Novel Inducers of Fetal Globin Identified through High Throughput Screening (HTS) Are Active In Vivo in Anemic Baboons and Transgenic Mice

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Abstract

High-level fetal (γ) globin expression ameliorates clinical severity of the beta (β) hemoglobinopathies, and safe, orally-bioavailable γ-globin inducing agents would benefit many patients. We adapted a LCR-γ-globin promoter-GFP reporter assay to a high-throughput robotic system to evaluate five diverse chemical libraries for this activity. Multiple structurally- and functionally-diverse compounds were identified which activate the γ-globin gene promoter at nanomolar concentrations, including some therapeutics approved for other conditions. Three candidates with established safety profiles were further evaluated in erythroid progenitors, anemic baboons and transgenic mice, with significant induction of γ-globin expression observed in vivo. A lead candidate, Benserazide, emerged which demonstrated >20-fold induction of γ-globin mRNA expression in anemic baboons and increased F-cell proportions by 3.5-fold in transgenic mice. Benserazide has been used chronically to inhibit amino acid decarboxylase to enhance plasma levels of L-dopa. These studies confirm the utility of high-throughput screening and identify previously unrecognized fetal globin inducing candidates which can be developed expediently for treatment of hemoglobinopathies.

Introduction

The β-thalassemias and sickle cell disease (SCD), genetic disorders affecting the β-chain of adult hemoglobin A, are serious anemias and comprise a growing global health burden [1–24]. Fetal hemoglobin (HbF, α2γ2, HBG) is an endogenous hemoglobin present in all humans which is normally suppressed in infancy. Pharmacological augmentation of fetal hemoglobin...
is a commercial organization, which provided partial funding for this work (through NIH grants), and did not play a role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript and only provided financial support in the form of authors’ salaries and/or research materials. The funder provided support in the form of partial salaries for authors [SPP, DVF] and experimental expenses, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the “author contributions’ section”.

Competing Interests: SPP and DVF, authors of this manuscript have the following competing interests: employment, equity interest in, and research funding from Phoenicia Biosciences, Inc. GLW and RFW have received research funding from Phoenicia Biosciences, Inc. The other authors have declared that no competing interests exist. Drs. Perrine and Faller are Founders and have employment support from Phoenicia BioSciences. This does not alter the authors’ adherence to PLOS ONE policies on sharing data and materials.

**Methods**

High throughput screening (HTS) assay

A high throughput screening (HTS) assay was developed using a cell-based reporter, stably transfected with a construct containing the 1.4-kilobase (kb) KpnI-BglIII fragment of the human HS2 (hypersensitive sire 2) of the locus control region (LCR) linked to the γ-globin promoter and the enhanced green fluorescent protein (EGFP) reporter gene, as illustrated in Fig 1. Because EGFP messenger RNA (mRNA) is very stable, positive changes average 1.2- to 2-fold, and weak inducers are not detectable in this system [30]. Two-fold or higher induction over control indicates strong inducers of γ-globin gene activity[30]. The HTS assay was developed in a 96-well format on a Tecan SpectraFluor Plus, incorporating multiple positive and negative control wells in each plate, generating 40–80 assay points for each. Optimization of the number of cells per well in a 96-well format was carried out and ideal time points for optimal fluorescence...
Fig 1. Schema of the high-throughput screening assay (HTS). (A.) A construct containing the 1.4-kilobase (kb) KpnI-BgIII fragment of HS2 of the locus control region (LCR) linked to the γ-globin gene promoter driving the enhanced green fluorescent protein (EGFP) reporter gene stably transfected in K562 cells. Transfected cells were treated with compounds from diverse chemical libraries in an HTS format. (B.) Example of a hit in the high-throughput system is shown for compound MS-275. Fluorescent microphotographs of untreated cells are shown in the left panel; cells treated with MS-275 are shown in the right panel. (C.) Relative activity (relative fluorescence units) of new candidates identified by the HTS screen: Ambroxol (ABX), Desloratadine (DLT), MS-275, Resveratrol (RSV), Benserazide (BEN), NSC-95397 (NSC), and Idarubicin (IDA) are shown.

doi:10.1371/journal.pone.0144660.g001
measurements were identified. In this 96-well format, a positive signal of intensity of 9000 RFUs was demonstrated in a volume of 100 μl. A signal-to-background ratio of at least 7 was demonstrated. The mean and standard deviations for the two controls were calculated, and the Z' factor was generated (Z' = 0.71) Two campaigns of 10,000 compounds were performed.

Erythroid progenitor cultures and globin expression analyses

Erythroid progenitors were cultured from de-identified cord blood obtained from the New York Blood Center using CD34+ cells enriched using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) and EasySep (StemCell Technologies, Vancouver, BC), as previously described [28,30,31]. RNA was extracted and quantitative real time (qRT)-PCR was performed as previously described [28,30]. Briefly, cDNA was generated from equal amounts of total RNA extracted using the PerfectPure RNA purification kit (5 Prime Inc. Gaithersburg, MD) or RNA STAT-60 isolation reagent (Teltest, Friendswood, TX). Real-time PCR was performed using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA), with γ-globin mRNA calculated by the ΔΔCt method. The following primer set was used for γ-globin gene amplification: TCAAGAGAGGAGCAACAGCTA and GAGATCATCCAGGTGCTTT. GAPDH and 18S mRNA levels were used for standardization.

For immunoblotting, erythroid progenitor cells on day 14 of Phase 2 culture were lysed in Laemmli sample buffer and subjected to 12% SDS-PAGE with constant voltage at 30 V for 3 hr. Proteins were transferred to a nitrocellulose membrane and probed with antibodies to HbF (sc-21756, Santa Cruz Biotechnology, Dallas, Texas) and β-actin (Sigma A, St. Louis, MO)[34]. Proteins were visualized with the GE Imaging System (Image Quant, LSD4010 (GE Healthcare, Piscataway, NJ)) and quantified with ImageJ software (NIH, Bethesda, MD)[34].

In vivo studies in anemic non-human primates

Studies to evaluate globin expression with treatment with the lead test candidates were performed with approval of the Institutional Animal Care and Use committee of the University of Oklahoma Health Science Center, employing juvenile baboons (Papio hamadryas anubis) from the breeding colony of the University of Oklahoma. Only baboons which adapt well to research environment are used. The baboons are maintained with indwelling vascular catheters, placed under general anesthesia, which are protected in a vest and swivel that allows free movement and in an environment with enrichment toys and companionship. Briefly, animals were chronically phlebotomized (by 3.7 to 5 mls/kg/day, to achieve and maintain stable anemia with total hemoglobin of 7.0 to 7.5 g/dl, as previously described [15]. This magnitude of phlebotomy reproduces stress erythropoiesis which occurs in the hemoglobinopathies, and exchanged the blood volume approximately every 10–20 days. Test drug candidates were administered based on the more rapid metabolism of the baboon compared to humans and projected human equivalent doses, based on previously used human doses as follows: Desloratadine was administered orally (0.5 mg/kg/dose), three times a week over two weeks. MS-275 (Selleckchem, Houston, TX) was administered orally three times a week for two weeks at doses of 0.2 mg/kg/dose. Benserazide (Enzon, Farmingdale, NY) was administered orally, at 1 mg/kg for 4 days/for one week or 2 mg/kg, 4 days per week, for two weeks. A washout period was provided between administration of different compounds in the same baboon, and each drug candidate was tested in at least 2 baboons. Results were compared to Hydroxyurea administered for 4 days/week at 25 mg/kg/dose (125 mg/day) over 3 weeks. Complete blood counts were performed 3 times per week. Assays of γ-globin mRNA, total hemoglobin, and % F-cells were assessed before and during treatment with test compounds. No animals were sacrificed for these studies.
Analysis of F-cells by flow cytometry

Flow cytometry was performed on the baboons' peripheral blood as previously described[15]. Briefly, cells were washed with PBS containing 0.1% BSA, fixed with 0.05% glutaraldehyde (Polysciences Inc Warrington, PA) for 10 minutes at room temperature, washed twice in 0.1% BSA in PBS, and cells were permeabilized with 0.1% Triton-X100 (American Bioanalytical, Natick, MA) for 3 minutes at room temperature. Cells were then washed, re-suspended in 0.1% BSA in PBS and dispensed in 5 x10⁵ cells/tube. PerCP isotype-labeled and unstained cells were used as controls. Thiazole orange was used to identify proportions and populations of reticulocytes. A custom-synthesized PerCP mouse anti-human antibody (BD Biosciences, San Jose, CA) that detects HbF-containing cells in the baboon was used to label fetal globin-containing cells. Samples were incubated in the dark at room temperature for 30 minutes, washed several times with 0.1% BSA in PBS, and analyzed by flow cytometry, using a FACS Calibur (Beckton Dickinson, San Jose, CA) and CellQuest software.

In vivo studies in transgenic mice

Mice transgenic for the human β-globin gene locus including the locus control region (LCR) in a yeast artificial chromosome (YAC) were previously described with the approval of the Institutional Animal Care and Use Committee of Georgia Regents University[29]. Mice were treated with water as a vehicle control, or Hydroxyurea, 100 mg/kg/dose, administered once daily for 5 days/week, or the lead candidate (Benserazide) administered at 20 mg/kg/dose, (a human equivalent dose of 3 mg/kg), 3 times per week for 5 weeks. Dosing was performed by intraperitoneal injection to ensure consistent drug delivery. Water was administered in the same volume (100 microliters) as the drug candidates as a control. Blood was sampled for complete blood counts and for F-cell quantitation by flow cytometry as previously described[15].

Statistical Analyses

Data was analyzed by paired t-tests and by a Wilcoxon signed rank test; a level of 0.05 was considered significant.

Results

HTS-identified compounds

A schema and read-out example of a positive "hit" are shown in Fig 1. The HTS identified multiple candidates which induced γ-globin gene expression, including some drugs from the EMA- and FDA-approved library (Fig 1), with highly diverse structures. Candidates with oral activity, and/ or benign safety profiles are shown in Fig 2. The structure of a known positive inducer, butyric acid is shown for comparison. High activity was found with Idarubicin, as previously reported[19], which validated the HTS system. The magnitude of induction was similar to the range reported with other high throughput screening campaigns[18]. As only a few
candidates did not inhibit erythroid cell growth at concentrations that induced γ-globin expression, and/or had routes of administration and safety profiles which appeared suitable for potential therapeutics for hemoglobinopathies, further analyses were focused on those candidates which are orally bioavailable.

γ-globin induction in erythroid progenitors

The non-cytotoxic candidates were assayed for their ability to induce γ-globin mRNA in treated erythroid cells compared to untreated progenitors from the same sample. Treatment with the new candidates induced γ-globin mRNA by 2 to 3.9-fold above untreated controls and
was significantly different by paired t-tests, p < 0.01, as shown in Fig 3A. A positive control, arginine butyrate, induced by a mean of 1.9-fold, p = 0.01, in the same cells. Expression of HbF at the protein level was confirmed by Western blot; representative examples of Benserazide- and MS-275-treated erythroid cells demonstrated 3.2-fold and 2.5-fold greater HbF, respectively, than in untreated control cells from the same source, shown in Fig 3B.
γ-globin mRNA induction in non-human primates

Three candidates, Desloratadine (DLT), MS-275 (Etinostat), and Benserazide, which are known to have benign safety profiles with long-term clinical use, were selected for further investigation in vivo in anemic baboons. The candidates were compared over 2 weeks of administration following a two-week wash-out period between each drug exposure. Administration of MS-275 (0.2 mg/kg given 3 times/week for two weeks) produced a 2.7-fold peak increase above baseline in γ-globin mRNA, an increase in F-cells from 18% to 26%, and an increase in F-reticulocytes from 40% to 70% (Fig 4). An increase in total hemoglobin from 7.5 to 9.0 gm/dl was observed, despite the ongoing phlebotomy (Fig 4). In an anemic baboon treated with DLT (0.5 mg/kg given three times/week for two weeks), γ-globin mRNA increased by 5.5-fold over baseline in the second week of treatment, and the effect persisted for four days after administration of the last dose, consistent with the time-frame required for erythroid cell differentiation in the baboon (Fig 5). Proportions of F-reticulocytes increased by 19%, from 39% to 58%, (33% of the baseline value), and the increase persisted for 5–10 days following drug administration. Administration of Benserazide (1 mg/kg given once/day orally, 3 times/week for 2 weeks, or 2 mg/kg, 5 days/week) produced the most dramatic increase in γ-globin mRNA. A 12-fold induction in γ-globin mRNA was observed after 1 mg/kg dose, and 27-to 33-fold increase above baseline levels was observed after 2 mg/kg/doses (p<0.01) (Fig 6A). Although changes in HbF levels are difficult to detect with brief 2-week treatment courses, increases in proportions of red blood cells expressing HbF protein, F-reticulocytes, increased from 14 to 36% after 2 mg/kg doses (61% of baseline) (Fig 6B). Total hemoglobin increased from 7.6 g/dl at treatment initiation to 9.0 g/dl, despite continued phlebotomy of 4 mls blood/
Fig 5. Fetal globin induction in an anemic baboon treated with Desloratidine (DLT). (A) Treatment with DLT (once per day, 5 days/week), shown by the black bars above the graph, resulted in 5-to 11-fold induction in γ-globin mRNA, (B) a 15% increase in F-reticulocytes, and (C) a 1.0 gm/dL increase in total hemoglobin.

doi:10.1371/journal.pone.0144660.g005
kg/day, which exchanged the baboon’s blood volume approximately every 12 days (Fig 6C). Fig 7 shows a comparison of peak induction of γ-globin mRNA and F-reticulocytes observed in the anemic baboons treated with these agents and compared to effects of Hydroxyurea (HU) or sodium 2,2 dimethylbutyrate (ST20). Small changes in total HbF were detected by HPLC after the brief 2-week treatment courses as follows: with DLT, from 2.5 to 5.6%, MS-275 from 0.6 to 1.3%, and Benserazide from 0.6 to 4.35%.

Fig 6. Fetal globin induction in an anemic baboon treated with Benserazide. (A.) Treatment with Benserazide resulted in a dose-dependent increase in γ-globin mRNA up to 34-fold. Two doses were tested, 1 mg/kg/dose (shown by the open squares) and 2 mg/kg/dose, (shown by the dark squares). (B.) F-reticulocytes increased by 15% during treatment with 2 mg/kg, and (C.) total hemoglobin increased by 1.5 gm/dl, despite the phlebotomy which exchanged the blood volume every 12 days.

doi:10.1371/journal.pone.0144660.g006
Effects of HTS drugs in β-globin locus transgenic mice

Mice transgenic for a YAC containing the human LCR-β-globin gene locus have been shown to faithfully recapitulate the human fetal to adult switch, and to respond to prior generation γ-globin inducing agents [29, 35]. Analyses of F-cells in this human β-globin complex transgenic murine model treated with Hydroxyurea or Benserazide at baseline, week 2 (top left panel) and week 5 of treatment (top right panel) are shown in Fig 8A–8D. Mean values in three animals are compared. Proportions of F-cells and mean fluorescence intensity (MFI), both measures of HbF protein, increased significantly within the first week of Hydroxyurea treatment; F-cells increased from 1.2% to 5.7% and MFI increased from 24% to 47% (Fig 8A and 8C); these values declined to 2% F-cells and 13% MFI by week 5, perhaps associated with marrow suppression (Fig 8B and 8D). Treatment with Benserazide (BEN) resulted in an increase in F-cells from 0.7% to 7.3% at week 2, and this level persisted at week 5; MFI increased from 24 to 41% at week 2 and to 50% at week 5 with Benserazide treatment. The changes with the two drug treatments were statistically significant compared to the (water) control values, \( p < 0.0001 \) with Benserazide treatment and \( p < 0.04 \) with Hydroxyurea (Wilcoxon signed rank test). Hemoglobin increased from 12 g/dL to 14 g/dL by week 2 with both treatments, and by another 1 to 1.5 gm/dL by week 5 with Benserazide treatment (Fig 8E), suggesting an independent effect on erythropoiesis.

Discussion

Multiple compounds are reported to induce expression of fetal globin, although only one therapeutic, Hydroxyurea (HU), is currently FDA-approved for treatment of sickle cell disease, and considered ameliorating in approximately 50% of adult patients, causing a mean 3% rise in HbF which reduces morbidity in many treated subjects, and increases survival in those who obtain total HbF of 0.5 g/dl[3,6,8, 11–39]. Additional therapeutics should beneficial for treatment of the diverse global hemoglobinopathy population[4–6]. Developing therapies for orphan conditions requires lengthy trials to identify safe dose limits and doses which produce efficacy, with high costs required for clinical development of new chemical entities. This high-throughput screening effort identified structurally- and functionally-unrelated drug candidates with HbF-inducing activity, although many candidates are not suitable or optimal for long-
Fig 8. Responses of transgenic mice to treatment with Benserazide or Hydroxyurea. (A. & C.) Mice containing the human non-alpha globin genes in a YAC were treated with vehicle (water) as a control, Hydroxyurea (HU), or Benserazide. % F-cells and mean fluorescent intensity are shown pretreatment vs. 2 weeks after initiation of treatment, and (B. & D) pretreatment vs. 5 weeks after initiation of treatment. Changes with treatment were significant, (p<0.001 for Benserazide and p<0.05 for Hydroxyurea). (E.) Total hemoglobin levels are shown pre-treatment vs. 2 weeks and 5 weeks after initiation of each treatment.

doi:10.1371/journal.pone.0144660.g008
term treatment. For example, the cytotoxic anticancer drug Idarubicin was identified to have demonstrated high activity, as previously reported[19], which validated the HTS system, but this and several other candidates have side-effect profiles which are undesirable for treatment of a nonmalignant chronic condition. However, a few previously unrecognized small molecules were found to efficiently induce the fetal globin gene promoter, including two candidates approved for treatment of other conditions which have decades of clinical use. Their activity was verified in erythroid cell progenitors and predictive animal models.

Desloratadine, an oral, long-acting tricyclic antihistamine which is approved virtually world-wide, is used to treat symptoms of allergic reactions by blocking the histamine receptor (H1), and preventing activation of H1 receptor-containing cells. Benserazide, an inhibitor of decarboxylation of aromatic amino acids in peripheral (extracerebral) tissues, is used medically to enhance the pharmacokinetic profile of Levodopa, which results in higher concentrations of dopamine in the brain, thus lessening the side-effects observed with higher doses of Levodopa alone[40]. Benserazide is commercially provided in an oral combination tablet with Levodopa for treatment for Parkinson’s disease, and has been approved and widely utilized in this combination for more than 40 years[40] Three other inducers which were not pursued in animal models included: Ambroxol, a drug with secretolytic and secretomotoric actions that restores respiratory physiological clearance mechanisms in bronchopulmonary diseases, which is used as an inhalant. Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a naturally-occurring compound with diverse actions, is rapidly metabolized to multiple metabolites in humans, and therefore was not evaluated in these models in vivo. The bioactive NSC-95397 showed potent inducing activity in vitro, but blocks G2/M cell cycle phase transition and cell growth, which is undesirable in beta thalassemia, so it was also not evaluated in vivo.

The three candidates which have favorable human safety profiles were compared in the anemic baboon model, and their activity was compared to fetal globin induction observed with the established fetal globin inducers Hydroxyurea and sodium 2, 2 dimethylbutyrate (ST20 or HQK-1001)[29–31]. Desloratadine induced γ-globin mRNA up to 11-fold above baseline, MS-275 induced by 2.7-fold, while Benserazide induced by 20- to 33-fold above baseline in two different treated baboons. In beta YAC transgenic mice, F-cells increased within 2 weeks of initiation of Benserazide treatment at 20 mg/kg, compared to HU at 100 mg/kg/dose, and higher proportions of F-cells persisted with Benserazide treatment after 5 weeks than with HU. Human equivalent doses projected from effective doses of Benserazide in the baboon are 0.5 to 1.5 mg/kg/dose for an adult human, which is a lower range than the standard daily doses of 200–300 mg total typically used in an average 70 kg adult with Parkinson’s disease [40]. The benign safety profile of Benserazide, utilized chronically for >40 years[40], with strong fetal globin-inducing activity in baboons and transgenic mice, strongly suggest that clinical evaluation of this therapeutic in patients with hemoglobin disorders is warranted. HbF levels have not been monitored in these patients, to our knowledge.

Many compounds have been found to stimulate γ-globin gene expression, in preclinical systems, acting through a variety of cellular and molecular mechanisms[4–6, 11–13, 17, 18, 37, 38]. The cytotoxic agents, were initially thought to accelerate the maturation of erythroid precursors and produce progeny which maintained the “fetal stage” of globin switching. Other investigators have generated evidence that these drugs may produce a cellular stress response or other signaling pathways that contribute to fetal globin expression[34–37]. Agents which act “directly” on the γ-globin promoter include a variety of compounds of which some disrupt repressor complexes regulating γ-globin gene expression[3–6,11–13,17]. The down-regulation of BCL11A by butyrate[41], and other short-chain fatty acid derivatives such as sodium 2,2 dimethylbutyrate, may alter the ability of BCL11A to recruit the LCR to specific β-globin complex genes[6,11–13, 17]. HDAC inhibitors, particularly inhibitors of HDACs 1, 2 and 3, block
the enzymatic activity of these HDAC isotypes[38] contained in the NURD/CoREST repressor complexes which silence γ-globin expression, releasing this repression and restoring transcription[3–6,38]. Other molecules, including short-chain fatty acid derivatives which lack HDAC activity, cause dissociation of HDAC3 from these same complexes, also releasing transcriptional repression[12–13]. Initial studies of mechanisms of action indicate Benserazide treatment of erythroid progenitors results in displacement of HDAC-3, LSD-1, and suppression of BCL-11A [6] (manuscript in press). Translation of potential therapies in the diverse hemoglobinopathy population is likely affected by underlying molecular mutations, genetic modifier traits[42–50], and by physiologic factors such as erythrokinetics and endogenous erythropoietin levels[6]. Multiple therapeutics which act through different, potentially complementary mechanisms, may be required to successfully translate this approach in severely affected patients.

The fetal globin inducing activity demonstrated in two animal species here strongly suggests that Benserazide should have activity in human patients, because these animal models have been predictive of activity in subsequent human clinical trials of 5-azacytidine, Butyrate, Isobutyramide, and sodium 2,2 dimethylbutyrate. These earlier generation candidates induced fetal globin expression in baboons generally by 1.5 to 2-fold, and subsequently in clinical trials induced fetal globin protein in patients in thalassemia patients up to 20%. Benserazide emerged as a lead candidate for clinical evaluation due to its efficacy in vivo and because it has shown safety with chronic human use for decades in many countries[40]. MS-275 also offers particular interest due to its long half-life, allowing once/week administration, and because histone acetylation, which enhances chromatin accessibility, is likely to facilitate efficacy of other types of γ-globin inducers, offering an approach for combination therapies.

In summary, drug candidate hits from a high-throughput screen employing the human fetal globin gene promoter linked to an EGFP reporter were validated with in vivo responses demonstrated in two predictive animal models, including nonhuman primates. Whether select populations of patients with certain mutations or genetic modifiers will respond better to any single agent than others must be evaluated in clinical trials. Previously unrecognized, yet approved, therapeutic candidates discovered in this system offer a development route with lower risk than new chemical entities for which safety profiles and effective administration schedules must be determined.

Acknowledgments

We thank Sarah Haigh, Ada Kane, Nicole Reuter, David Carey, and Marilyn Perry Carey for dedicated and expert technical assistance and Cloret Carl for assistance with preparation of the manuscript.

This work was supported by grants from the National Institutes of Health, R01 DK-52962, (SPP, Boston University), R41 HL-105816 (SPP, Phoenicia BioSciences), and R42 HL-110727 (Phoenicia BioSciences), 2 P40 ODO010988-16 (GLW, University of Oklahoma) and UL1-TR000157 (RFW, University of Oklahoma). SMN was supported by P50 HL-118006. The funders had no role in study design, data collection or analysis, decision to publish, or preparation of the manuscript.

Author Contributions

Conceived and designed the experiments: SPP DVF. Performed the experiments: MSB JS LS YD BL LM EW RFW GLW. Analyzed the data: BSP MSB SPP DVF MN. Wrote the paper: SPP DVF BSP.
References

1. Weatherall DJ. The inherited diseases of hemoglobin are an emerging global health burden. Blood. 2010; 115:4331–4336. doi: 10.1182/blood-2010-01-251348 PMID: 2023970

2. Vichinsky EP, MacKlin EA, Waye JS, Lorey F, Olivieri NF. Changes in the epidemiology of thalassemia in North America: a new minority disease. Pediatrics. 2005; Dec; 116(6):e818–25. PMID: 16291734

3. Steinberg MH, Rodgers GP. Pharmacologic modulation of fetal hemoglobin. Medicine. 2001; 80: 328–344. PMID: 11552087

4. Wilbur A, Neinhuis AW, Person DA. Transcriptional regulation of fetal to adult hemoglobin switching: new therapeutic opportunities. Blood. 2012; 120(15):2949–2953. doi: 10.1182/blood-2012-06-292078 PMID: 22904296

5. Bauer DE, Kamran SC, Orkin SH. Reawakening fetal hemoglobin: prospects for new therapies for the beta-globin disorders. Blood. 2012; 120(15):2949–2953. doi: 10.1182/blood-2012-06-292078 PMID: 22904296

6. Perrine SP, Pace BS, Faller DV. Targeted fetal hemoglobin induction for treatment of beta hemoglobinopathies. In: Vichinsky EP, editor. Emerging therapy in hemoglobinopathies: lessons from the past and optimism for the future. Hematol Oncol Clin North Am. pp 233–48. Philadelphia, PA: Elsevier, Inc.; 2014. doi: 10.1016/j.hoc.2013.11.009

7. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med. 1994; 330:1639–1644. PMID: 7993409

8. Steinberg MH, McCarthy WF, Castro O, Ballas SK, Armstrong FD, Smith W, et al. Therisks and benefits of long-term use of hydroxyurea in sickle cell anemia: A 17.5 year follow-up. Am J Hematol. 2010; 85:403–408. doi: 10.1002/ajh.21699 PMID: 20513116

9. Schrier SL. Pathobiology of thalassemic erythrocytes. Curr Opin Hematol. 1997; 4:75–78. PMID: 9107522

10. Taher AT, Musallam KM, Karimi M, El-Beshlawy A, Blhoul K, Daar S, et al. Overview on practices in thalassemia intermedia management aiming for lowering complications rates across a region of endemicity: the Optimal Care Study. Blood. 2010; 11:1886–1892.

11. Bohacek R, Boosalis MS, McMartin C, Faller DV, Perrine SP. Identification of novel small-molecule inducers of fetal hemoglobin using pharmacophore and ‘PSEUDO’ receptor models. Chem Biol Drug Des. 2006; 67:318–328. PMID: 16784456

12. Perrine SP, Mankidy R, Boosalis MS, Bieker JJ, Faller DV. Erythroid Kruppel-like factor (EKLF) is recruited to the gamma-globin gene promoter as a co-activator and is required for gamma-globin gene induction by short-chain fatty acid derivatives. Eur J Haematol. 2009; 82:466–476. doi: 10.1111/j.1600-0609.2008.01234.x PMID: 19220418

13. Mankidy R, Faller DV, Mabaera R, Lowrey CH, Boosalis MH, White GL, et al. Short-chain fatty acids induce gamma-globin gene expression by displacement of an HDAC3-NCoR repressor complex. Blood. 2006; 108:3179–3186. PMID: 16949648

14. Constantinouakis P, Knitter G, Stamatoyannopoulos G. On the induction of fetal hemoglobin by butyrate. Blood. 2002; 100:4640–4648. doi: 10.1182/blood-2002-04-0143 PMID: 12393583

15. Taher AT, Musallam KM, Karimi M, El-Beshlawy A, Blhoul K, Daar S, et al. Overview on practices in thalassemia intermedia management aiming for lowering complications rates across a region of endemicity: the Optimal Care Study. Blood. 2010; 11:1886–1892.

16. Bohacek R, Boosalis MS, McMartin C, Faller DV, Perrine SP. Identification of novel small-molecule inducers of fetal hemoglobin using pharmacophore and ‘PSEUDO’ receptor models. Chem Biol Drug Des. 2006; 67:318–328. PMID: 16784456

17. Perrine SP, Mankidy R, Boosalis MS, Bieker JJ, Faller DV. Erythroid Kruppel-like factor (EKLF) is recruited to the gamma-globin gene promoter as a co-activator and is required for gamma-globin gene induction by short-chain fatty acid derivatives. Eur J Haematol. 2009; 82:466–476. doi: 10.1111/j.1600-0609.2008.01234.x PMID: 19220418

18. Mankidy R, Faller DV, Mabaera R, Lowrey CH, Boosalis MH, White GL, et al. Short-chain fatty acids induce gamma-globin gene expression by displacement of an HDAC3-NCoR repressor complex. Blood. 2006; 108:3179–3186. PMID: 16949648

19. Constantinouakis P, Knitter G, Stamatoyannopoulos G. On the induction of fetal hemoglobin by butyrate. Blood. 2002; 100:4640–4648. doi: 10.1182/blood-2002-04-0143 PMID: 12393583

20. DeSimone J, Heller P, Hall L, Zwiets D. 5-azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. Proc Natl Acad Sci USA. 1982; 79(14):4428–4431 PMID: 6181507
21. Perrine SP, Ginder GD, Faller DV, Dover GH, Ikuta T, Witkowska HE, et al. A short-term trial of butyrate to stimulate fetal-globin-gene expression in the beta-globin disorders. *N Engl J Med.* 1993; 328:81–86. PMID: 7679666

22. Collins AF, Pearson HA, Giardina P, McDonagh KT, Brusilow SW, Dover GJ. Oral sodium phenylbutyrate therapy in homozygous beta thalassemia: a clinical trial. *Blood.* 1995; 85:43–49. PMID: 7528572

23. Atweh GF, Sutton M, Nassif I, Boosalis V, Dover GJ, Wallenstein S, et al. Sustained induction of fetal globin by pulse butyrate therapy in sickle cell disease. *Blood.* 1999; 93:1790–1797. PMID: 10068649

24. Resar LM, Segal JB, Fitzpatrick LK, Friedmann A, Brusilow SW, Dover GJ. Induction of fetal globin hemoglobin synthesis in children with sickle cell anemia on low-dose oral sodium phenylbutyrate therapy. *J Pediatr Hematol Oncol.* 2002; 24:737–741. PMID: 12468915

25. Perrine SP, Dover GH, Daftari P, Walsh CT, Jin Y, Mays A, Faller DV. Isobutyramide, an orally bioavailable butyrate analogue, stimulates fetal globin gene expression in vitro and in vivo. *Br J Haematol.* 1994; 88:555–561. PMID: 7529533

26. Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, et al. Effect of hydroxyurea on frequency of painful crises in sickle cell anemia. Investigators of the multicenter study of hydroxyurea in sickle cell anemia. *N Engl J Med.* 1995; 332(2):1317–1322.

27. Wang WC, Ware RE, Miller ST, Iyer RV, Casella JF, Minniti CP, et al. Hydroxycarbamide in very young children with sickle-cell anemia: a multicentre, randomized, controlled trial (BABY HUG). *Lancet.* 2011; 377:1663–1672. doi: 10.1016/S0140-6736(11)60355-3 PMID: 21571150

28. Saunthararajah Y, Hillery CA, Lavelle D, Molokie R, Dorn L, Bressler L, et al. Oral isobutyramide reduces transfusion requirements in some patients with homozygous beta thalassemia. *Br J Haematol.* 2013; May; 161(4): 587–93. doi: 10.1111/bjh.12304 PMID: 23530969

29. Fuchareon S, Inati A, Siritanaraku N, Thein SL, Wargin WC, Koussa S, et al. A randomized Phase II/II trial of HQK-1001, an oral foetal globin gene inducer, in beta thalassemia intermedia and HBe beta thalassemia. *Br J Haematol.* 2013; 161(4): 587–93. doi: 10.1111/bjh.12304 PMID: 23530969

30. Kutlar A, Reid ME, Ataga K, Inati A, Taher AT, Abboud MR, et al. A dose escalation phase IIa study of 2, 2-dimethylbutyrate (HQK-1001), an oral fetal globin inducer, in sickle cell disease. *Am J Hematol.* 2013;(11 ):E255–260. doi: 10.1002/ajh.23533 PMID: 23828223

31. Patthamalai P, Fuchareon S, Chaneiam N, Ghalie R, Chui DHK, Boosalis MS, et al. Aphase 2 trial in patients with thalassemia intermedia: results of a phase II open study. *Blood Cells, Mols, Dis.* 2000; 26:105–111.

32. Reich S, Buhrer C, Henze G, Ohlendorf D, Mesche M, Sinha P, et al. Oral isobutyramidereduces transfusion requirements in some patients with homozygous beta thalassemia. *Blood.* 2000; 96:3357–3363. PMID: 11071627

33. Dai Y, Ngo D, Forman LW, Qin DC, Jacob J, Faller DV. Sirtuin 1 is required for an antagonist induced transcriptional repression of androgen responsive genes by the androgen receptor. *Mol Endocrinol.* 2007;: 21:807–811.

34. Mabaera R, West RJ, Conine SJ, Boyd CD, Engman CA, Lowrey CH. A cell stress signaling model of fetal hemoglobin induction: what doesn't kill red blood cells may make them stronger. *Blood.* 2014; 123(12):1956–7. doi: 10.1182/blood-2013-11-538470 PMID: 24652964

35. Cappelini MD, Graziaidei G, Ciceri L, Comino A, Bianchi P, Porcella A, et al. Oral isobutyramide therapy in patients with thalassemia intermedia: results of a phase II open study. *Blood Cells, Mols, Dis.* 2000; 26:105–111.

36. Chen J-J. Regulation of protein synthesis by the heme-regulated eIF2α kinase: relevance to anemias. *Blood.* 2007; 109:2693–2699. PMID: 17110456

37. Schaeffer EK, West RJ, Conine SJ, Lowrey CH. Multiple physical stresses induce y-globin gene expression and fetal hemoglobin production in erythroid cells. *Blood Cells Mol Dis.* 2014; 52:214–221. doi: 10.1016/j.bcmd.2013.10.007 PMID: 24314748

38. Bradner JE, Mak R, Tanquutri SK, Mazitschef R, Haggarty SJ, Ross, et al. Chemical genetic strategy identifies histone deacetylase 1 (HDAC1) and HDAC2 as therapeutic targets in sickle cell disease. *Proc Natl Acad Sci USA.* 2010; 107(28):12617–12622. doi: 10.1073/pnas.1006774107 PMID: 20616024

39. Testa U. Fetal hemoglobin chemical inducers for treatment of hemoglobinopathies. *Ann Hematol.* 2009; 88:505–528. doi: 10.1007/s00277-008-0637-y PMID: 19011856

40. Hoffman-La Roche Limited. Product Monograph levodopa and benserazide combination Capsules 50–12.5, 100–25, 200–50 Pharmaceutical standard: professed Antiparkinson Agent. Submission control No. 128706. Available: http://www.rochecanada.com/fmfiles/re7234008/Research/ClinicalTrialsForms/
41. Chen Z, Luo HY, Steinberg MH, et al. BCL11A represses HBG transcription in K562 cells. *Blood Cells Mol Dis.* 2009; 42:144–149. doi: 10.1016/j.bcmd.2008.12.003 PMID: 19153051

42. Labie D, Pagnier J, Lapoumeroulie C, Rouabhi F, Dunda-Belkhodja O, Chardin P, et al. Common haplotype dependency of high G gamma-globin gene expression and high Hb F levels in beta-thalassemia and sickle cell anemia patients. *Proc Natl Acad Sci USA.* 1985; 82: 2111–2114. PMID: 2580306

43. Thein SL, Menzel S. Discovering the genetics underlying foetal haemoglobin production in adults. *Br J Haematol.* 2009; 145:455–467. doi: 10.1111/j.1365-2141.2009.07650.x PMID: 19344402

44. Sheehan VA, Luo Z, Flanagan JM, Howard TA, Thompson BW, Wang WC, et al. Genetic modifiers of sickle cell anemia in the Baby HUG cohort: influence on laboratory and clinical phenotypes. *Am J Hematol.* 2013 Apr 20; doi: 10.1002/ajh.23457

45. Uda M, Galanello R, Sanna S, Lettre G, Sankaran VG, Chen W, et al. Genome-wideassociation study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. *Proc Natl Acad Sci USA.* 2008; 105:1620–1625. doi: 10.1073/pnas.0711566105 PMID: 18245381

46. Nuinoon M, Makarasara W, Mushiroda T, Setianingsih I, Wahidiyat PA, Sripichai O, et al. A genome-wide association identified the common genetic variants influence disease severity in beta 0-thalassemia/hemoglobin E. *Hum Genet.* 2010; 127:303–314.

47. Lettre G, Sankaran VG, Bezerra MA, Araujo AS, Uda M, Sanna S, Cao A, et al. DNA polymorphisms at the BCL11A, HBS1L-MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. *Proc Natl Acad Sci USA.* 2008; 105(33):11869–11874. doi: 10.1073/pnas.0804799105 PMID: 18667698

48. Liu D, Zhang X, Yu L, Cai R, Ma X, Zheng C, et al. KLF1 mutations are relatively more common in a thalassemia endemic region and ameliorate the severity of β-thalassemia. *Blood.* 2014; 124:803–811. doi: 10.1182/blood-2014-03-561779 PMID: 24829204

49. Manwani D, Bieker JJ. KLF1: when less is more. *Blood.* 2014; 124:672–673. doi: 10.1182/blood-2014-05-576967 PMID: 25082863

50. Bank A. Regulation of human fetal hemoglobin: new players, new complexities. *Blood.* 2006; 107:435–443. PMID: 16109777