassemias worldwide [4, 5]. Current treatments for β-thalassemia are primarily based on supportive therapies including regular, lifelong blood transfusions combined with iron chelators. Hematopoietic stem cell transplantation remains the only curative treatment; however, it is accessible to only a small fraction of patients. More recently, gene therapy for β-thalassemia

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appears to be more promising [6], but it is unlikely to be widely applied, again due to clinical accessibility.

Patients with β-thalassemia/HbE show remarkable variability in clinical severity, ranging from nearly asymptomatic to severe, transfusion-dependent thalassemia [7–9]. It is known that increased levels of HbF expression is an important modifying factor that can ameliorate the clinical severity of β-thalassemia/HbE patients due to improved α/β-globin chain imbalance [7, 9].

Hydroxyurea was the first US Food and Drug Administration (FDA)-approved HbF inducer for sickle cell disease (SCD) and/or β-thalassemia [10]. However, the response to hydroxyurea treatments is highly variable in SCD patients [10] and limited in β-thalassemia patients [11–14]. Moreover, hydroxyurea poses undesirable side effects including myelosuppression and possible long-term carcinogenesis [15]. L-glutamine has more recently been approved by the FDA for treatment of SCD by reducing oxidative stress [16], exhibiting quite modest clinical benefit. More effective and less toxic HbF-inducing agents are thus warranted.

γ-Globin gene repression requires several epigenetic modifying enzymes, including DNA methyl transferase 1 (DNMT1), histone deacetylases (HDAC), lysine-specific demethylase 1 (LSD1), euchromatin histone lysine methyltransferases 1/2 (EHMT1/2), and protein arginine *N*-methyltransferase 5 (PRMT5), which have each been investigated as potential therapeutic targets for HbF induction [17–19]. A number of pharmacological agents such as the DNMT1 inhibitor decitabine [20], HDAC inhibitors (e.g., HQK-1001 [21]), LSD1 inhibitors (e.g., tranylcypromine [22] and RN-1 [23]), and EHMT1/2 inhibitors (e.g., UNC0638 [24, 25]) have been shown to induce HbF expression. Moreover, the immunomodulatory drug pomalidomide has been reported to be a potent HbF inducer partly by downregulation of the key γ-globin repressors, BCL11A and SOX6 [26–28].

It has been demonstrated that γ-globin gene repression is associated with accumulation of the repressive chromatin mark histone H3 dimethyl-lysine 9 (H3K9me2) at the γ-globin loci [29]. Addition of H3K9me2 is mediated by the EHMT1 (GLP) and EHMT2 (G9a) histone methyltransferases [30]. Selective inhibition of EHMT1/2 by the small chemical molecule UNC0638 has been shown to induce γ-globin mRNA and HbF expression in erythroid progenitor cells from normal individuals [24, 25, 31]. The stimulation of HbF expression by UNC0638 treatment was associated with diminished accumulation of H3K9me2 near the γ-globin loci and with increased loop formation between the locus control region (LCR) and the γ-globin promoters through recruitment of the LDB1 complex to the γ-globin promoters [24, 25].

In this study, the HbF-inducing activity of UNC0638, either alone or in combination with other pharmacological HbF inducers, was investigated in erythroid progenitor cells isolated from β^0-thalassemia/HbE patients. These data confirm earlier studies examining *HBG* induction in tissue culture cells and demonstrate that UNC0638, alone or in combination with pomalidomide or decitabine, potently induces elevated HbF expression, suggesting that inhibition of EHMT1/2 holds therapeutic potential for β-thalassemia treatment.

Materials and methods

Ex vivo differentiation of human CD34$^+$ cells

Studies of human erythroid progenitor cell culture were approved by Institutional Review Boards of Mahidol University; written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. CD34$^+$ hematopoietic stem/progenitor cells (HSPC) were isolated from the peripheral blood of β^0-thalassemia/HbE patients. Hematological parameters and the percentages of HbF in the peripheral blood of these patients are shown in Supplementary Table S1. Briefly, peripheral blood mononuclear cells were isolated using Lymphoprep (Axis-Shield, Oslo, Norway), and CD34$^+$ cells were purified using the CD34 microbead kit with cell separation columns according to the manufacturer's instructions (Miltenyi Biotec, Gladbach, Germany). CD34$^+$ cells were differentiated toward the erythroid lineage using a 3-phase liquid culture system employing three different erythroid differentiation culture media. The composition of the basal medium is Iscove's modified Dulbecco medium (Biochrom GmbH, Berlin, Germany) supplemented with 20% fetal bovine serum (Merck, Temecula, CA, USA), 300 μg/mL holo-transferrin (ProSpec, Rehovot, Israel), and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). CD34$^+$ cells were cultured in the basal medium supplemented with either 10 ng/mL human interleukin-3 (Miltenyi Biotec), 50 ng/mL human stem cell factor (SCF; Miltenyi Biotec), and 2 U/mL erythropoietin (EPO; Janssen-Cilag, Bangkok, Thailand) during phase I (days 0–4) or 10 ng/mL SCF and 2 U/mL EPO during phase II (days 4–8) or 4 U/mL EPO during phase III (days 8–14). Cell concentration was maintained at 1–2 × 10^6 cells/mL during phase III of culture. Cells were incubated at 37 °C, 5% CO_2 in a 100% humidified atmosphere.

Chemical treatment of primary human erythroid progenitor cells

All compounds were purchased from Sigma-Aldrich. UNC0638 (U4885), pomalidomide (P0018), and decitabine (A3656) were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich). Compounds were freshly diluted and added to cells at the designated concentrations and durations. Culture medium containing 0.1% v/v of DMSO served as a concentration-matched vehicle control.

Hemoglobin analysis by HPLC

The proportion of HbF (%HbF) was determined by the Bio-Rad Variant II Hemoglobin Testing System (Bio-Rad) with β-Thalassemia Short Program. At least one million differentiating erythroid cells were subjected to HPLC analysis. The Lyphochek Hemoglobin A2 control (Bio-Rad) was used for normalization. The percentage of HbF was reported relative to total Hb (HbF + HbE) in β^0-thalassemia/HbE erythroid cells. The increase in HbF percentage after treatment from the baseline level (DMSO control) was expressed as Δ%HbF (%HbF [compound treatment] − %HbF [DMSO control]).

Cell proliferation, viability, and morphology

Cell number and viability of erythroid cells were analyzed by trypan blue staining and counted with a hemocytometer. Erythroid cell morphology was examined by modified Giemsa staining (Sigma-Aldrich) of cytospins.

Flow cytometry analysis

To assess erythroid differentiation, erythroid cells at day 12 of culture were stained with antibodies against erythroid surface markers, including a phycoerythrin (PE)-conjugated anti-human CD71 (clone CY1G4; Biolegend, San Diego, CA, USA) and an allophycocyanin (APC)-conjugated anti-human CD235a (clone GA-R2; BD Biosciences, San Jose, CA, USA) and analyzed on BD Accuri C6 Plus cytometer (BD Biosciences). To assess HbF-containing cells (F-cells), erythroid cells at day 14 of culture were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were then stained with either a fluorescein isothiocyanate (FITC)-conjugated anti-human fetal hemoglobin antibody (clone 2D12; BD Biosciences) or a FITC-conjugated mouse IgG1, κ isotype control (clone MOPC-21, BD Biosciences). The stained cells were analyzed on the Accuri C6 Plus cytometer. Data analysis was performed using FlowJo version 10.3.0 (FlowJo LLC, Ashland, OR, USA) software.

RNA isolation and gene expression analysis

Total RNA was isolated from erythroid cells at day 12 of culture using TRIzol Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were treated with DNase I (ThermoFisher Scientific, Waltham, MA, USA) and subsequently subjected to cDNA synthesis using RevertAid First-Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's instruction. Quantitative real-time PCR was carried out in CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) using FastStart Essential DNA Green Master (Roche, Mannheim, Germany) according to the manufacturer's instruction. Relative expression was calculated using the ΔΔCT method by normalizing to β-actin (*ACTB*) expression. Primer sequences are provided in Supplementary Table S2.

Statistical analysis

Data are presented as mean ± standard deviation (SD). All statistical analyses were performed using unpaired Student's *t* test by GraphPad Prism version 8.2.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was assumed at a *P* value less than 0.05 ($P < 0.05$).

Results

UNC0638-mediated induction of HbF in β^0-thalassemia/HbE erythroid cell culture

To determine the therapeutic potential of UNC0638 as a HbF inducer, we employed a 3-phase liquid culture system to promote erythroid differentiation of primary human $CD34^+$ hematopoietic stem/progenitor cells (HSPC) isolated from peripheral blood of β^0-thalassemia/HbE patients. During phase I (days 0–4) and phase II (days 4–8) of culture, $CD34^+$ cells differentiate into erythroid lineage progenitors. By day 8 of differentiation, the majority of cells are basophilic erythroblasts as evidenced by high expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a) (Supplementary Fig. S1). During phase III (days 8–14) of differentiation, cells undergo terminal erythroid maturation.

We initially determined the time-dependent effect of UNC0638 on HbF induction in β^0-thalassemia/HbE erythroid progenitor cells. UNC0638 at a concentration of 1.0 μM (previously determined [24]) was added to cells during days 4–14 or days 8–14 of culture. Because of the normally highly variable HbF baseline levels in β^0-thalassemia/HbE erythroid cells, the effects of UNC0638 on HbF induction are presented as increases in HbF percentage from the baseline level in DMSO-treated cells from the same donor (Δ%HbF). We found that addition of UNC0638 during days 4–14 induced a higher increase in HbF percentage (Δ%HbF = 36.5 ± 3.4) compared with when the addition was performed during days 8–14 (Δ%HbF = 9.3 ± 1.6) (Fig. 1a). This suggested that in order to achieve the maximal effect on HbF induction, UNC0638 should be added during early stages of erythroid differentiation. The addition of 1.0 μM UNC0638 to cells during days 4–14 did not affect cell viability (Fig. 1b); however, it was associated with a significant reduction of erythroid proliferation (Fig. 1c). We next assessed the effects of varying the concentrations of UNC0638, when added during days 4–14, on the induction of HbF in β^0-thalassemia/HbE erythroid progenitor cells. We found that UNC0638 induced HbF production in a dose-

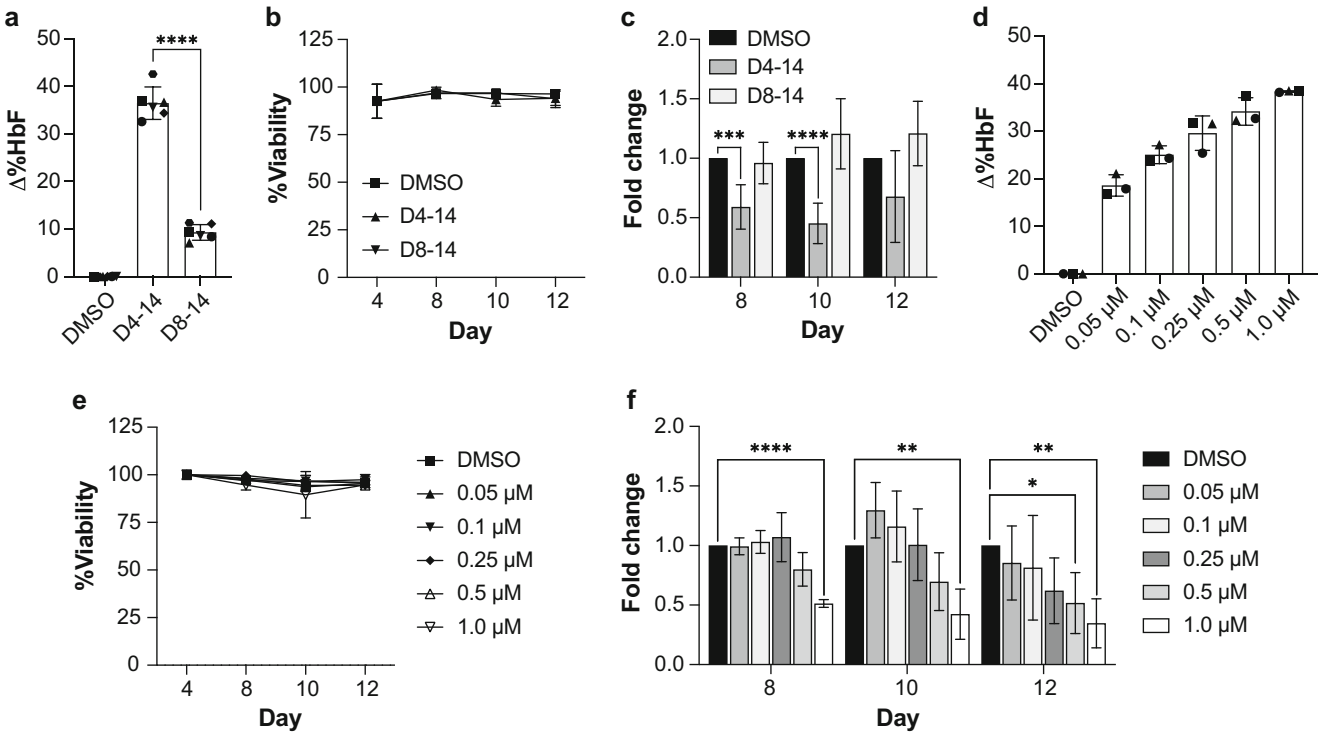

Fig. 1 UNC0638 induces HbF expression in a time- and dose-dependent manner in erythroid progenitor cells from β^0-thalassemia/HbE patients. **a**–**c** β^0-Thalassemia/HbE erythroid progenitor cells recovered from the peripheral blood of random patients ($n = 6$) were cultured in the presence of 1.0 μM UNC0638 for the indicated length of time. **a** The increase in HbF percentage analyzed by HPLC at day 14 was expressed as Δ%HbF (%HbF [compound treatment] − %HbF [DMSO control]). **b** Cell viability and **c** proliferation during erythroid differentiation. The cell proliferation was expressed as a fold change relative to DMSO control. **d**–**f** β^0-Thalassemia/HbE erythroid progenitor cells ($n = 3$) were cultured in the presence of UNC0638 at the indicated concentrations during 4–14 days of culture. **d** The increase in HbF percentage analyzed by HPLC at day 14 (Δ%HbF). **e** Cell viability and **f** proliferation during erythroid differentiation. Data are presented as mean ± standard deviation (SD). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

dependent fashion without affecting erythroid cell viability at any tested dose (Fig. 1d, e). However, a significant reduction in cell proliferation during erythroid differentiation was quite evident at 1.0 and 0.5 μM of UNC0638 (Fig. 1f). We thus chose to treat cells with 0.25 μM UNC0638 during days 4–14 of erythroid culture in the subsequent investigations.

We next determined the therapeutic potential of UNC0638 in erythroid progenitor cells derived from 6 individual compound heterozygous β^0-thalassemia/HbE patients carrying different β^0-thalassemia mutations, including 4 cases of $\beta^{codon17(A>T)}/\beta^E$ and 2 cases of $\beta^{codon41/42(-TCTT)}/\beta^E$ (Supplementary Table S1). Based on the optimal concentration and duration of treatment, we found that UNC0638 increased HbF significantly and reproducibly in β^0-thalassemia/HbE erythroid progenitor cells. Although the %HbF baseline levels in individual cases varied from 15 to 50%, all of them responded to virtually the same extent upon UNC0638 addition (Fig. 2a, b and Supplementary Table S3). The increase of HbF (Δ%HbF) achieved by UNC0638 treatments was 25.5 ± 4.2% above the DMSO control baseline levels (Fig. 2b). These results demonstrated that β^0-thalassemia/HbE erythroid progenitor cells bearing a variety of different β^0-thalassemia mutations with divergent HbF baseline levels were all susceptible to significantly elevated HbF induction upon UNC0638 treatment. Moreover, the significant increase in HbF levels analyzed by HPLC paralleled the increase in the percentage of cells expressing HbF (F cells) analyzed by flow cytometry. The average percentage of F-cells was elevated from 50.2 ± 7.3% in DMSO-treated cells to 76.0 ± 10.4% in UNC0638-treated cells (Fig. 2c). HbF expression induced by UNC0638 correlated with the increase in γ-globin mRNA analyzed by quantitative RT-PCR. The results revealed that UNC0638 significantly induced γ-globin (*HBG*) mRNA accumulation, resulting in a 2.1 ± 0.6-fold increase compared with DMSO-treated cells (Fig. 2d). The increase in γ-globin mRNA was coordinated with reduced β-globin (*HBB*) transcription without a significant change in α-globin (*HBA*) expression. Erythroid cell viability and proliferation were not significantly altered after UNC0638 treatments (Fig. 2e, f), suggesting that there are no significant cytotoxic effects of UNC0638 under these culture conditions.

The erythroid differentiation pattern and morphology were also analyzed by flow cytometry and modified Giemsa-stained cytospins, respectively, in the absence or presence of UNC0638. The results showed that erythroid

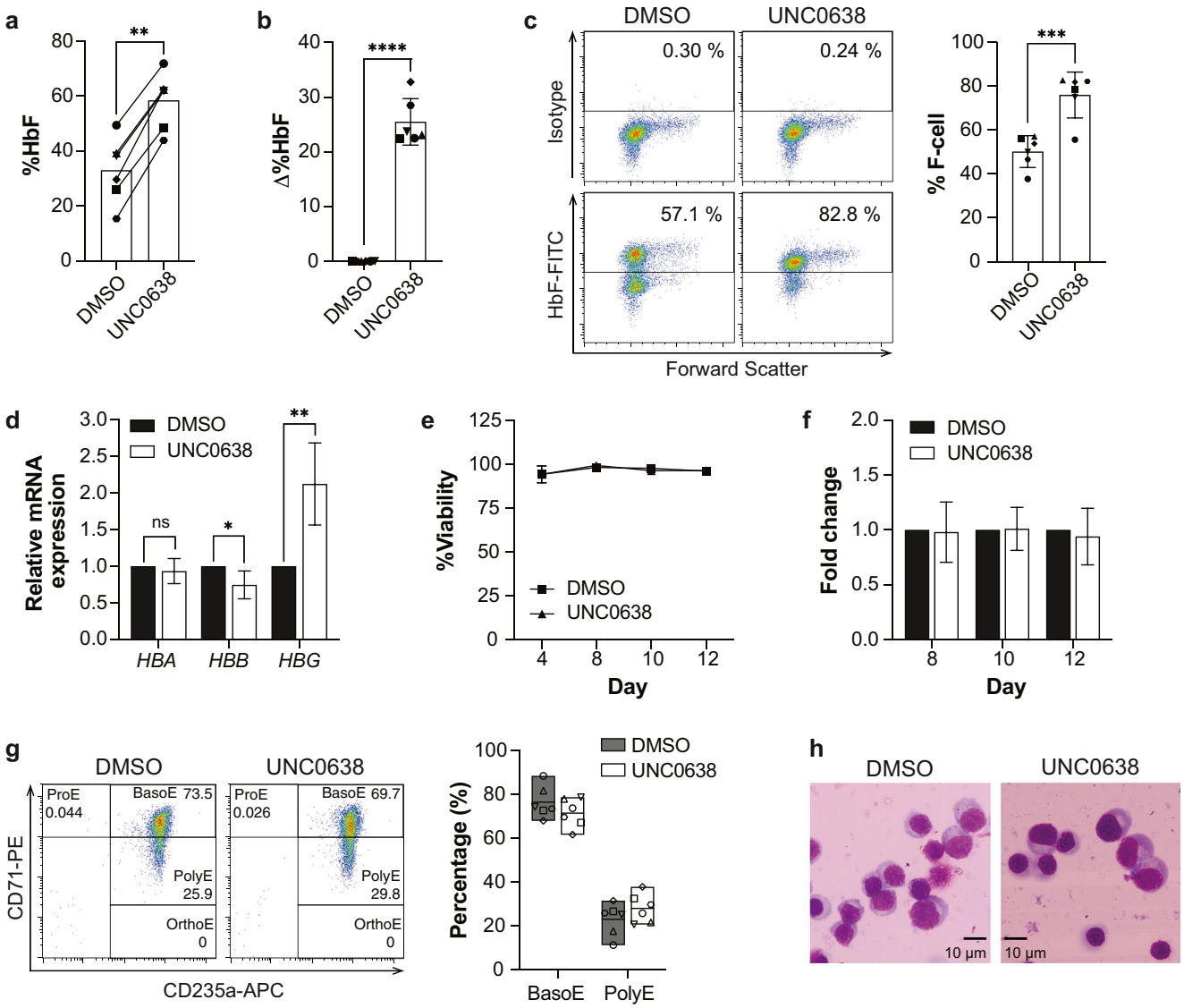

Fig. 2 UNC0638 potently increases γ-globin gene expression and HbF production in erythroid progenitor cells from β^0-thalassemia/HbE patients. β^0-Thalassemia/HbE erythroid progenitor cells were treated with 0.25 μM UNC0638 during days 4–14 of culture. **a** The percentage of HbF analyzed by HPLC at day 14 of culture. **b** The increase in HbF percentage (Δ%HbF) in UNC0638-treated cells from the baseline level in DMSO-treated cells. **c** Representative flow cytometry dot plots and quantitative analysis of the percentage of F-cells. **d** Quantitative RT-PCR showing the relative fold change of *HBA*, *HBB*, and *HBG* mRNA expression levels normalized to β-actin (*ACTB*) after 12 days of culture. **e** Cell viability and **f** proliferation during erythroid differentiation. The fold change of erythroid proliferation represents the cell number in UNC0638-treated samples versus DMSO controls. **g** Representative flow cytometry dot plots and quantitative analysis of erythroid subpopulations assessed by the expression levels of CD71 and CD235a surface markers at day 12 of culture. BasoE, basophilic erythroblasts; PolyE, polychromatophilic erythroblasts. (h) Representative images of modified Giemsa-stained cytospins at day 12 of culture. Scale bar = 10 μm. Data are presented as mean ± SD ($n = 6$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$

cells in the presence or absence of UNC0638 exhibited a comparable differentiation pattern and morphology by 12 days of culture (Fig. 2g, h). However, we noted a trend toward accelerated erythroid differentiation for cells treated with UNC0638 as evidenced by the higher number of polychromatophilic erythroblasts. These results suggest that UNC0638 is a potent inducer of γ-globin mRNA expression and HbF production, in the absence of significant cytotoxicity, in β^0-thalassemia/HbE erythroid progenitor cells.

UNC0638 treatment reveals additive effects with pomalidomide and decitabine

To investigate the possibility for even more robust HbF induction for therapeutic purposes, the combined use of UNC0638 with two other potentially therapeutic HbF agents, pomalidomide and decitabine, was evaluated in β^0-thalassemia/HbE erythroid progenitor cells. Based on preliminary data, the maximum HbF-inducing activities for pomalidomide and decitabine were observed when 4.0 μM pomalidomide

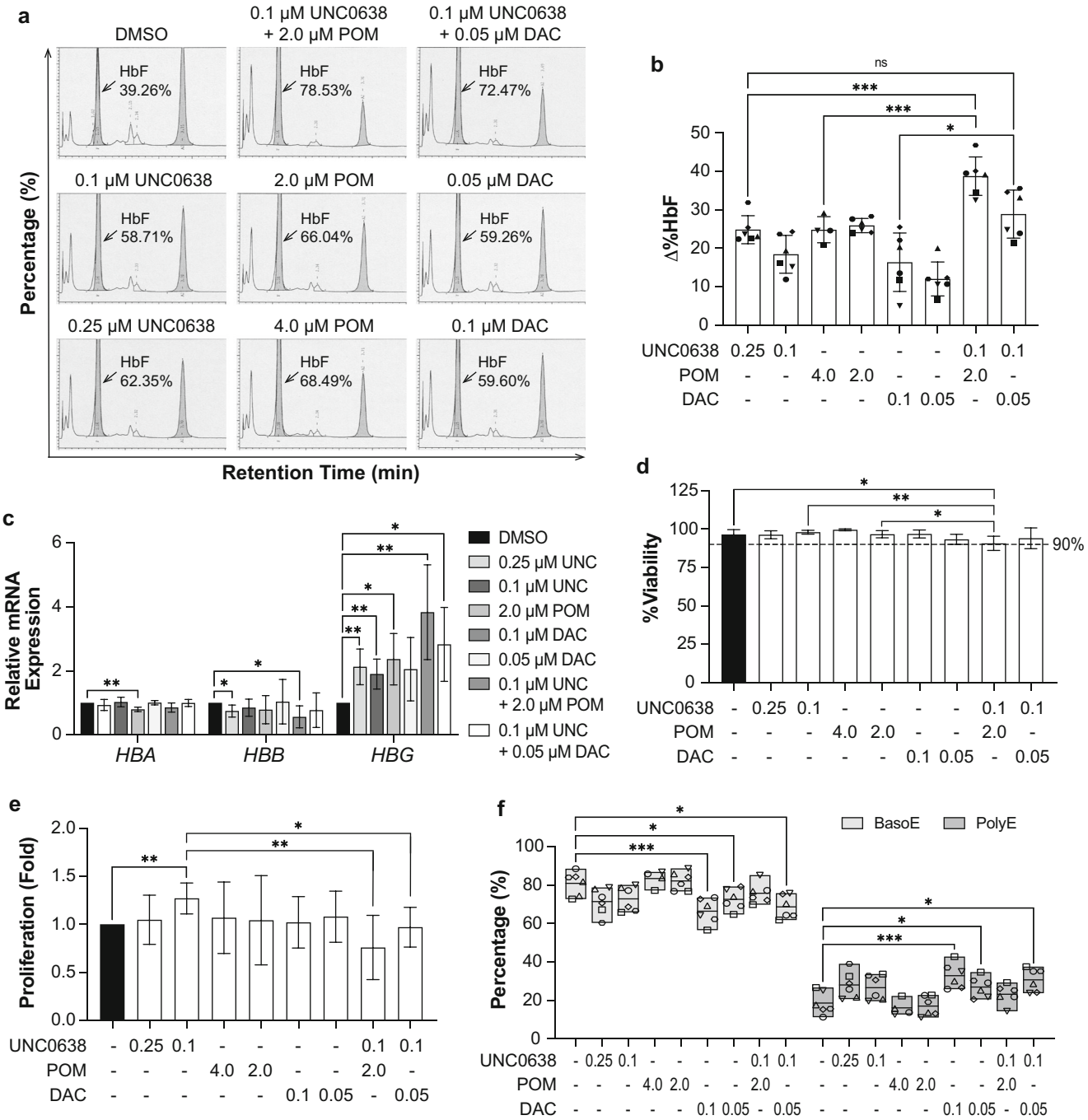

Fig. 3 UNC0638 plus pomalidomide or decitabine additively induces HbF production in erythroid progenitor cells from β^0-thalassemia/HbE patients. β^0-Thalassemia/HbE erythroid progenitor cells were treated with UNC0638 alone (UNC, from days 4–14), pomalidomide alone (POM, from days 4–14), decitabine alone (DAC, from days 8–14), UNC0638 + POM, or UNC0638 + DAC at the indicated concentrations. **a** Representative HPLC chromatograms depicting hemoglobin composition at day 14 of culture. **b** The increase in HbF percentage (Δ%HbF) in compound-treated cells from the baseline level in DMSO-treated cells. (mean ± SD, $n = 4$ for 4.0 μM POM and $n = 6$ for other treatments). **c** Quantitative RT-PCR showing relative fold change of *HBA*, *HBB*, and *HBG* mRNA expression levels normalized to β-actin (*ACTB*) at day 12 of culture. (mean ± SD, $n = 4$). **d** Cell viability and **e** proliferation of erythroid cells at day 10 of culture. The fold change of erythroid proliferation represents the cell number in compound-treated samples versus DMSO controls. **f** The histogram represents the quantitative analysis of erythroid subpopulations assessed by the expression levels of CD71 and CD235a surface markers at day 12 of culture. BasoE, basophilic erythroblasts; PolyE, polychromatophilic erythroblasts. (mean ± SD, $n = 4$ for 4.0 μM POM and $n = 6$ for other treatments). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

and 0.1 μM decitabine were added to erythroid progenitor cells during days 4–14 and 8–14 of culture, respectively. Under these conditions, 4.0 μM pomalidomide and 0.25 μM UNC0638 induced comparable levels of HbF while 0.1 μM

decitabine showed less effective HbF-inducing activity (Fig. 3a, b). We used half of these doses for all compounds in the combination treatments to minimize cytotoxicity. We found that the combination of 0.1 μM UNC0638 with either 2.0 μM pomalidomide or 0.05 μM decitabine additively increased HbF levels in all samples. In particular, the strongest response was observed when 0.1 μM UNC0638 was combined with 2.0 μM pomalidomide, resulting in a 38.8 ± 5.0% HbF increase (Fig. 3a, b and Supplementary Table S3). This increase was significantly higher than that observed with any single compound at both full and partial doses. We found that 0.1 μM UNC0638 plus 2.0 μM pomalidomide significantly induced a 3.8-fold increase in γ-globin mRNA above baseline in DMSO control with a coincident decrease in β-globin mRNA level (Fig. 3c) in the absence of α-globin mRNA change. To a lesser extent, we observed a similar result for the combination of 0.1 μM UNC0638 and 0.05 μM decitabine. The Δ%HbF increased from 18.5 ± 4.9% for 0.1 μM UNC0638 and 12.0 ± 4.4% for 0.05 μM decitabine to 28.9 ± 6.3% in combination.

Erythroid cell viability and proliferation in the combination treatment of 0.1 μM UNC0638 and 2.0 μM pomalidomide was significantly but modestly reduced when compared to that of single-molecule treatments (Fig. 3d, e), suggesting minimal cytotoxic effects of this combinatorial regimen. Additionally, the combination of 0.1 μM UNC0638 and 0.05 μM decitabine slightly decreased proliferation but not viability of erythroid cells. We next determined erythroid differentiation after 12 days of culture by flow cytometry. We found that treatment with UNC0638 or in combination with pomalidomide did not change the erythroid differentiation pattern compared with DMSO treatment (Fig. 3f). Interestingly, cells treated with decitabine alone or decitabine plus UNC0638 exhibited a significant increase in erythroid differentiation as shown by an increase in polychromatophilic erythroblasts with a diminished number of basophilic erythroblasts (Fig. 3f). This result suggested that decitabine may accelerate the differentiation of β^0-thalassemia/HbE erythroid progenitor cells.

Taken together, these results strongly suggest that UNC0638 is a potent HbF inducer without cytotoxicity under these tested conditions. UNC0638 exhibits additive effects with pomalidomide and decitabine, implying that it induces γ-globin mRNA and HbF expression through a mechanism of action that differs from either pomalidomide or decitabine. Further analyses of UNC0638 alone or in combination with pomalidomide or decitabine may lead to improved treatments for β-thalassemia.

Discussion

The induction of γ-globin and increase in HbF expression has been shown to ameliorate the pathophysiology and severity of β-thalassemia patients by reducing excess unmatched α-globin chains in red blood cells [9, 32, 33]. A current FDA-approved HbF inducer, hydroxyurea, is not effective in more than 50% of β-thalassemia patients [14], and therefore, more effective HbF inducers are sorely needed. Several epigenetic modifying enzymes involved in γ-globin repression, including DNMT1, LSD1, and EHMT1/2 histone methyltransferases, are attractive therapeutic targets for induction of HbF [19]. Inhibition of EHMT1 (GLP) and EHMT2 (G9a) histone methyltransferases by the small chemical compound UNC0638 has shown to potently induce γ-globin and HbF expression in erythroid progenitor cells from normal adult donors by decreasing the repressive histone H3K9Me2 mark at the γ-globin promoters and by facilitating loop formation between the LCR and the γ-globin promoters [24, 25, 31]. Moreover, UNC0638 treatment does not affect the expression of key erythroid transcription factors (GATA1, KLF1, and NFE2), or key γ-globin repressors (BCL11A and MYB) [25], suggesting that it does not induce HbF expression via an effect on delayed erythroid differentiation or downregulation of γ-globin repressors.

In this report, we further evaluated the effects of UNC0638 on HbF induction in β^0-thalassemia/HbE erythroid progenitor cells; as is well appreciated, these cells have very high HbF baseline levels. We found that UNC0638 robustly increased γ-globin mRNA, HbF, and F-cells in β^0-thalassemia/HbE erythroid progenitor cell cultures. The HbF induction achieved by UNC0638 treatment was 25.5 ± 4.2% above baseline levels, which is comparable to previous reports in normal erythroid progenitor cells [24, 25]. Moreover, UNC0638 exhibited HbF-inducing activity similar to pomalidomide, while exhibiting even stronger induction than decitabine under these culture conditions. Interestingly, erythroid precursor cells from different β^0-thalassemia/HbE patients with varying HbF baseline levels all exhibited a similar degree of HbF induction in response to UNC0638 treatment. These data demonstrated that UNC0638 potently induced HbF expression in β^0-thalassemia/HbE erythroid progenitor cells regardless of specific β^0-thalassemia mutations or HbF baseline levels.

In agreement with a previous study [24], the level of HbF induction was more pronounced when UNC0638 was added at an early stage (day 4) than when added in a late stage (day 8) of erythroid differentiation. Since the pattern of globin gene expression is highly regulated and still reversible during early erythroid differentiation stages, UNC0638 was shown to act more effectively during this period. Thus, the time of addition of UNC0638 in erythroid cell culture was critical for inhibiting EHMT1/2 and reactivating HbF expression.

Combinatorial therapy by multiple HbF inducers is a promising therapeutic strategy to achieve clinical improvement in patients. Given that the major limitation of combination therapy is the increase in toxicity, we therefore used half the amount determined to be the most effective concentration of each compound in combination to achieve greater HbF

induction and avoid adverse effects in comparison to single-drug treatment. Pomalidomide, an FDA-approved immunomodulatory drug for the treatment of multiple myeloma, has been shown to induce γ-globin and HbF expression, at least partially through the downregulation of BCL11A and SOX6 [26–28, 34]. Decitabine, a DNMT1 inhibitor, has been shown to induce γ-globin and HbF expression in patients with SCD [35, 36] and β-thalassemia [20]. Here, we found that the combination of UNC0638 with pomalidomide or with decitabine increased HbF expression more than any of the three individually. Although, we observed a statistically significant reduction in viability and proliferation of cells treated with UNC0638 plus pomalidomide, these reductions were modest when compared to treatment with any single agent (full or half doses) or DMSO alone. Further studies examining precise dose titration of these compounds in a combination regimen would reduce the negative effect on cell viability and proliferation while potentially maintaining the high level of HbF induction. Interestingly, the differential HbF generated by the combination of UNC0638 and pomalidomide was greater than 30% of total hemoglobin, which is the level that has been demonstrated to achieve a significant clinical improvement of patients with SCD [37–40] and β-thalassemia [14]. These results suggest that the combination of compounds that have different mechanisms of action has the potential to additively increase HbF expression.

Although UNC0638 has demonstrated a strong HbF-inducing activity in ex vivo erythroid cell culture systems, its in vivo pharmacokinetic properties are poor due to a lack of drug-like properties [41]. Further development of EHMT1/2 inhibitors with higher potency and improved in vivo pharmacokinetic properties should reveal novel HbF inducers that would be suitable for clinical applications.

In summary, the present study confirms that inhibition of EHMT1/2 histone methyltransferases by a small molecule, UNC0638, exhibits strongly elevated HbF induction in β-thalassemia/HbE erythroid progenitor cells. Furthermore, UNC0638 was shown to have additive effects on HbF induction when combined with either pomalidomide or decitabine. Therefore, searching for novel potent and selective EHMT1/2 inhibitors with improved drug-like properties may lead to clinical application in the treatment of β-thalassemia.

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Authors' contributions Contribution: T.N., P.K., and N.J. designed the research; T.N., P.K., P.P., and W.K. performed the experiments; T.N., P.K., and N.J. analyzed the data; D.S., K.P., S.H., and S.F. provided the samples and resources; T.N., J.D.E., and N.J. wrote the manuscript; O.S., J.D.E., S.H., S.F., and N.J. conceptualized the idea and supervised the project; and all the authors read and approved the final manuscript.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethics approval The study was approved by Institutional Review Boards of Mahidol University and was conducted in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all participants before being included in the study.

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doses).[11] This adds weight to the argument of starting chelation with deferasirox early in the course of MDS when lower doses may suffice and improve compliance.

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High-level induction of fetal haemoglobin by pomalidomide in β-thalassaemia/HbE erythroid progenitor cells

Studies have shown that increased expression of fetal haemoglobin (HbF; $\alpha_2\gamma_2$) can ameliorate red blood cell deficiencies in patients with β-thalassaemia and sickle cell disease (SCD).[1–3] Pharmacological induction of HbF expression in β-thalassaemia has been investigated using several classes of small molecules,[4] including 5-azacytidine,[5] decitabine,[6] hydroxyurea,[7] LSD1 inhibitors (tranylcypromine and RN-1),[8,9] and short chain fatty acid derivatives.[10,11] Among these molecules, hydroxyurea is the only U.S. Food and Drug Administration (FDA) currently approved drug for the treatment of SCD and/or β-thalassaemia. However, hydroxyurea has shown modest and variable responses with potential myelosuppression in β-thalassaemia patients. Therefore, more robust and safer HbF therapeutics are highly desired.

Pomalidomide, an FDA-approved immunomodulatory drug for the treatment of multiple myeloma,[12,13] stimulates γ-globin mRNA and HbF expression in erythroid progenitor cells by downregulating factors involved in γ-globin repression, including *BCL11A, SOX6, GATA1, KLF1* and *LSD1*.[14–16] In addition, treatment of a humanized mouse model of SCD with pomalidomide induced comparable HbF expression to hydroxyurea, but without myelosuppressive effects.[15]

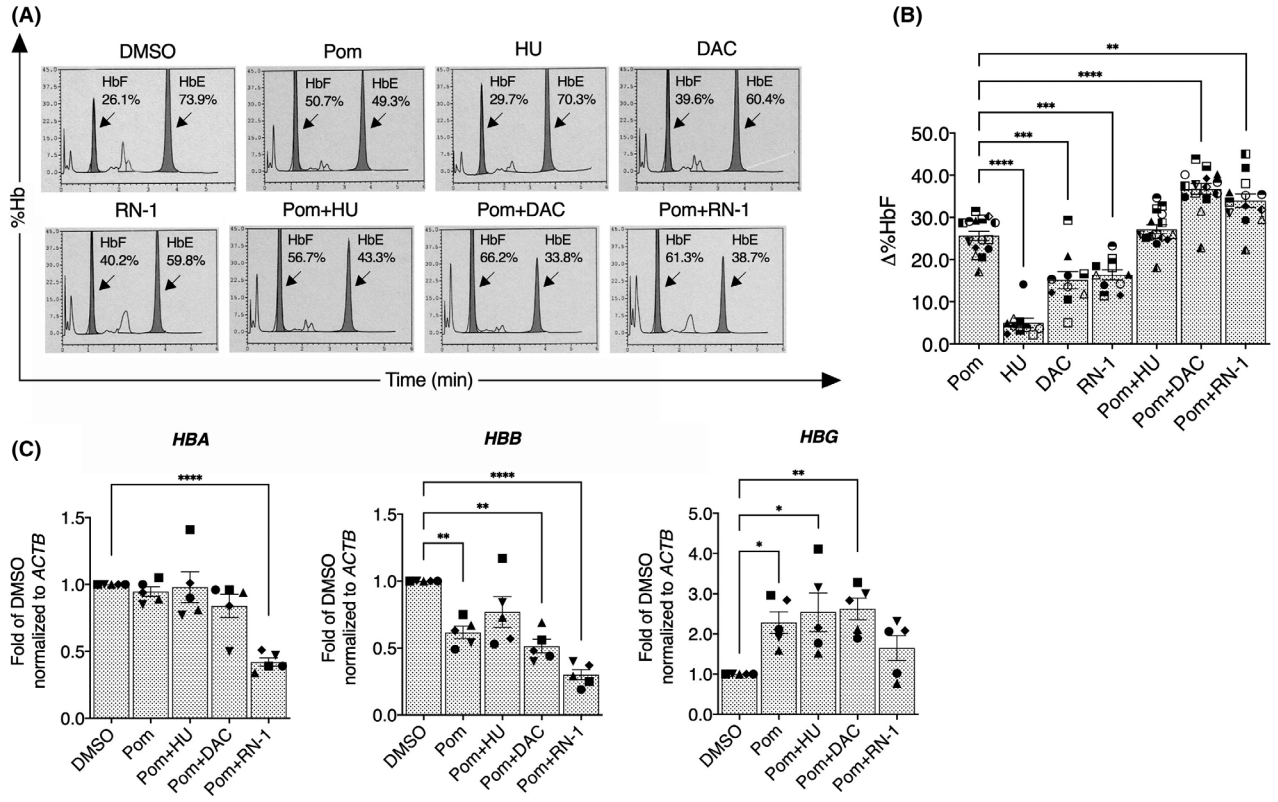

Fig 1. Robust HbF induction in β^0-thalassaemia/HbE erythroid cells by pomalidomide alone or in combination with other HbF inducers. β^0-Thalassaemia/HbE erythroblasts were treated with 4·0 μmol/l pomalidomide only (Pom, from day 4–14), 1·0 μmol/l hydroxyurea only (HU, from day 8–14), 0·1 μmol/l decitabine only (DAC, from day 8–14), 0·02 μmol/l RN-1 only (from day 8–14), or in combination of Pom with HU, with DAC, or with RN-1. (A) Representative high-performance liquid chromatograms (HPLC) showing haemoglobin composition at day 14 of erythroid differentiation. (B) The percentage of HbF relative to total Hb (%HbF + %HbE) determined by HPLC at day 14 of erythroid differentiation. The increase in HbF percentage after treatment from the baseline level in dimethyl sulfoxide (DMSO) control was expressed as Δ%HbF (% HbF [compound treatment] − %HbF [DMSO control]). Mean ± standard error of the mean [SEM], n = 10 for HU, DAC and RN-1; n = 15 for Pom, Pom + HU and Pom + DAC; and n = 13 for Pom + RN-1. (C) Quantitative reverse transcription polymerase chain reaction analysis showing relative *HBA*, *HBB* and *HBG* mRNA expression levels normalised to β-actin (*ACTB*) at day 12 of erythroid differentiation. Data are presented as the mean (± SEM) of relative fold change of DMSO (n = 5) *P < 0·05; **P < 0·005; ***P < 0·0005; ****P < 0·0001.

Here, we investigated the therapeutic potential of pomalidomide and its combined effects with other HbF inducers, including hydroxyurea, decitabine and RN-1, in erythroid progenitor cells from compound heterozygous β^0-thalassaemia/HbE (*HBB*:c.79G>A) patients (Table SI) using a three-phase liquid culture system that supports terminal maturation of erythroid cells (Data S1 and Fig S1). Comparison of results using optimal conditions for each compound (Figs S2 and S3) revealed that pomalidomide was much more effective in inducing HbF expression than hydroxyurea, decitabine or RN-1 (Fig 1A,B). The greatest increase in HbF percentage from the baseline level was observed in pomalidomide-treated cells, achieving 25·6 ± 1·1% as determined by high-performance liquid chromatography (HPLC) (Fig 1A,B). β^0-thalassaemia/HbE precursors from patients of different β^0-thalassemic mutations (Table SI) showed similarly increased levels of HbF induction in response to pomalidomide treatment. This result suggested that deficient progenitors, regardless of specific β^0-thalassemic mutation or baseline HbF level, are all susceptible to strong induction with pomalidomide (Fig 1A,B and Tables SI and SII). The percentage of cells expressing HbF (F cells) increased from 49·8 ± 4·7% for dimethyl sulfoxide (DMSO) controls to 60·6 ± 2·5% after pomalidomide treatment (Fig S4). By quantitative reverse transcription polymerase chain reaction (RT-PCR), we found that pomalidomide significantly increased γ-globin (*HBG*) mRNA expression, achieving a 2·3 ± 0·3-fold increase over control cells, with coincidentally diminished β-globin (*HBB*) expression, without significant change in α-globin (*HBA*) expression (Fig 1C).

To enhance the level of HbF induction, we investigated the effects of combined treatment of pomalidomide either with or without other pharmacological HbF inducers. The combination of pomalidomide and decitabine had an additive effect on induction, as shown by the differential HbF level (Δ% HbF = 36·7 ± 1·3) when compared to treatment with any single agent (Fig 1A,B). Hydroxyurea did not generate any additional increase in HbF when combined with pomalidomide. The

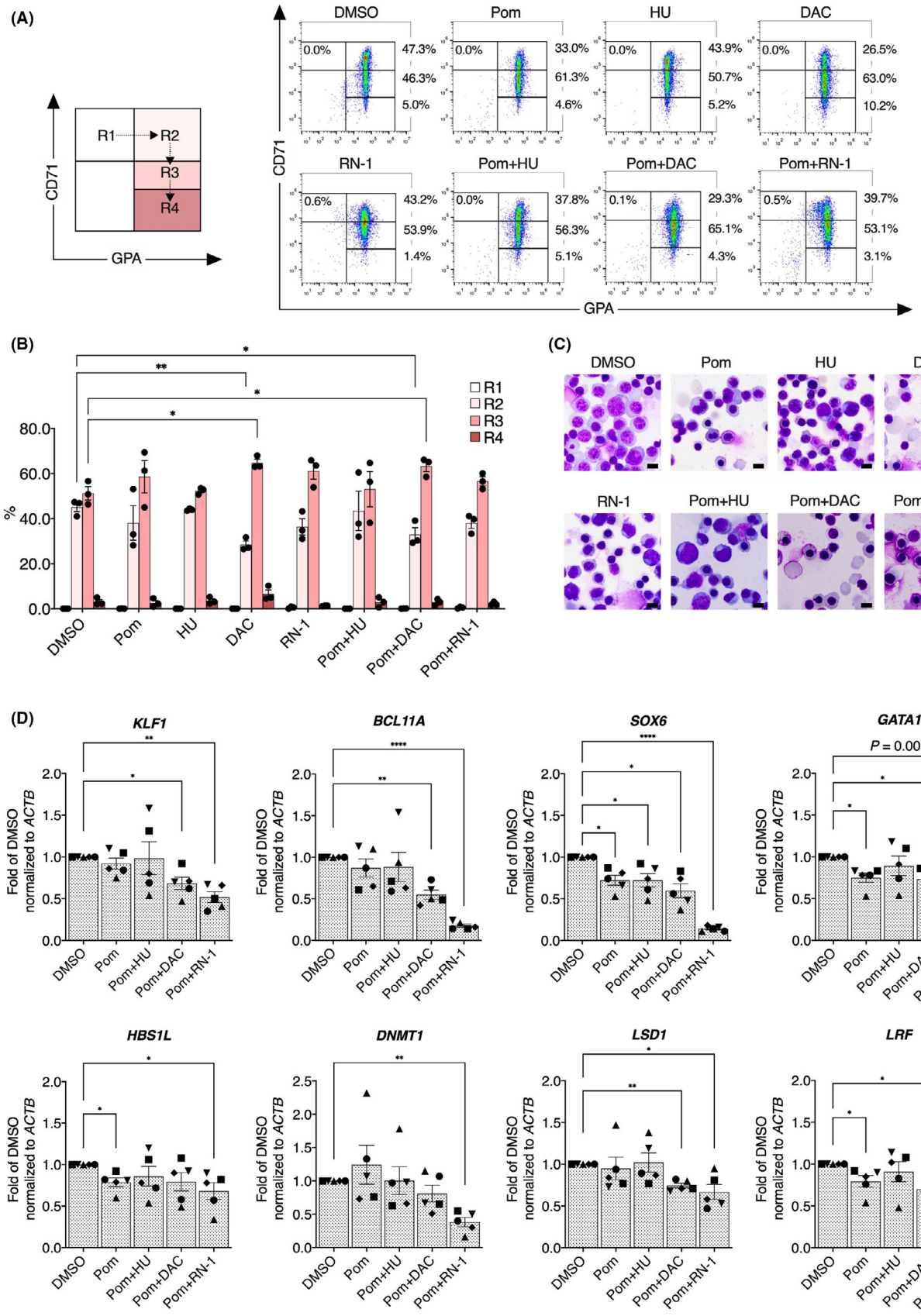

Fig 2. Effect of pomalidomide and its combinations on erythroid differentiation and mRNA expression of HbF regulators in cultured erythroid cells from β^0-thalassaemia/HbE patients. β^0-Thalassaemia/HbE erythroblasts were treated with 4·0 μmol/l pomalidomide only (Pom, from day 4 –14), 1·0 μmol/l hydroxyurea only (HU, from day 8–14), 0·1 μmol/l decitabine only (DAC, from day 8– 14), 0·02 μmol/l RN-1 only (from day 8–14), the combination of Pom with HU, with DAC, or with RN-1. (A) Representative flow cytometry dot plots for erythroid differentiation analysis on day 12 of culture. Erythroid cells were gated into R1 to R4 populations according to the expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a). (B) The histogram represents the quantitation of erythroid subpopulations analysed by flow cytometry. (mean ± SEM, $n = 3$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$, relative to dimethyl sulfoxide (DMSO) control. (C) Representative modified Giemsa-stained cytospins at day 12 of culture showing erythroid morphology after DMSO or compound treatments. Scale bar = 10 μm. (D) Relative mRNA abundance of known HbF regulators normalized to β-actin (*ACTB*) determined by quantitative reverse transcription polymerase chain reaction at day 12 of erythroid cell culture. Gene names are shown at the top of each histogram. Data are presented as the mean (± SEM) of relative fold change of DMSO . ($n = 5$) $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$; $^{****}P < 0{\cdot}0001$.

combination of pomalidomide and RN-1 did increase the percentage of HbF (Fig 1A,B) and at the same time reduced *HBA*, *HBB* and *HBG* mRNA expression (Fig 1C), suggesting that this combination negatively affected total globin mRNA expression. Taken together, these results suggest that pomalidomide and decitabine act through independent pathways to induce, additively, high-level HbF expression, implying a cooperative therapeutic potential for the treatment of β-thalassaemia.

We next determined the cytotoxicity of treatments and found that pomalidomide did not significantly affect erythroid cell proliferation (Fig S5A) or viability (Fig S5B). However, pomalidomide plus decitabine showed a reduction in cell proliferation on day 12 of culture without affecting cell viability. Erythroid cell proliferation and viability were significantly reduced in cells exposed to pomalidomide plus RN-1 (Fig S5A,B), suggesting toxicity of the latter combination. Analysis of erythroid differentiation of cells treated with hydroxyurea or pomalidomide plus hydroxyurea was similar to that of DMSO-treated cells (Fig 2A,B), suggesting that these treatments did not affect erythroid terminal differentiation. We noted a trend towards increased differentiation of cells treated with pomalidomide, RN-1 and pomalidomide plus RN-1, compared with the controls. Interestingly, significantly accelerated erythroid differentiation was observed in decitabine alone and pomalidomide plus decitabine, as evidenced by elevated transferrin receptor $(CD71)^{medium}$/(glycophorin A $(GPA)^{high}$ population and decreased $CD71^{high}$/GPA^{high} cells (Fig 2A,B). Similarly, modified Giemsa-stained cytospins showed an increased number of late-stage erythroblasts in cells exposed to decitabine alone and pomalidomide plus decitabine when compared to control cells, indicating a shift towards normal erythroid cell maturation (Fig 2C and Fig S1). These results suggested that the differentiation of β^0-thalassaemia/HbE progenitor cells significantly improved after treatment with either decitabine alone or pomalidomide plus decitabine.

To investigate the effects of pomalidomide plus or minus these effectors on transcriptional regulation in β-thalassaemic erythroid progenitor cells, quantitative RT-PCR analyses revealed that one key γ-globin repressor mRNA, *BCL11A,* was only slightly reduced after treatment with pomalidomide or pomalidomide plus hydroxyurea. *BCL11A* was significantly downregulated (by 1·8- and 5·6-fold) after treatment with pomalidomide plus decitabine or pomalidomide plus RN-1 respectively (Fig 2D). Moreover, the expression of *SOX6*, *GATA1*, *HBS1L* and *LRF* were modestly but significantly downregulated by pomalidomide, whereas other erythroid regulators were unaffected (Fig 2D and Fig S6). In addition, combined pomalidomide and decitabine treatment, which showed additive effects on HbF induction, reduced the expression of *KLF1*, *LSD1* and *CHD4*. The combination of pomalidomide plus RN-1 significantly affected the expression of several key regulators, including *KLF1*, *SOX6*, *GATA1*, *HBS1L*, *DNMT1*, *LSD1*, *ID2*, *CHD4*, *FOXO3*, *NRF2* and *MYB* (Fig 2D and Fig S6), consistent with the fact that this same combination significantly reduced cell proliferation and viability (Fig S5). Taken together, these results indicate that the mechanisms of action of pomalidomide and several co-effectors in induction of HbF expression partly involve transcriptional regulation of key HbF repressors and/or co-repressors.

In summary, the present data show that pomalidomide is a potent HbF inducer and is more potent than hydroxyurea. The combination of pomalidomide and decitabine provide additive effects in inducing HbF expression in erythroid cells from β^0-thalassaemia/HbE patients. Despite these promising results, it must be emphasized that the potential risks associated with the use of pomalidomide include developmental defects (if taken during pregnancy), thrombosis and pancytopenia,[17] which are similar to the toxicities of the parental drugs, lenalidomide and thalidomide. Development of pomalidomide structural refinements or analogues with similar biological effects may lead to future, fully effective, reduced adverse effects and possible clinical application.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

PK, TN, OS and NJ designed the research; PK, TN, PP and WK performed the experiments; PK, TN and NJ analysed data; DS, KP, SH and SF provided samples and resources; PK, JDE, and NJ wrote the manuscript; JDE, SH, SF, OS and NJ conceptualized the idea and supervised the project. All the authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplemental methods.

Fig S1. Delayed erythroid differentiation is observed in β^0-thalassaemia/HbE comparing to normal erythroblast culture.

Fig S2. Time- and dose-dependent inducing effects of pomalidomide on HbF induction in erythroid cells from β^0-thalassaemia/HbE patients.

Fig S3. HbF-inducing effects of hydroxyurea (HU), decitabine (DAC), and RN-1 in erythroid cells from β^0-thalassaemia/HbE patients.

Fig S4. Pomalidomide and its combinations increase erythroid cells expressing HbF (F-cells).

Fig S5. Effect of pomalidomide and its combinations on cell proliferation, viability of cultured erythroid cells from β^0-thalassaemia/HbE patients.

Fig S6. Treatment of pomalidomide and its combinations alter the expression of HbF regulators in β^0-thalassaemia/HbE erythroid cells.

Table SI Haematological parameters of β^0-thalassaemia/HbE patients participated in this study.

Table SII Induction of HbF in erythroid progenitor cells from β^0-thalassaemia/HbE after treatments with pomalidomide, hydroxyurea, decitabine, RN-1 and their combinations.

Table SIII Primers used in this study.

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Clinical and biological correlates of the expression of select Polycomb complex genes in Brazilian children with acute promyelocytic leukaemia

De novo acute promyelocytic leukaemia (APL) is an aggressive subtype that accounts for 5–10% of all childhood acute myeloid leukaemia (AML). In some Latin American and European populations, APL is more frequent than in other geographic populations. Approximately 97% of patients with APL present t(15;17)(q22;q21.1)/promyelocytic leukaemia (PML)-retinoic acid receptor alpha (RARA) fusion protein and nearly all of the affected patients respond to therapy with all-*trans* retinoic acid (ATRA) combined with arsenic trioxide (ATO).[1] Additionally, studies in transgenic mice revealed that this transcript is necessary, but not sufficient, for APL development, suggesting that additional genetic or epigenetic changes are also required for the APL establishment.[2]

The FMS-like tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) has been frequently reported in patients with APL presenting a more aggressive disease course, with lower overall and disease-free survival rates. These mutations are known to co-operate with other initiating events to advance disease progression, but they do not initiate leukaemia independently.[3] Regarding paediatric APL, genomic studies reported that APL cells have fewer genetic alterations than those from other AML subtypes.[4] These data indicate that childhood APL development may require more than just genetic alterations to manifest the disease phenotype.

In this context, there are several reports on the epigenetic alterations implicated in AML development, and they are mainly presented in APL. For instance, patients with APL are characterised by a specific DNA methylation pattern, which may be due to relatively late events in APL leukaemogenesis, contributing to APL maintenance rather than leukaemia initiation.[5]

Besides, PML/RARA induces a multitude of alterations in chromatin architecture, including the recruitment of crucial epigenetic-modifying factors, such as histone deacetylase complexes and DNA methyltransferases. Moreover, pieces of evidence have revealed that the Polycomb repressor complex (PRC) could contribute to the alterations observed in the typical APL epigenetic landscape.[6]

Polycomb group (PcG) proteins are histone modifiers in two multiprotein complexes: Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). Radulovic et al.[6] highlighted the emerging implications of these genes in haematopoietic neoplasms, including myeloid neoplasia. Therefore, changes in the expression profile of individual PcG genes might yield novel information about APL pathogenesis.

We evaluated the PcG gene levels, namely, enhancer of zeste homologue 2 (*EZH2*), Yin and Yang 1 protein (*YY1*), BMI1 proto-oncogene, Polycomb ring finger (*BMI1*) and suppressor of zeste 12 protein homologue (*SUZ12*), in a cohort of 25 Brazilian children with APL, with and without a *FLT3*-ITD mutation. In addition, we compared them to those found in patients with other AML subtypes, with and without the *FLT3* mutation, to verify whether this mutation status could be associated with the APL epigenetic landscape.

Amongst the 25 patients with APL, eight had additional chromosome abnormalities that mostly involved chromosomes 6, 8, 20 and 21. In the AML group, 39 patients had their karyotype evaluated and the abnormalities detected were as follows: 15 (38·5%) presented with lysine methyltransferase 2A (*KMT2A*) gene abnormalities; eight (20·5%) presented with non-recurrent chromosome abnormalities; four (10·3%) presented with RUNX1 translocation partner 1 (*RUNX1*)/*RUNXT1* fusion genes; four (10·3%) presented with core binding factor subunit beta (*CBFB*)/myosin heavy chain 11 (*MYH11*) fusion genes; four (10·3%) presented with normal karyotypes; three (7·7%) presented with abnormalities in chromosomes 5 and 7; and one (2·4%) presented with no mitosis. In relation to *FLT3* status, 21 (30·9%) patients had *FLT3*-ITD mutations, 13 (61·9%) were patients with APL and eight (38·1%) were patients with AML [AML-M5 (five patients), AML-M2 (two) and AML-M6 (one)] (Clinical data are in Table S1).