Blood Cells, Molecules and Diseases 79 (2019) 102345

Contents lists available at ScienceDirect

Blood Cells, Molecules and Diseases

journal homepage: www.elsevier.com/locate/bcmd

Conjugate prodrug AN-233 induces fetal hemoglobin expression in sickle erythroid progenitors and β-YAC transgenic mice

Aluya R. Oseghale[a], Xingguo Zhu[b], Biaoru Li[b], Kenneth R. Peterson[c], Abraham Nudelman[d], Ada Rephaeli[e], Hongyan Xu[f], Betty S. Pace[a,b,g,*]

[a] Vascular Biology Center, Augusta University, Augusta, GA, USA
[b] Department of Pediatrics, Augusta University, Augusta, GA, USA
[c] Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA
[d] Chemistry Department, Bar Ilan University, Ramat Gan, Israel
[e] Felsenstein Medical Research Center, Sackler Medical School, Tel Aviv University, Petach Tikva, Israel
[f] Department of Population Health Sciences, Augusta University, Augusta, GA, USA
[g] Department of Biochemistry and Molecular Biology, Augusta University, Augusta, GA, USA

ARTICLE INFO

Editor: Mohandas Narla

Keywords:
Fetal hemoglobin
AN-233
Butyric acid
δ-Aminolevulinate
β-YAC mice

ABSTRACT

Pharmacologic induction of fetal hemoglobin (HbF) is an effective strategy for treating sickle cell disease (SCD) by ameliorating disease severity. Hydroxyurea is the only FDA-approved agent that induces HbF, but significant non-responders and requirement for frequent monitoring of blood counts for drug toxicity limit clinical usefulness. Therefore, we investigated a novel prodrug conjugate of butyric acid (BA) and δ-aminolevulinate (ALA) as a potential HbF inducing agent, using erythroid precursors and a preclinical β-YAC mouse model. We observed significantly increased γ-globin gene transcription and HbF expression mediated by AN-233 in K562 cells. Moreover, AN-233 stimulated mild heme biosynthesis and inhibited expression of heme-regulated eIF2α kinase involved in silencing γ-globin expression. Studies using primary erythroid precursors generated from sickle peripheral blood mononuclear cells verified the ability of AN-233 to induce HbF, increase histone H3 and H4 acetylation levels at the γ-globin promoter and reduce erythroid precursor sickling by 50%. Subsequent drug treatment of β-YAC transgenic mice confirmed HbF induction *in vivo* by AN-233 through an increase in the percentage of HbF positive red blood cells and HbF levels measured by flow cytometry. These data support the potential development of AN-233 for the treatment of SCD.

1. Introduction

Sickle cell disease (SCD) is a group of hematologic disorders that arise from mutations in the structural gene encoding a subunit of hemoglobin (Hb). A single point mutation, A to T at the sixth codon of the β-globin gene results in the production of HbS [1]. The homozygous form of the mutation produces sickle cell anemia, in which HbS polymerizes under deoxygenated conditions leading to formation of sickle-shaped red blood cells (RBCs). In the United States, approximately 100,000 individuals are affected with SCD and worldwide over 330,000 babies are born annually [2]. The different forms of SCD are characterized by chronic hemolysis, anemia and impair blood flow by sickle RBCs, leading to recurrent painful vaso-occlusive episodes and other complications such as infection, acute chest, splenic sequestration, and end organ damage [3,4].

The development of pharmacologic agents that induce HbF expression is an effective strategy for treating people with SCD because HbF exerts anti-sickling effects through formation of HbS/HbF hybrid molecules [5]. Studies from the Comprehensive Study of Sickle Cell Disease demonstrated higher HbF levels improve long-term survival of persons with sickle cell anemia [6]. After demonstrated efficacy in the Multicenter Hydroxyurea Study, in 1998 hydroxyurea (HU) became the only Food and Drug Administration-approved drug proven to induce HbF in SCD patients. The use of HU is limited by a significant non-responder rate, the need for close monitoring of blood counts for bone marrow toxicity, infertility, and patient concerns with taking a chemotherapy class agent [7,8]. Therefore, a need for the development of additional less toxic and effective therapies exists.

Several groups have previously reported robust HbF induction in SCD patients by the histone deacetylase inhibitor butyric acid (BA)

* Corresponding author at: Department of Pediatrics, Augusta University, 1120 15th Street, CN4112, Augusta, GA 30912, USA.
E-mail address: bpace@augusta.edu (B.S. Pace).

https://doi.org/10.1016/j.bcmd.2019.102345
Received 8 July 2019; Accepted 8 July 2019
Available online 09 July 2019

[9–11], however rapid metabolism of BA when given by oral administration hindered clinical development. Subsequent studies by our group and others demonstrated the ability of BA to activate p38 MAPK signaling [12,13] and CREB1 phosphorylation to achieve γ-globin gene trans-activation [14]. Individuals treated with intravenous BA showed robust HbF induction [10], but continuous treatment produced anti-proliferative effects in the bone marrow [11], requiring intermitted drug dosing [9]. Clinical trials using the oral analogue dimethylbutyrate induced HbF in β-thalassemia patients, but proved less effective in SCD [15].

To address the need for additional safe and effective oral HbF inducers, we investigated a novel oral active conjugate of BA and δ-aminolevulinate (ALA). The prodrug 1-(butyryloxy)ethyl-5-amino-4-oxopentanoate (AN-233) undergoes intracellular hydrolysis in an esterase-dependent manner to yield two active drugs BA and ALA [16] thus avoiding intra-hepatic drug inactivation. Oral administration of the prodrug to anemic C57/BL6 mice increased total Hb levels [17], but the effects of AN-233 on HbF was not previously tested. Therefore, using K562 cells and sickle primary erythroid precursors, we demonstrated HbF induction by AN-233 and observed an anti-sickling effect. Mechanistic studies showed the ability of AN-233 to mediate hyperacetylation of histones in the γ-globin gene promoter, p38 MAPK phosphorylation and heme biosynthesis to repress heme-regulated eIF2α kinase (HRI) expression. Subsequent preclinical studies in β-YAC transgenic mouse confirmed that AN-233 increased HbF levels and the percent of HbF positive cells (F-cells) *in vivo* without significant anti-proliferative effects in the bone marrow. These findings support the potential development of AN-233 as an HbF inducer for SCD.

2. Materials and methods

2.1. Synthesis of AN-233 prodrug

The conjugate prodrug AN-233 was synthesized as a precursor compound of BA attached to ALA with a protective BOC (*tert*-butyloxycarbonyl) group on the N terminal by Drs. Rephaeli and Nudelman [16]. Purified AN-233 was obtained by the removal of the BOC protective group under acidic conditions to yield the acyloxyethyl esters of ALA and BA. Synthesized AN-233 was made available with > 95% purity in two lots. The first lot was reconstituted in 10% ethanol (vehicle control) while the second lot was reconstituted in water for *in vivo* studies to avoid potential toxicities in mice.

2.2. Tissue culture and reagents

K562 cells were cultured in Iscove's Modified Dulbecco medium (IMDM) with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (0.1 mg/mL). Drug inductions for K562 cells were conducted for 48 h and cell viability evaluated with 0.4% Trypan blue exclusion. Cell counts were performed a dual chamber apparatus and the percentage viability obtained using an Automated Cell Counter (Bio-Rad).

For primary cultures, erythroid precursors were generated from peripheral blood mononuclear cells isolated from discard blood of sickle patients under an IRB exempt protocol. These cells were cultured in a two-phase liquid culture system previously published by our group [18]. During phase 1, cells were grown in Iscove's Dulecco Media with 15% fetal bovine serum, 15% human AB serum, 10 ng/mL interleukin-3, 50 ng/mL stem cell factor and 2 IU/mL of erythropoietin (Peprotech, Rocky Hill NJ). Phase 2 of culture initiated on day 7 with a similar medium without stem cell factor and interleukin-3. On day 8, erythroid precursors were treated with AN-233 (0.125 mM and 0.25 mM), ethanol (EtOH; 0.0008% and 0.016%) and the positive control HU (100 μM) for 48 h and harvested for the various analyses.

2.3. Reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from cells using Trizol (Ambion, Carlsbad CA) and analyzed by RT-qPCR as previously published by our group [12]. Gene-specific primers were used to quantify mRNA levels for γ-globin, β-globin and internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All mRNA levels were normalized to GAPDH before analysis.

2.4. Western blot analysis

Western blot analysis was performed as previously published by our group [19] using whole cell lysates generated with RIPA buffer (ThermoScientific, Rockford, IL) supplemented with proteinase and phosphatase inhibitor cocktails. For histone acetylation studies, nuclear lysates were prepared by suspending cells in buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 420 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and protease inhibitor mixture for 30 min, followed by centrifugation. Antibodies against HbF (51–7), HbA (37–8), and Tata binding protein (TBP; N-12) were purchased from Santa Cruz Biotechnology (Dallas TX); antibodies against β-actin (A5316) and rabbit IgG (I8140) were purchased from Sigma (St. Louis MO). Acetylated histone H3 (AcH3; 06–599) and AcH4 (06–866) antibodies were purchased from Millipore (Burlington, MA).

2.5. Flow cytometry analysis

To measure percent HbF positive cells (F-cells), K562 cells and erythroid precursors were fixed with 1% formaldehyde, permeabilized with ice-cold acetone:methanol (4:1 ratio) and stained with fluorescein isothiocyanate (FITC) anti-HbF antibody (ab19365, Abcam Cambridge MA) and isotype control IgG antibody (MBS524511, MyBioSource, San Diego CA) was used to detect non-specific staining. The F-cells levels and HbF protein levels measured by mean fluorescence intensity (MFI) were analyzed on an LSR-II flow cytometer (BD Biosciences, San Jose CA) and FlowJo analysis to generate quantitative data.

2.6. Heme quantitation assay

The total cellular heme content was determined using the QuantiChrom™ Heme Assay Kit (DIHM-250, BioAssay Systems, Hayward, CA) per the manufacturer's instructions. Briefly, 25 μL of cellular lysate was mixed with 100 μL of detection reagent. The mixture was incubated at room temperature for 5 min followed by measuring the absorbance at 400 nm on a microplate reader [20]. The total heme concentration was calculated based on a formula provided by the manufacturer: Heme concentration = $(OD^{sample} - OD^{blank})/ (OD^{calibrator} - OD^{blank})$ x 125 x dilution factor. This value was normalized by total protein in each sample.

2.7. Sickling assay

In vitro sickling studies were conducted as previously published by our group [21]. Briefly, after drug inductions of sickle erythroid precursors for 48 h, cells were incubated in 2% oxygen overnight and then fixed with 2% formaldehyde. Erythroid morphology was evaluated microscopically and the number of sickled cells per high power field manually counted for 1000 cells, per triplicates per condition.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed as previously published by our group [22] with immunoprecipitations for AcH3, AcH4 and TBP antibodies (Santa Cruz Biotechnology) and rabbit IgG as a control. Primers used to quantify *in vivo* chromatin modifications are as follows: locus control region DNase I hypersensitive site 2 (LCR-HS2) forward CCTTCTGGC

TCAAGCACAGC and reverse ATAGGAGTCATCACTCTAGGC, γ-globin promoter (forward CTGAAACGGTCCCTGGCTA, reverse CTGTGAAAT GACCCATGGCG), and β-globin promoter (forward TGGAGCCACACCC TAGGGTTGGC, reverse CTTGTAACCTTGATACCAACCTG).

2.9. β-YAC transgenic mouse treatment protocol

The β-YAC is a transgenic mouse model containing the full-length 81 kb human β-globin gene locus including the LCR and surrounding region. The five functional human globin genes 5′-ε-Gγ-Aγ-δ-β-3′ are present and undergo normal developmental regulation with the γ-globin gene silenced shortly after birth [23]. β-YAC mice (5–6 months old) were administered AN-233 suspended in water (200 or 300 mg/kg) 5 days/week for 4 weeks by intraperitoneal injection; we treated five mice per group with 3 males and 2 females. Hydroxyurea (100 mg/kg) was included as a positive control. We collected blood by tail bleed at week 0, 2 and 4 and analyzed for automated complete blood counts with differential using a Micros 60 machine (HORIBA Medical/ABX Diagnostics). The level of F-cells and MFI were performed by flow cytometry as previously published by our group [24]. For reticulocyte counts, whole blood was stained with acridine orange and flow cytometry performed on an LSR-II flow cytometer (BD Biosciences).

2.10. Statistical analysis

For tissue culture studies, data for at least 3–5 independent experiments performed in triplicate were reported as the mean ± standard error of the mean (SEM). The Student's *t*-test was performed to determine significance and $p < 0.05$ was considered statistically significant. For β-YAC studies, untreated (water), HU and AN-233 treated mice were analyzed by paired *t*-tests to compare week 0 (baseline) to week 2 and week 4. Data were normalized based on 100 x [activity (therapeutic)-mean activity (negative control)]/[Activity(positive control)-mean activity (negative control)]. Finally, changes across treatment groups were compared using ANOVA with post-hoc Tukey HSD test for pairwise comparison at week 2 and week 4.

3. Results

3.1. AN-233 induces γ-globin transcription and HbF expression in K562 cells

Studies by Rephaeli and Nudelman demonstrated the prodrug conjugate AN-233 undergoes intracellular hydrolysis by esterase enzymes in K562 cells into two active metabolites BA and ALA [16]. Therefore, our initial studies were conducted in K562 cells to determine the ability of AN-233 to induce γ-globin transcription and HbF expression. K562 cells display characteristics of erythroid cells including expression of the ε, γ and α globin genes [25,26] and these cells are useful for initial drug screening and discovery of potential HbF inducers [27].

K562 cells were treated with AN-233 for 48 h and globin gene transcription was analyzed by RT-qPCR. We observed a significantly increase γ-globin mRNA levels of 1.8-fold and 2.0-fold by AN-233 0.125 and 0.25 mM respectively (Fig. 1A) compare to 1.7-fold induction by HU. Control studies with BA and ALA alone directly added in culture at 0.125 mM, increased γ-globin mRNA 3-fold and 1.5 fold respectively. The next set of studies determined the effects of AN-233 on HbF protein expression by flow cytometry. Similar to mRNA levels, treatment with AN-233 (0.125 and 0.25 mM) increased the F-cells to a maximum of 19% compared to 10% in EtOH treated controls (Fig. 1B and C). To substantiate HbF protein levels, we performed Western blot confirming a dose-dependent 3 to 4-fold increase in HbF ($p < 0.05$) by AN-233 (Fig. 1D). These levels compare to a 2-fold and 6-fold increase in HbF by BA and ALA respectively ($p < 0.05$).

3.2. AN-233 stimulates heme biosynthesis and regulates cellular protein targets

Since the biosynthesis of heme prosthetic group requires ALA as a precursor [28,29] and AN-233 is hydrolyzed to BA and ALA, we next determined whether heme levels are altered after treatments of K562 cells. Using a colorimetric quantitative assay, we observed a 1.5-fold and 1.8-fold increase ($p < 0.05$) in intracellular heme after 0.25 mM and 0.5 mM AN-233 treatment respectively (Fig. 2A). As would be expected, ALA (0.5 and 2.0 mM) and hemin (75 μM) increased heme levels in control experiments. Cellular heme is known to directly modulate the activity of Heme-regulated inhibitor (HRI) kinase [30], which under iron deficient states is activated to mediate eIF2αP (eukaryotic translation initiation factor 2 α phosphorylation) and inhibition of protein synthesis [31]. Therefore, we treated K562 cells with AN-233 and measured phosphorylated and total HRI and eIF2αP levels. We observed a dose-dependent maximal 52% decrease in HRI and 50% decrease in eIF2αP levels by AN-233 (Fig. 2B and C).

The second active metabolite of AN-233 hydrolysis is BA. We previously reported HbF induction by BA through p38 MAPK activation [12,32]. Subsequent, Western blot analysis showed that 0.25 mM AN-233 induced a 1.4-fold increase in phosphorylated p38 MAPK (Fig. 2D). By contrast, pretreatment with the p38 MAPK inhibitor SB203580 (10 μM) followed by 0.25 mM AN-233, decreased F-cell levels by 30% (Supplemental Fig. S1) suggesting p38 signaling is partially involved in mechanisms of HbF induction by AN-233.

3.3. AN-233 induces HbF synthesis in sickle erythroid precursors and inhibits sickling

While K562 cells serve as an initial screening model system for HbF inducers, we sought to confirm our findings in physiologically relevant cells. Thus, we generated sickle erythroid precursors from peripheral blood mononuclear cells of SCD patients using a 2-phase liquid culture system. After AN-233 (0.125 and 0.25 mM) treatment, we observed a maximal 2-fold increase in F-cells from 16.31% to 32.5% (Fig. 3A and B). Similarly, HbF measured by MFI increased 1.5-fold, levels comparable to HU treated cells (Fig. 3C). Pretreatment with SB203580 reduced F-cells and MFI levels at both AN-233 concentrations. To substantiate these findings in sickle precursors, Western blot confirmed the ability of 0.25 mM AN-233 to increase HbF protein by 2.6-fold ($p < 0.05$) without changing HbS expression (Fig. 3D), which was inhibited by SB203580 treatment.

While HbF induction is a good indicator of drug efficacy, it is also desirable to achieve an anti-sickling effect under hypoxic conditions. Therefore, sickle erythroid precursors were incubated in 2% oxygen overnight, fixed with formaldehyde and examined by light microscopy. As shown in Fig. 3E AN-233 reduced the percentage of sickled erythroid precursors up to 56% ($p < 0.05$) similar to HU, supporting an anti-sickling effects of the prodrug. Furthermore, pretreatment with SB203580 produced higher sickled erythroid progenitor levels. These findings support the ability of the AN-233 to induce HbF in sickle erythroid cells.

3.4. AN-233 increased histone acetylation in β-globin locus

Previous work from our lab and others, demonstrated the ability of BA to induce HbF *via* inhibition of histone deacetylases [33,34]. Therefore, we determined AcH3 and AcH4 levels in nuclear lysates of sickle erythroid cells after AN-233 treatment, where we observed increased global AcH3 levels (Fig. 4A). We next asked the question whether histone acetylation levels are enhanced in the β-globin locus as part of mechanisms of γ-globin gene activation. To answer this question, ChIP assay demonstrated that AN-233 mediated a dose-dependent 12-fold and 30-fold enrichment for AcH3 in the γ-globin promoter compared to IgG control studies (Fig. 4B); likewise, AcH4 levels were

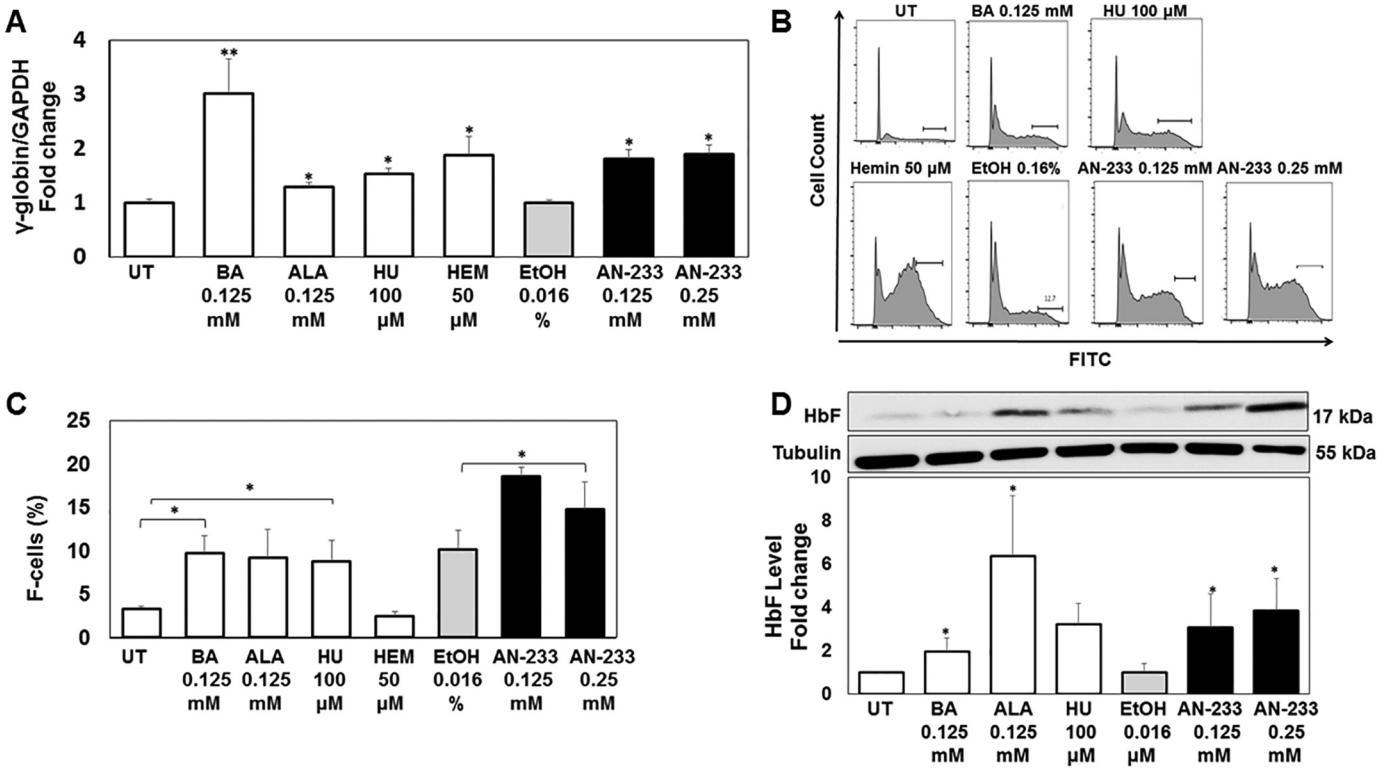

Fig. 1. AN-233 increased γ-globin transcription and HbF synthesis. K562 cells were treated with AN-233 and ethanol (EtOH) vehicle control for 48 h. Total RNA and whole cell lysates were isolated for RT-qPCR and Western blot analysis. After treatments, K562 cells were fixed and stained for flow cytometry. All data are shown as the mean ± SEM ($N = 5$) and $^{*}p < 0.05$ was considered statistically significant; $^{**}p < 0.01$. A) Shown in the bar graph is mRNA data generated by RT-qPCR under the different treatment conditions. B and C) K562 cells were stained with fluorescein isothiocyanate (FITC) conjugated anti-HbF antibody and analyzed by flow cytometry. Shown are representative histograms of cell populations that stained positive for HbF (F-cells) and quantitiative data in the graph. D) Western blot analysis determined HbF levels with tubulin as internal loading control. Shown is a representative blot and quantitative data generated by densitometry analysis.

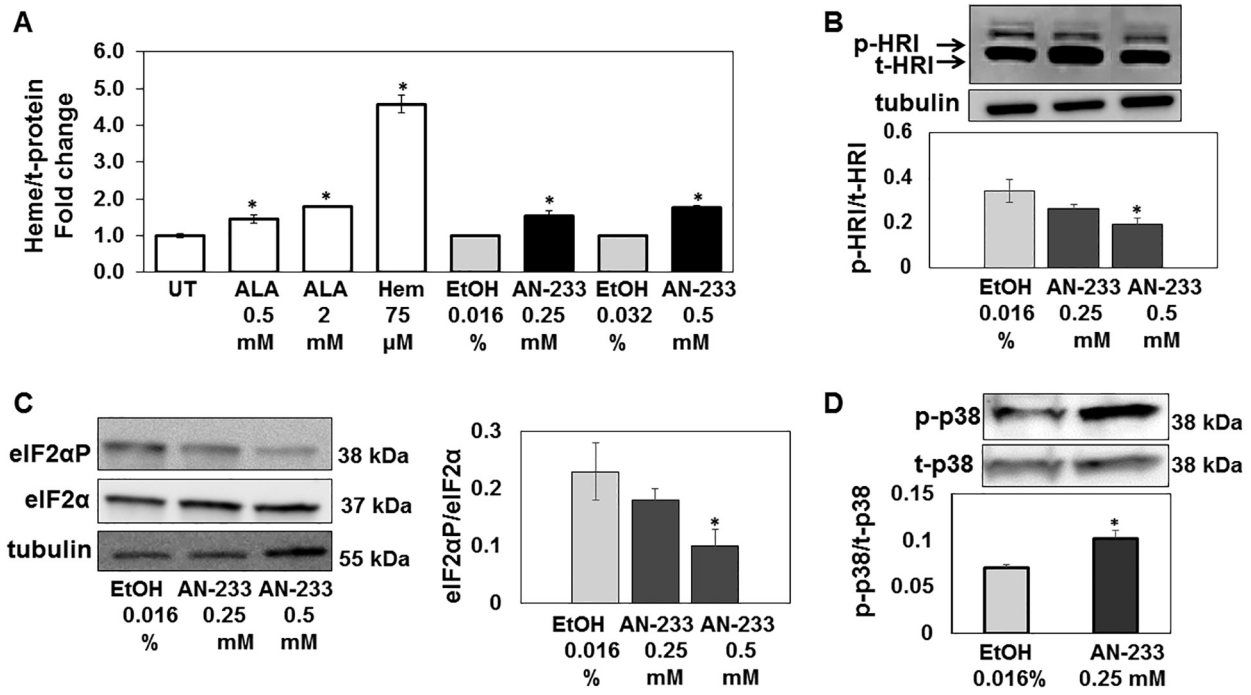

Fig. 2. AN-233 mediates heme biosynthesis and modulates several targets *in vitro*. K562 cells were induced with the prodrug AN-233 for 48 h and then analyzed for heme levels and HRI, eIF2α, and p38 MAPK protein levels. All data are shown as the mean ± SEM ($N = 3$ per group) and $^{*}p < 0.05$ was considered statistically significant. A) Shown are intracellular heme levels measured using a colorimetric QuantiChrom™ Heme Assay Kit for K562 cells under the different treatment conditions; heme values were normalized by total protein. B) Western blot analysis of phosphorylated HRI (*p*-HRI) and total HRI (t-HRI) levels. C) Western blot analysis of phosphorylated eukaryotic translation initiation factor (eIF2αP) levels. D) Shown is a representative gel and quantitative data for Western blot of p38 MAPK expression after AN-233 treatment.

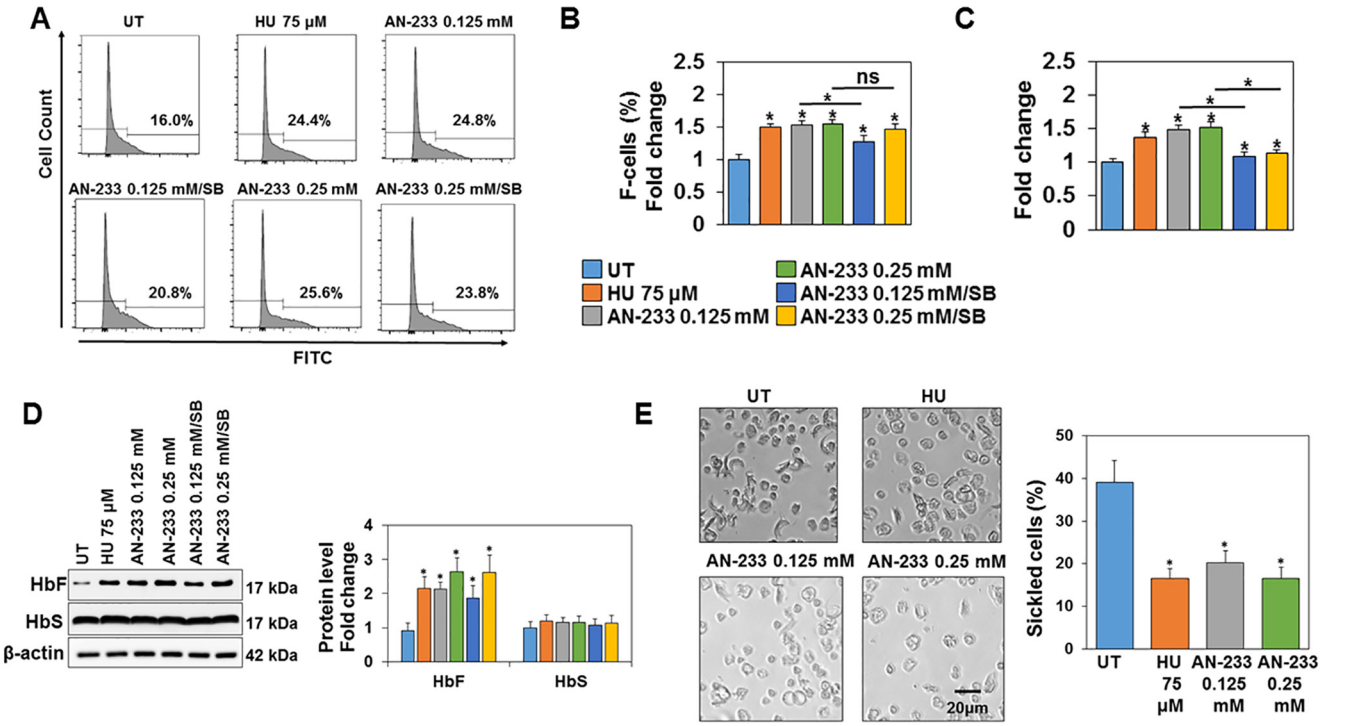

Fig. 3. AN-233 induces HbF without changing HbS levels in sickle erythroid precursors. A second lot of AN-233 dissolved in water vehicle were used to treat primary sickle erythroid progenitors on day 8 for 48 h and used for the various studies. All data are shown as the mean ± SEM (N = 3 per group) and *$p < 0.05$ was considered statistically significant. A) Shown is representative histograms from flow cytometry analysis of sickle erythroid precursors stained with FITC-HbF antibody (See Materials and Methods). B) F-cell levels were determined by flow cytometry and quantitative data generated by FlowJo analysis. C) The level of HbF protein was quantified by MFI generated by flow cytometry. D) Total protein lysates were isolated and used for Western blot for HbF and HbS protein; β-actin was the loading control. Shown is a representative blot and the quantitative data generated by densitometry. E) To determine if AN-233 mediates anti-sickling effects, erythroid precursors were treated with the various drugs and then incubated in 2% oxygen overnight, fixed in 2% formaldehyde and the number of sickle-shaped erythroid progenitors counted by light microscopy. Shown are images of sickle precursors for different conditions and summary of quantitative data for 1000 cells per triplicate for N = 3 donors.

increased. Similar effects were observed in the LCR-HS2 region where AN-233 mediated 12.5-fold and 5-fold increase in AcH3 and AcH4 respectively (Fig. 4C). By contrast, there were no significant changes in histone acetylation at the β-globin gene promoter (Fig. 4D).

3.5. AN-233 induces HbF expression in β-YAC transgenic mice

While our research group and many others have shown drug-mediated HbF induction in tissue culture systems, these findings do not

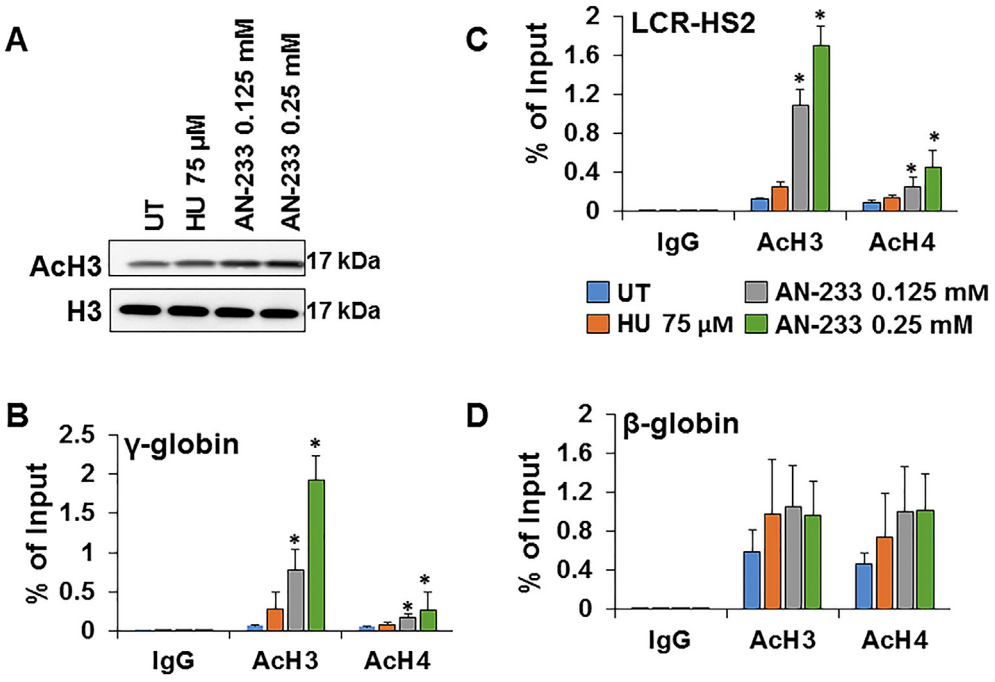

Fig. 4. AN-233 enhances histone acetylation in the γ-globin gene promoter. A) Nuclear protein lysates were isolated from sickle erythroid precursors and used for Western blot analysis of acetylated histone H3 (AcH3) levels (N = 3). Sickle erythroid progenitors treated under the different conditions = were used for ChIP assay as recently published by our group [52]. Shown is AcH3 and AcH4 levels in the (B) proximal γ-globin gene promoter, (C) locus control region DNAse I hypersensitivity site 2 (LCR-HS2), and (D) β-globin gene promoter; *$p < 0.05$ was considered statistically significant.

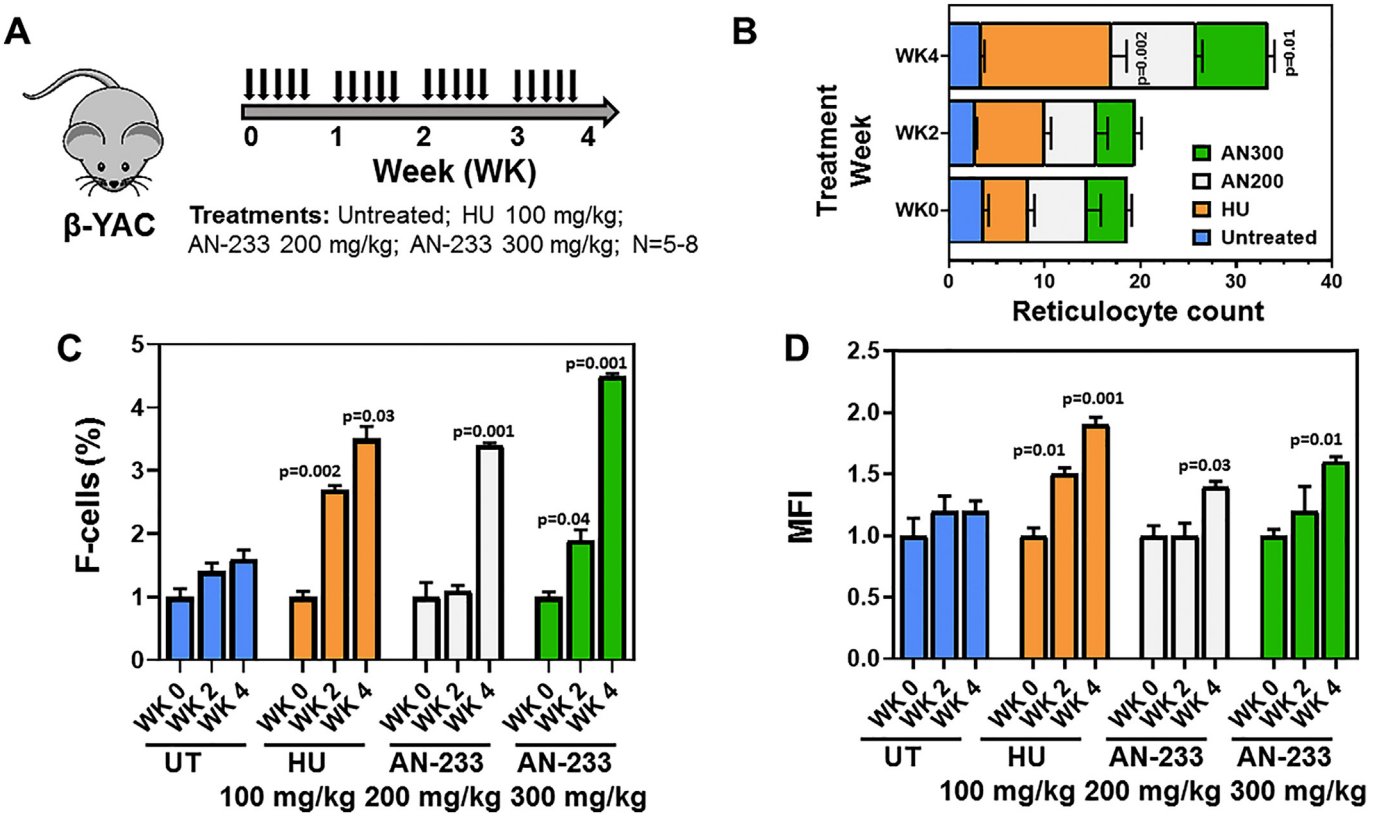

Fig. 5. AN-233 increases HbF expression in β-YAC transgenic mice. A) To assess the *in vivo* effect of AN-233, 5–6 months old β-Y*AC* transgenic mice were treated with 200 or 300 mg/kg of AN-233 dissolved in water for 4 weeks by intraperitoneal injections; water (vehicle) and hydroxyurea (HU) treatments were completed as controls ($N = 5$ per group; 3 males and 2 females). B) Blood samples collected at week 0, 2 and 4 were stained with acridine orange for reticulocyte percent by flow cytometry. Data are shown as the mean ± SEM and $p < 0.05$ was considered significant; exact *p*-values are shown. C) Peripheral blood was stained with FITC-conjugated anti-HbF antibody and flow cytometry performed to quantify the F-cells by FlowJo data analysis. D) The level of HbF expression was measured by MFI data generated by flow cytometry analysis.

always translate *in vivo*. Therefore, our final preclinical studies evaluated the potential of AN-233 to induce HbF using β-YAC transgenic mice, in which γ-globin to β-globin switching occurs during development [23]. We previously demonstrated HbF induction by α-amino butyric acid in this model [34]. Mice 5–6 months old were administered AN-233 dissolved in water (200 mg/kg and 300 mg/kg) or HU (100 mg/kg) by intraperitoneal injections, 5 days per week for 4 weeks with five mice per treatment group; an untreated water control group was also analyzed (Fig. 5A). At week 0, 2 and 4, mice were weighed and blood samples collected by tail bleed for automated complete blood counts and reticulocyte percent, percentage of F-cells and MFI by flow cytometry. Over 4 weeks of treatment, no drug toxicity occurred and normal body weights were maintained for all groups (data not shown). Untreated control mice had no significant change in blood counts over the 4-week treatment period (Supplemental Table S1), however, HU decreased total Hb, hematocrit, and white blood cell and platelet counts. Treatment with AN-233 at both doses produced a mild decrease in Hb and hematocrit, but the platelet count remained normal. To gain insights into the effects of AN-233 on erythropoiesis we measured reticulocyte count by acridine orange staining and flow cytometry. As shown in Fig. 5B, treatment with 300 mg/kg AN-233 increased reticulocytes 1.8-fold ($p = 0.01$) at week 4, compared to a 1.9-fold increase for HU ($p = 0.002$) suggesting AN-233 stimulated erythropoiesis.

We next analyzed the ability of AN-233 to induce HbF expression *in vivo*. As shown in Fig. 5C, the F-cells increased 3.4-fold (from 15.46% to 52.5%) in mice treated with 200 mg/kg AN-233, while 300 mg/kg increased F-cells 4.5-fold (from 13.5% to 60.6%). The levels of HbF measured by MFI increased 1.4-fold (from 588 to 824 units) and 1.7-fold (from 447 to 760 units) respectively at the two AN-233 doses (Fig. 5D). With five mice per treatment group, we performed ANOVA, which showed significant difference between untreated (water) control and all other treatment groups for Hb ($p = 0.0359$), reticulocytes ($p = 0.0003$), F-cells ($p = 0.0109$) and MFI ($p = 0.0369$) by week 4. These findings support the ability of AN-233 to induce HbF *in vivo* in β-YAC transgenic mice.

4. Discussion

Over the last three decades, numerous pharmacologic agents have been tested and shown to display HbF inducing properties *in vitro*, but few have translated into clinical efficacy. However, HbF induction by small molecules is an important therapeutic approach for treatment of the β-hemoglobinopathies and continues to be an intense area of investigation. Agents such as 5-azacytidine [35], decitabine [36] arginine butyrate [9,37] and short chain fatty acid derivatives [38,39] were shown to induce HbF in clinical trials. These drugs act by diverse mechanisms including inhibition of DNA methyl transferases and histone deacetylases, enhanced DNA binding of transcription factors and cell signaling activation. Recent studies of combined oral treatment with decitabine and tetrahydrouridine showed HbF induction in a Phase 1 clinical trial [40]. Thus, development of additional safe and effective oral agents that induce HbF without bone marrow toxicity when combined with HU, offer the potential for improved outcomes in β-hemoglobinopathies.

We first performed studies using K562 cells to provide *in vitro* evidence of HbF induction by the novel prodrug conjugate AN-233 composed of BA and ALA. We observed increased γ-globin transcription at

the mRNA level and HbF protein synthesis after AN-233 treatment. While our findings in K562 cell support efficacy, these cells arguably possess inherent features that make them less likely to recapitulate findings in erythroid cells [41]. Therefore, we next performed drug induction studies in human primary sickle erythroid precursors undergoing terminal differentiation to determine the ability of AN-233 to induce HbF under oxidative stress conditions.

Treatment with AN-233 significantly increased γ-globin mRNA and HbF levels in sickle erythroid cells. Interestingly, evaluation of changes in HbS protein revealed that the prodrug did not induce synthesis of adult globin chains. These effects of AN-233 are clinically desirable since drugs that either have no effect or decrease HbS levels would produce an anti-sickling result. Indeed, under hypoxic conditions we observed a lower number of sickle precursors after AN-233 treatment confirming an anti-sickling effect similar to that produced by HU.

After intracellular hydrolysis of AN-233 by esterase enzymes, two active metabolites BA and ALA are released. Therefore, to verify activity of these agents, we tested mechanisms of HbF induction through pathways regulated by both compounds. Butyric acid is a pan-histone deacetylase inhibitor, which mediates histones acetylation causing epigenetic changes in chromatin structure allowing accessibility of DNA binding proteins to activate gene transcription [42]. By ChIP assay, we confirmed that histone H3 and H4 acetylation levels were increased in the γ-globin gene promoter and LCR-HS2; by contrast, no significant change of histone acetylation occurred in the β-globin gene. A second mechanism by which BA induces HbF expression is through p38 MAPK phosphorylation to stimulate cell signaling and activation of CREB1 to achieve γ-globin gene transcription [12,14]. To support this mechanism of AN-233, we observed γ-globin gene silencing and a decrease in anti-sickling effects in primary erythroid precursors when the p38 MAPK inhibitor SB203580 was added.

The second active metabolite of AN-233 hydrolysis is ALA, a known precursor of heme biosynthesis involving eight enzymes in the mitochondrion and cytoplasm of cells [43]. Among them, δ-aminolevulinate synthase catalyzes the first and rate limiting reaction to produce ALA. The addition of exogenous ALA accelerates heme production [44] and enhances globin mRNA translation and Hb synthesis in cell culture systems [28,45]. To gain insights into heme-related mechanisms of HbF activation by AN-233, we investigated the HRI/eIF2αP signaling axis, normally regulated by heme levels *in vivo*. We observed a significant increase in heme levels by AN-233 and simultaneous silencing of the protein kinases HRI and eIF2αP, which normally block global protein synthesis. Recent studies from Blobel and colleagues [46] showed HRI depletion markedly increased HbF production and reduced sickling in human primary erythroid cells. Furthermore, diminished expression of the major γ-globin repressor BCL11A accounted in part for the effects of HRI depletion.

Studies have shown that activation of the eIF2α stress pathway mediates HbF induction through post-transcriptional mechanisms. For example, salubrinal activates eIF2α signaling to enhance HbF production in primary human erythroid cells [47]. Salubrinal selectively increased the number of actively translating ribosomes on γ-globin mRNA. Translational regulation of hemoglobin synthesis is mediated by HRI, which is an intracellular heme sensor that coordinates heme and globin synthesis during erythropoiesis [48]. In iron deficient states, HRI is activated and inhibits synthesis of globin chains and heme biosynthetic enzymes. The HRI-eIF2αP-ATF4 stress signaling pathway is important for regulating excess globin synthesis during erythropoiesis, and for adaptation to oxidative stress. Modulation of this signaling pathway with small chemicals may provide a novel therapy for β-hemoglobinopathies.

To translate novel HbF inducers into clinical trials requires evidence of efficacy in preclinical animal models. The *in vivo* safety and efficacy of oral AN-233 was previously explored in an anemic C57BL mouse model; mice were treated for 4 weeks with up to 400 mg/kg without toxicity [17]. In fact, hemoglobin levels improved and tissue harvested 4 to 6 h after one oral dose of AN-233 confirmed histone hyperacetylation in spleen tissue. We treated β-YAC mice, 5 days per week for 4 weeks, without observing anti-proliferative effects of AN-233 on erythropoiesis. The mild increase in reticulocyte counts and decrease in hemoglobin suggest mild hemolysis, which requires additional studies.

The β-YAC mouse model has been used to test different agents for their capacity to induce HbF *in vivo*. We demonstrated the ability of α-amino butyric acid to activate γ-globin transcription when combined with 5-azacytidine [34]. Subsequently, we tested the histone deacetylase inhibitor, Scriptaid, which activated γ-globin without affecting β-globin gene transcription [49]. Others agents analyzed in β-YAC mice that induce γ-globin include tranylcypromine (LSD1 inhibitor), sodium dimethyl butyrate (histone deacetylase inhibitor) and benserazide (DOPA decarboxylase inhibitor) [24,50,51] which support potential *in vivo* efficacy. Subsequent human trials with sodium dimethylbutyrate (HQK-1001) demonstrated HbF induction in 70% of patients with β-thalassemia, but was less effective in SCD [15,38]. Even though β-YAC mice undergo hemoglobin switching and serve as an excellent preclinical model for drug screening, limitation of the model included the lack of anemia and oxidative stress present in SCD and β-thalassemia. SCD mice or baboons provide additional animal models to test pharmacological agents for their potential to induce HbF. Therefore, additional studies in these models will provide evidence for clinical safety and efficacy.

5. Conclusion

The prodrug AN-233 is a novel oral active conjugate of BA (histone deacetylase inhibitor) and ALA (heme precursor). The ability of AN-233 to activate γ-globin transcription, induce HbF and produce an anti-sickling effect underscores benefits as a therapeutic agent. The data presented support potential development of AN-233 as a candidate drug for treatment of SCD.

Declaration of Competing Interest

Authors have no conflict of interests.

Acknowledgement

This work was funded through grants from the National Heart Lung and Blood Institute, HL069234 to BSP and HL111264 to KRP and the National Institute of Diabetes and Digestive and Kidney Diseases, DK100595 to KRP.

Author contributions

ARO contributed to experimental design, performed experiments, analyzed data and wrote the manuscript; XZ and BL contributed to primary culture studies and ChIP assay; KRP established the β-YAC transgenic mouse, drug design and revised paper; AN and AR synthesized AN233 and contributed to drug treatment design and writing paper; HX created database and performed statistical data analysis for tissue culture and mouse treatments and edited paper; BSP conceived and designed the study and wrote and revised the manuscript. Authors approve the final version of manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2019.102345.

References

[1] L. Pauling, H.A. Itano, et al., Sickle cell anemia a molecular disease, Science 110 (1949) 543–548.

[2] B. Modell, M. Darlison, Global epidemiology of hemoglobin disorders and derived service indicators, Bull. World Health Organ. 86 (2008) 480–487.
[3] F.B. Piel, M.H. Steinberg, D.C. Rees, Sickle cell disease, N. Engl. J. Med. 376 (2017) 1561–1573.
[4] P. Sundd, M.T. Gladwin, E.M. Novelli, Pathophysiology of sickle cell disease, Annu. Rev. Pathol. 14 (2019) 263–292.
[5] W.N. Poillon, B.C. Kim, G.P. Rodgers, C.T. Noguchi, A.N. Schechter, Sparing effect of hemoglobin F and hemoglobin A2 on the polymerization of hemoglobin S at physiologic ligand saturations, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 5039–5043.
[6] O.S. Platt, D.J. Brambilla, W.F. Rosse, P.F. Milner, O. Castro, M.H. Steinberg, P.P. Klug, Mortality in sickle cell disease. Life expectancy and risk factors for early death, N. Engl. J. Med. 330 (1994) 1639–1644.
[7] S. Charache, M.L. Terrin, R.D. Moore, G.J. Dover, F.B. Barton, S.V. Eckert, R.P. McMahon, D.R. Bonds, Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia, N. Engl. J. Med. 332 (1995) 1317–1322.
[8] L.K. Sahoo, B.K. Kullu, S. Patel, N.K. Patel, P. Rout, P. Purohit, S. Meher, Study of seminal fluid parameters and fertility of male sickle cell disease patients and potential impact of hydroxyurea treatment, J. Assoc. Physicians India 65 (2017) 22–25.
[9] G.F. Atweh, M. Sutton, I. Nassif, V. Boosalis, G.J. Dover, S. Wallenstein, E. Wright, L. McMahon, G. Stamatoyannopoulos, D.V. Faller, S.P. Perrine, Sustained induction of fetal hemoglobin by pulse butyrate therapy in sickle cell disease, Blood 93 (1999) 1790–1797.
[10] S.P. Perrine, G.D. Ginder, D.V. Faller, G.H. Dover, T. Ikuta, H.E. Witkowska, S.P. Cai, E.P. Vichinsky, N.F. Olivieri, A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders, N. Engl. J. Med. 328 (1993) 81–86.
[11] G.D. Sher, G.D. Ginder, J. Little, S. Yang, G.J. Dover, N.F. Olivieri, Extended therapy with intravenous arginine butyrate in patients with beta-hemoglobinopathies, N. Engl. J. Med. 332 (1995) 1606–1610.
[12] J. Sangerman, M.S. Lee, X. Yao, E. Oteng, C.H. Hsiao, W. Li, S. Zein, S.F. Ofori-Acquah, B.S. Pace, Mechanism for fetal hemoglobin induction by histone deacetylase inhibitors involves gamma-globin activation by CREB1 and ATF-2, Blood 108 (2006) 3590–3599.
[13] O. Witt, K. Sand, A. Pekrun, Butyrate-induced erythroid differentiation of human K562 leukemia cells involves inhibition of ERK and activation of p38 MAP kinase pathways, Blood 95 (2000) 2391–2396.
[14] V. Ramakrishnan, B.S. Pace, Regulation of gamma-globin gene expression involves signaling through the p38 MAPK/CREB1 pathway, Blood Cells Mol. Dis. 47 (2011) 12–22.
[15] M.E. Reid, A. El Beshlawy, A. Inati, A. Kutlar, M.R. Abboud, J. Haynes Jr., R. Ward, B. Sharon, A.T. Taher, W. Smith, D. Manwani, R.G. Ghalie, A double-blind, placebo-controlled phase II study of the efficacy and safety of 2,2-dimethylbutyrate (HQK-1001), an oral fetal globin inducer, in sickle cell disease, Am. J. Hematol. 89 (2014) 709–713.
[16] G. Berkovitch, D. Doron, A. Nudelman, Z. Malik, A. Rephaeli, Novel multifunctional acyloxyalkyl ester prodrugs of 5-aminolevulinic acid display improved anticancer activity independent and dependent on photoactivation, J. Med. Chem. 51 (2008) 7356–7369.
[17] A. Rephaeli, N. Tarasenko, E. Fibach, G. Rozic, I. Lubin, J. Lipovetsky, S. Furman, Z. Malik, A. Nudelman, Bi-functional prodrugs of 5-aminolevulinic acid and butyric acid increase erythropoiesis in anemic mice in an erythropoietin-independent manner, European Journal of Pharmaceutical Sciences : Official Journal of the European Federation for Pharmaceutical Sciences 91 (2016) 91–97.
[18] B. Li, X. Zhu, M.A. Hossain, C.R. Guy, H. Xu, J. Bungert, B.S. Pace, Fetal hemoglobin induction in sickle erythroid progenitors using a synthetic zinc finger DNA-binding domain, Haematologica 103 (2018) e384–e387.
[19] C.M. Ward, B. Li, B.S. Pace, Original research: stable expression of miR-34a mediates fetal hemoglobin induction in K562 cells, Exp. Biol. Med. 241 (2016) 719–729.
[20] S. Aggarwal, A. Lam, S. Bolisetty, M.A. Carlisle, A. Traylor, A. Agarwal, S. Matalon, Heme attenuation ameliorates irritant gas inhalation-induced acute lung injury, Antioxid. Redox Signal. 24 (2016) 99–112.
[21] X. Zhu, C. Xi, B. Thomas, B.S. Pace, Loss of NRF2 function exacerbates the pathophysiology of sickle cell disease in a transgenic mouse model, Blood 131 (2018) 558–562.
[22] L. Liu, S. Karmakar, R. Dhar, M. Mahajan, A. Choudhury, S. Weissman, B.S. Pace, Regulation of Ggamma-globin gene by ATF2 and its associated proteins through the cAMP-response element, PLoS One 8 (2013) e78253.
[23] K.R. Peterson, C.H. Clegg, C. Huxley, B.M. Josephson, H.S. Haugen, T. Furukawa, G. Stamatoyannopoulos, Transgenic mice containing a 248-kb yeast artificial chromosome carrying the human beta-globin locus display proper developmental control of human globin genes, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7593–7597.
[24] M.S. Boosalis, J.I. Sangerman, G.L. White, R.F. Wolf, L. Shen, Y. Dai, E. White, L.H. Makala, B. Li, B.S. Pace, M. Nouraie, D.V. Faller, S.P. Perrine, Novel inducers of fetal globin identified through high throughput screening (HTS) are active in vivo in anemic baboons and transgenic mice, PLoS One 10 (2015) e0144660.
[25] L. Cioe, A. McNab, H.R. Hubbell, P. Meo, P. Curtis, G. Rovera, Differential expression of the globin genes in human leukemia K562(S) cells induced to differentiate by hemin or butyric acid, Cancer Res. 41 (1981) 237–243.
[26] T. Rutherford, J.B. Clegg, D.R. Higgs, R.W. Jones, J. Thompson, D.J. Weatherall, Embryonic erythroid differentiation in the human leukemic cell line K562, Proc. Natl. Acad. Sci. U. S. A. 78 (1981) 348–352.
[27] N. Bianchi, F. Ongaro, C. Chiarabelli, L. Gualandi, C. Mischiati, P. Bergamini, R. Gambari, Induction of erythroid differentiation of human K562 cells by cisplatin analogs, Biochem. Pharmacol. 60 (2000) 31–40.
[28] D. Chiabrando, S. Mercurio, E. Tolosano, Heme and erythropoieis: more than a structural role, Haematologica 99 (2014) 973–983.
[29] S. Liu, R.N. Suragani, F. Wang, A. Han, W. Zhao, N.C. Andrews, J.J. Chen, The function of heme-regulated eIF2alpha kinase in murine iron homeostasis and macrophage maturation, J. Clin. Invest. 117 (2007) 3296–3305.
[30] J.J. Chen, Regulation of protein synthesis by the heme-regulated eIF2alpha kinase: relevance to anemias, Blood 109 (2007) 2693–2699.
[31] S.H. Back, D. Scheuner, J. Han, B. Song, M. Ribick, J. Wang, R.D. Gildersleeve, S. Pennathur, R.J. Kaufman, Translation attenuation through eIF2alpha phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells, Cell Metab. 10 (2009) 13–26.
[32] B.S. Pace, X.H. Qian, J. Sangerman, S.F. Ofori-Acquah, B.S. Baliga, J. Han, S.D. Critz, p38 MAP kinase activation mediates gamma-globin gene induction in erythroid progenitors, Exp. Hematol. 31 (2003) 1089–1096.
[33] T. Ikuta, Y.W. Kan, P.S. Swerdlow, D.V. Faller, S.P. Perrine, Alterations in protein-DNA interactions in the gamma-globin gene promoter in response to butyrate therapy, Blood 92 (1998) 2924–2933.
[34] B. Pace, Q. Li, K. Peterson, G. Stamatoyannopoulos, Alpha-amino butyric acid cannot reactivate the silenced gamma gene of the beta locus YAC transgenic mouse, Blood 84 (1994) 4344–4353.
[35] T.J. Ley, J. DeSimone, C.T. Noguchi, P.H. Turner, A.N. Schechter, P. Heller, A.W. Nienhuis, 5-Azacytidine increases gamma-globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia, Blood 62 (1983) 370–380.
[36] Y. Saunthararajah, C.A. Hillery, D. Lavelle, R. Molokie, L. Dorn, L. Bressler, S. Gavazova, Y.H. Chen, R. Hoffman, J. DeSimone, Effects of 5-aza-2′-deoxycytidine on fetal hemoglobin levels, red cell adhesion, and hematopoietic differentiation in patients with sickle cell disease, Blood 102 (2003) 3865–3870.
[37] S.P. Perrine, G.D. Ginder, D.V. Faller, G.H. Dover, T. Ikuta, H.E. Witkowska, S.P. Cai, E.P. Vichinsky, N.F. Olivieri, A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders, N. Engl. J. Med. 328 (1993) 81–86.
[38] S. Fucharoen, A. Inati, N. Siritanaratku, S.L. Thein, W.C. Wargin, S. Koussa, A. Taher, N. Chaneim, M. Boosalis, R. Berenson, S.P. Perrine, A randomized phase I/II trial of HQK-1001, an oral fetal globin gene inducer, in beta-thalassaemia intermedia and HbE/beta-thalassaemia, Br. J. Haematol. 161 (2013) 587–593.
[39] M.E. Reid, A. El Beshlawy, A. Inati, A. Kutlar, M.R. Abboud, J. Haynes Jr., R. Ward, B. Sharon, A.T. Taher, W. Smith, D. Manwani, R.G. Ghalie, A double-blind, placebo-controlled phase II study of the efficacy and safety of 2,2-dimethylbutyrate (HQK-1001), an oral fetal globin inducer, in sickle cell disease, Am. J. Hematol. 89 (2014) 709–713.
[40] R. Molokie, D. Lavelle, M. Gowhari, M. Pacini, L. Krauz, J. Hassan, V. Ibanez, M.A. Ruiz, K.P. Ng, P. Woost, T. Radivoyevitch, D. Pacelli, S. Fada, M. Rump, M. Hsieh, J.F. Tisdale, J. Jacobberger, M. Phelps, J.D. Engel, S. Saraf, L.L. Hsu, V. Gordeuk, J. DeSimone, Y. Saunthararajah, Oral tetrahydrouridine and decitabine for non-cytotoxic epigenetic gene regulation in sickle cell disease: a randomized phase 1 study, PLoS Med. 14 (2017) e1002382.
[41] N. Komatsu, Culture of megakaryocytic cell lines: uses and limitations, Methods Mol. Biol. 272 (2004) 361–373.
[42] C.A. Mizzen, C.D. Allis, Linking histone acetylation to transcriptional regulation, Cell. Mol. Life Sci. 54 (1998) 6–20.
[43] R.S. Ajioka, J.D. Phillips, J.P. Kushner, Biosynthesis of heme in mammals, Biochim. Biophys. Acta 1763 (2006) 723–736.
[44] Z. Malik, H. Lugaci, J. Hanania, Stimulation of friend erythroleukemic cell cytodifferentiation by 5-amino levulinic acid; porphyrins, cell size, segregation of sialoglycoproteins, and nuclear translocation, Exp. Hematol. 16 (1988) 330–335.
[45] T. Fujiwara, K. Okamoto, R. Niikuni, K. Takahashi, Y. Okitsu, N. Fukuhara, Y. Onishi, K. Ishizawa, R. Ichinohasama, Y. Nakamura, M. Nakajima, T. Tanaka, H. Harigae, Effect of 5-aminolevulinic acid on erythropoiesis: a preclinical in vitro characterization for the treatment of congenital sideroblastic anemia, Biochem. Biophys. Res. Commun. 454 (2014) 102–108.
[46] J.D. Grevet, X. Lan, N. Hamagami, C.R. Edwards, L. Sankaranarayanan, X. Ji, S.K. Bhardwaj, C.J. Face, D.F. Posocco, O. Abdulmalik, C.A. Keller, B. Giardine, S. Sidoli, B.A. Garcia, S.T. Chou, S.A. Liebhaber, R.C. Hardison, J. Shi, G.A. Blobel, Domain-focused CRISPR screen identifies HRI as a fetal hemoglobin regulator in human erythroid cells, Science 361 (2018) 285–290.
[47] C.K. Hahn, C.H. Lowrey, Induction of fetal hemoglobin through enhanced translation efficiency of gamma-globin mRNA, Blood 124 (2014) 2730–2734.
[48] J.J. Chen, Translational control by heme-regulated eIF2alpha kinase during erythropoiesis, Curr. Opin. Hematol. 21 (2014) 172–178.
[49] J. Johnson, R. Hunter, R. McElveen, X.H. Qian, B.S. Baliga, B.S. Pace, Fetal hemoglobin induction by the histone deacetylase inhibitor, scriptaid, Cell Mol Biol (Noisy-le-grand) 51 (2005) 229–238.
[50] B.S. Pace, G.L. White, G.J. Dover, M.S. Boosalis, D.V. Faller, S.P. Perrine, Short-chain fatty acid derivatives induce fetal globin expression and erythropoiesis in vivo, Blood 100 (2002) 4640–4648.
[51] L. Shi, S. Cui, J.D. Engel, O. Tanabe, Lysine-specific demethylase 1 is a therapeutic target for fetal hemoglobin induction, Nat. Med. 19 (2013) 291–294.
[52] X. Zhu, B. Li, B.S. Pace, NRF2 mediates gamma-globin gene regulation and fetal hemoglobin induction in human erythroid progenitors, Haematologica 102 (2017) e285–e288.