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RESEARCH ARTICLE

Pharmacologic Therapies in Beta Hemoglobinopathies: Fetal Globin Gene Induction in the First Molecular Diseases

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ABSTRACT
Beta hemoglobinopathies and thalassemias are caused by diverse molecular mutations of the β^A globin chains and modulated by polymorphisms. These are important disorders in medical history, as they became prevalent globally in regions where P. falciparum malaria was endemic. Heterozygous or carrier states conferred a survival advantage; however, doubly heterozygous or homozygous states cause severe hemolytic anemia and multi-organ complications.
These disorders are somewhat unique in that all humans have alternate genes for fetal globin chains, which are expressed in fetal life but are silenced on a developmental clock in infancy. An established approach to ameliorating conditions of abnormal adult globin genes is to reactivate, or increase expression, of the endogenous fetal globin genes to replace the missing protein chains in beta thalassemias or inhibit polymerization of hemoglobin S and reduce the red cell abnormalities.
This review provides a high-level overview of cell and molecular mechanisms that mediate fetal to adult globin gene switching and pharmacologic therapies that can reactivate fetal globin through different actions. Intermittent or metronomic dosing regimens have overcome challenges of undesirable off-target effects by agents that cause erythroid cell growth arrest.
Multiple pharmacologic candidates reactivate or increase the expression of fetal globin protein and proportions of red blood cells containing HbF (F-cells). Hydroxyurea maintains high levels of HbF if begun in infancy, with some decline in mid-childhood. It elicits lower responses in HbF in adult patients, but still has broad clinical benefit in reducing many complications. However, many adult patients do not tolerate optimal hydroxyurea dosing due to cytopenias. Parenterally administered therapies with differing molecular actions, such as demethylating agents and histone deacetylase inhibitors, have shown proof-of-principle in reactivating HbF. Orally bioavailable candidates with complementary molecular mechanisms are in trials.
Combining fetal globin-inducing agents with other therapies with complementary mechanisms, such as recombinant erythropoietin that promotes the survival of red blood cells and therapeutics that promote erythroid cell metabolism, should have additive effects. These pharmaceutical candidates offer great clinical potential and global feasibility for ameliorating these serious diseases.

"After > 50 years' investigation of hemoglobin switching, targeted pharmacologic candidates have demonstrated potential to reverse silencing of the fetal globin genes by inhibiting the repressor machinery and complement our single approved therapy. This approach is well- established to substantially reduce many damaging clinical effects of the beta hemoglobinopathies."

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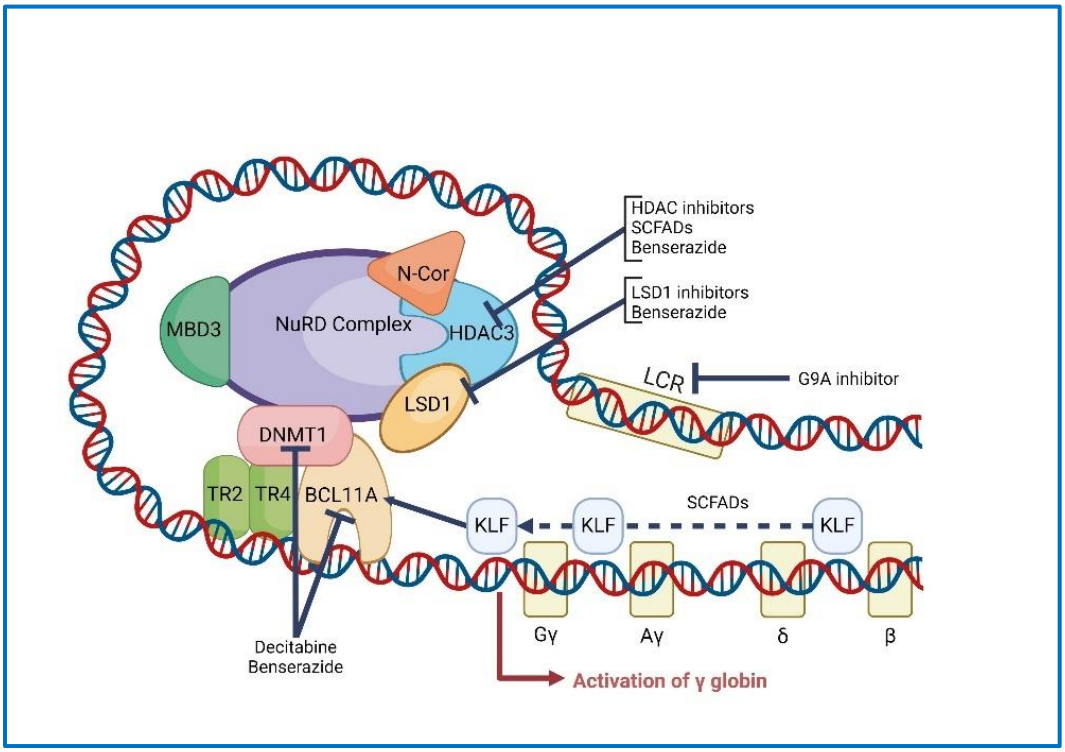
Hemoglobin disorders became the most prevalent monogenic conditions globally because their carrier states conferred a survival advantage by resistance to *P. falciparum* malaria through reduced red blood cell (RBC) size or early splenic removal with shortened RBC lifespan, preventing malarial parasites from completing their life cycle.[1-3] Beta hemoglobinopathies are characterized by mutations in the adult beta-globin genes, causing abnormal or diminished adult hemoglobin (HbA, $\alpha_2\beta^A{}_2$), hemolytic anemia, reduced RBC lifespan, and diverse organ complications.[3-5] Sickle cell disease (SCD) is caused by a mutation in the β^A globin gene that produces an abnormal hemoglobin (hemoglobin S, HbS, $\alpha_2\beta^S{}_2$), which polymerizes with repeated deoxygenation, causing red blood cell membrane abnormalities that adhere to the endothelium and cell deformity into sickled shapes.[1,3,4,6] Beta thalassemias are caused by reduced or absent synthesis of β^A globin chains, with a subsequent imbalance in the ratio of α to β chains, accumulation of excess α globin chains, which damage red blood cells and cause early apoptosis of erythroblasts and RBC hemolysis.[7,8] Depending on the severity of the imbalance, thalassemias are classified as major or intermedia, transfusion-dependent, or -independent.[8,9] Globally, 300,000 children are born annually with SCD and over 60,000 with β-thalassemia.[10,11]

The hemoglobinopathies have been subjects of intense medical interest and were often first to be characterized as new techniques were developed, as affected RBCs are more readily accessible to analysis than organs that require biopsies.[1,3] Observations by insightful physicians recognized abnormalities in peripheral RBCs and differences in disease severity between individual patients and populations worldwide, providing clues that led to extensive research by scientists who applied advances in biochemistry, cell, and molecular biology techniques to characterize differences and intricacies of gene regulation.[1,3,5,12] Therapies today have built on a vast body of work over decades. [3,12,13] Hydroxyurea and stem cell transplantation are now standard care for beta hemoglobin disorders, and gene therapies have been approved for these conditions, although many patients are not eligible for the latter.[14-17] The focus of this review is on selected pharmacologic therapies to induce fetal hemoglobin expression which have been investigated in clinical trials, solved challenges, and provide opportunities feasible for significantly reducing disease severity in patients globally.

A few mammalian species (sheep, non-human primates, humans) undergo a switch from embryonic to fetal to adult globin gene expression during development.[4,18,19] Fetal hemoglobin, HbF ($\alpha_2\gamma_2$, HBG), has a greater affinity for oxygen than HbA ($\alpha_2\beta_2$) but functions well before and after birth. HbF is developmentally silenced or switched off on a biological clock, beginning at approximately 32 weeks' gestation and completed by six months of age in healthy human infants.[18-20] Dr. Janet Watson, a pediatrician, noted that infants with SCD do not have sickled cells in their blood or become ill until after six months of age when fetal globin declines.[21] A large body of research has shown that persistent or renewed expression of fetal hemoglobin confers a clinical benefit in hemoglobinopathies by preventing polymerization of HbS, which reduces the severity of SCD, and by compensating for defective or deficient β^A globin chains in the β-thalassemias.[22,23] The ameliorating effects are mediated by the amount of HbF and the proportion of cells expressing Hb F (F-cells). Both parameters are essential, as only the cells containing HbF at a hypothesized threshold of 10 pg/cell have prolonged survival.[22,24] Patients undergoing rapid or "stress erythropoiesis" during recovery from blood loss or marrow transplant have increased HbF and F-cells, as HbF is expressed first during erythroblast differentiation, and both rapid differentiation and cytostasis also increases proportions of F-cells.[25-28] Studies of different patient populations, such as sickle cell disease (SCD) with hereditary persistence of fetal hemoglobin (S-HPFH), patients with SCD in Eastern Saudi Arabia, India, and Senegal who have deletions or point mutations in the fetal globin gene promoter which prevent binding of transcriptional repressors, and patients with β-thalassemia syndromes with the same thalassemia mutation but with specific genetic modifiers (e.g., *Xmn-I, Bcl-11A, HMIP, KLF-1*), have higher baseline levels of HbF and F-cells and milder clinical courses, even in high-risk geographic regions.[23,29-34] Even single nucleotide polymorphisms produce higher total hemoglobin levels, fewer complications, milder courses, and prolonged survival than in patients with lower levels of HbF and F-cells.[9,29-38]

Figure 1. Sites of action by pharmacologic agents on fetal globin gene transcription
Schema of repressors which bind the proximal γ-globin gene promoter, preventing the LCR (Locus Control Region) from activating transcription. Repressors inhibited, suppressed, or displaced from the γ-globin gene promoter by pharmacologic agents are indicated. A SCFAD (RB7) displaces EKLF from the β-globin to the γ-globin gene promoter to activate transcription.
Figure created with BioRender.com



Inducing HbF expression by suppressing silencing mechanisms and/or stimulating erythroid proliferation are generally accepted approaches for treating beta-globin disorders. Multiple pharmacological agents induce HbF *in vitro*, in animal models, and in patients, including HU, 5-azacytidine, decitabine, short-chain fatty acids and derivatives (SCFADs), HDAC inhibitors, molecularly-modeled compounds, and imids such as thalidomide and pomalidomide.[39-44] These agents act by different mechanisms – by inhibiting DNA methylation or specific inhibitors of repressor complexes which bind the fetal globin gene promoter, facilitate interactions with the locus control region (LCR), by recruiting binding of an activating factor and remodeling complex which typically promote beta-globin expression, or by altering erythroid cell differentiation through cytostasis or accelerating differentiation.[45]

Stem cell transplantation (SCT) with infusion of donor stem cells that produce normal hemoglobin into patients, either from partially to fully HLA-matched donors or a cord blood source, has been refined over decades; when available it is curative with excellent outcomes.[15] Gene therapies currently require myeloablative conditioning, with associated risks and some limitations on eligibility, although striking outcomes.[16,46,47,48] Pharmacologic therapies that are non-mutagenic and which can be administered in a dosing regimen that avoids cytopenias are broadly considered to be most feasible and accessible to patients globally currently.

The Basis for Enhancing Fetal Globin Gene Expression with Pharmacologic Agents

HbF induction involves chromatin remodeling of the fetal globin gene (HBG) to prevent repressors from binding to or enabling activating proteins to bind, the promoter of the γ globin gene (HBG).[13] The modification of gene expression without altering DNA sequences is referred to as epigenetic regulation. Chemical modifications of DNA and histones can activate or silence genes. In the context of hemoglobinopathies, epigenetic regulation plays a crucial role in the developmental expression of the γ and β-globin genes, which are reciprocally expressed. A complex interplay of transcription factors, chromatin looping, and epigenome modifications regulates the β-globin locus. These modifications include histone methylation/ demethylation, acetylation/ deacetylation, and

DNA methylation, which are associated with up-or down-regulation of gene expression.[13,48] Agents which directly inhibit epigenetic enzymes that repress the HBG have targeted DNA methyltransferase 1 (DNMT1), histone deacetylases (HDAC inhibitors), lysine demethylase 1 (KDM1A/LSD1), protein arginine methyltransferase 5 (PRMT5), euchromatic histone lysine methyltransferase 2 (EHMT2), and chromodomain helicase DNA binding protein 4 (CHD4).[13]

Multiple genetic modifier sequences, including single nucleotide polymorphisms (SNPs), contribute to baseline fetal globin expression in proportions of F-cells or % HbF, likely affecting responses to individual pharmacologic agents, even at low baseline levels in patients from different global regions.[29-34,49-50] A polymorphism with high modulating impact, referred to as Xmn-1, is associated with a higher proportion of Gγ than Aγ and is reported in select populations, including the Arabian Indian sickle cell haplotype in Eastern Saudi Arabia, the Senegal haplotype in Africa, and Asian β thalassemia subjects.[23,29,49-51,52] In HbE β-thalassemia populations in Thailand, the Xmn-I SNP is linked to the β^0 globin mutations.[52] Genome-wide association study (GWAS) of a large population identified BCL-11A, HMIP, and Xmn-I as important modulators of F-cell proportions.[29] Next generation sequencing, complemented by Sanger sequencing as needed, to examine exons and presumed regulatory regions of 49 genes in 1142 β-thalassemia patients also identified a polymorphism in DNMT1 as a cause of HPFH.[53] The single-nucleotide polymorphism (SNP) BCL-11A is associated with higher total Hb levels in Italian and Asian β-thalassemia patients with the same thalassemia mutations.[29-31] SNPs in KLF1 and ZBTB7A are associated with HPFH syndromes.[32,33,54] SNPs associated with higher baseline HbF and F-cells are associated with higher responses to some pharmacologic therapies.[108]

Table I summarizes many small molecule pharmacologic and epigenetic therapies developed from a progressive understanding of globin regulation and challenges of off-target cellular actions overcome by metronomic or intermittent dose regimens.

Hydroxyurea

Hydroxyurea is an oral antineoplastic agent that inhibits ribonucleotide reductase, a key enzyme in DNA synthesis, which slows cell division and thereby increases the total cell content of HbF, as HbF is expressed first before adult Hb A during differentiation of erythroid cells.[38] HU increases proportions of HbF-containing RBCs, an important criteria required for clinical benefit.[24] Other beneficial effects of HU include increasing mean cell volume (MCV), which dilutes HbS and reduces HbS polymerization,[35,55,56] reduction of leukocyte adhesion to endothelial cells and inflammatory cascades that contribute to vaso-occlusion, and increase in nitric oxide (NO) production by stimulating soluble cGMP and endothelial NO synthase, which enhance vasodilation and improves blood flow.[57] The combination of HU with Erythropoietin has shown additive effects in inducing F-cells and HbF in SCD and beta-thalassemia.[58-61]

In a pivotal trial supported by the U.S. National Institutes of Health, HU at 15-35 mg/kg administered for two years increased HbF in adults with SCD by a mean of 3.6% above average baseline levels 5-7%, up to >15%, resulting in higher total Hb levels, increased survival of F-cells, fewer vaso-occlusive pain crises and acute chest syndrome events, and improved quality of life.[14,62-64] Early initiation of HU therapy in children in the baby HUG study also demonstrated decreases in vaso-occlusive crises.[65] In subsequent trials, HU has been shown to reduce the risks for strokes, renal insufficiency, pulmonary hypertension and to improve neurocognitive functioning in adolescents.[56,65-69] A recent review of 208 children with SCD treated for >5 years at a large sickle cell center showed that 71% had a sustained response to HU, although mean levels gradually declined from HbF > 16-20% in infancy to approximately 10% around ten years of age, with fewer clinical complications at higher HbF levels.[64,69] With clear clinical benefit in reducing many complications, HU is the standard of care for SCD and generally well-tolerated and economically available worldwide. Monitoring and dose adjustments to prevent cytopenias are required, especially in adults who have experienced repeated infarcts and have reduced bone marrow reserve with lower absolute reticulocytes, and often cannot tolerate optimal doses.[70,71] A study of 606 children treated with HU in sub-Saharan Africa (REACH, NCT01966731) demonstrated that HU at 25-30 mg/kg/day can be safely administered with a dose escalation to the maximally tolerated dose (MTD) and that routine CBC monitoring every three months is then sufficient.[69] As many adults with SCD develop renal insufficiency and inappropriately low erythropoietin (EPO) levels for their degree of anemia, addition of EPO to HU in adults has been investigated.[59,72] Combined therapy has demonstrated additive increases in HbF, F-cells, and total Hb.[59-61]

Table 1: Therapeutics which induce HbF in clinical trials

Therapeutic	Mechanisms Of Action	Cellular Effects	Populations Studied	Observations On Reponses
HYDROXYUREA (HU) Oral Phase 3, Approved	Ribonucleotide reductase Inhibitor- slows cell division (cytostasis), cGMP stimulant increases NO, Bi-modal effects: caspase activation (high conc)	Leukopenia, thrombocytopenia Decreases reticulocytes	SCD BTI	HbF mean increase in adults 3.6% above baseline (mean BL in adults with SCD is 5-7%) HbF maintained 16- 20% when begun in infancy, declines at age 10 years to lower levels in SCD
ERYTHROPOIETIN (EPO) IV Ph2	Promotes erythroid proliferation & survival via STAT-5	Erythroid proliferation	SCD BTI	HU+ EPO: Allows higher HU dosing Particularly beneficial in SCD with low endogenous EPO levels, low reticulocytes, renal insufficiency Increases % HbF
HU + EPO Ph 2	As above	Erythroid proliferation	SCD	Combination allows higher HU dosing, increases HbF Beneficial in SCD subjects with reduced renal function
5-AZACYTIDINE IV 5 days/week Interval dosing, Ph 2	Hypomethylation at specific sites	Neutropenia	NTDT/TDT SCD	Increases HbF, fetal globin mRNA, F-cells, F-reticulocytes; total Hb increase 1-3 g/dl High and rapid responses
DECITABINE IV, SC Interval dosing, Ph 2	DNMT1 Inhibitor	Neutropenia, Thrombocytosis	SCD BTI	Increases HbF, F-cells, total Hb 1-3 g/dl Decrease in cell sickling, adhesion, thrombotic indicators. High and rapid responses
DECITABINE + THU Oral 1-3 times per week, Ph 2 (Separate & combined formulations)	DNMT1 Inhibitor + CDA Inhibitor to enhance pharmacokinetics	Promotes erythroid differentiation	SCD	Increases F-cells, F-reticulocytes, HbF, total Hb Metronomic dosing 1X/week prevents thrombocytosis High response levels and response rates Rapid responses - 2 weeks
Arginine BUTYRATE IV 4-5 day per week, Pulse 1-2/month Phase 2, up to 7 years	Pan-HDAC inhibitor, enhances γ globin transcription/ translation Suppresses BCL-11A	Inhibition of erythropoiesis	BTI TDT SCD	SCD: Mean HbF 3-fold increase from 7% to 21%, Total Hb 1 g/dl, F-reticulocytes, F-cells increase 1.5-2X BTI- Increase in total Hb 1- 4 g/dl NTDT/ TDT – Transfusion independence > 7 years
Arginine BUTYRATE + EPO, Phase 2	HDAC Inhibitor + growth factor (STAT-5 Activator)	Addition of rhu-EPO promotes erythroid cell survival	BTI	Total Hb increase by 2 to 4 g/dl (high in HbLepore) β^+ thal, BL EPO < 120 mU/ml: mean increase 2.7 g/dl Hb β^0 thal, BL EPO > 160 m/ml- mean Total Hb 2.8 g/dl
Sodium PHENYLBUTYRATE Oral, Ph2	HDAC Inhibitor SCFAD	Erythropoiesis inhibited	SCD BT	HbF, F-cells increase in SCD Responses in BTI with baseline EPO levels > 140 mU/ml, not in TDT Prolonged responses (years) in Hb Lepore
Isobutyramide Phase ½	SCFAD	None	NTDT TDT	HbF increase in 50% BTI TDT- decrease of transfusions in 50%

Sodium DIMETHYLBUTYRATE Oral Ph 2	Promoter activation HDAC2 Inhibitor BCL-11A reduced	Enhances erythropoiesis through STAT-5 signaling, increases survival proteins	SCD BTI HbE β Thal	SCD- Mean total Hb increased 0.8 gm/dl, HbF 4% Hb E-β thal, (24 Wks treatment) • HbF mean increase 10% (3-20%) in 7/10 pts • Mean Total Hb increase 1 g/dl • Higher response rate in patients with Xmn-1 SNP
POMALIDAMIDE Ph 2	Imid, increases fetal globin, reduces inflammatory cytokines	Promotes erythropoiesis	SCD	Increases HbF without cytopenias HbF response observed after 12 months at low dose
BENSERAZIDE Phase 1b dose-ranging, in progress	Suppresses /displaces 4 repressors BCL-11A, KLF-1, HDAC3, LSD-1	LSD-1 inhibition shifts differentiation from erythroid to myeloid	BTI SCD	F-cells, F-reticulocytes increase up to 14 x baseline HbF% increase 2-14% Total Hb increase 1-2 g/dl in some BTI SCD- Additive with HU in 3/3 (HbF to >30%, >70% F-cells)

BL: Baseline; BT: Beta-thalassemia; BTI: Beta-thalassemia intermedia; CDA: Cytidine deaminase; DNMT1: DNA (cytosine-5)-methyltransferase 1; EPO: Erythropoietin; HDAC: Histone deacetylase; HU: Hydroxyurea; IV: Intravenous; LSD1: Lysine-specific demethylase; NO: Nitric oxide; NTDT: Non-transfusion-dependent thalassemia; SC: Subcutaneous; SCFAD: Short-chain fatty acid derivative; SCD: Sickle cell disease; TDT: Transfusion-dependent thalassemia; THU: Tetrahydrouridine; Ph: Phase

In some subjects with β-thalassemia intermedia, HU increases HbF, reduces ineffective erythropoiesis, and reduces anemia and marrow fibrosis.[73-76] In a few patients with thalassemia intermedia treated with low doses of ≤10 mg/kg per day, four days per week, HU increased total Hb levels by preferentially increasing β-globin biosynthesis.[77] Interestingly, the presence of the Xmn-I T/T genotype or the BCL11A rs766432 C allele correlates strongly with response to HU ($p < 0.001$), allowing for targeted selection of patients who are most likely to benefit.[78]

DNA hypomethylation and DNMT-1 Inhibition with 5-Azacytidine and Decitabine

DNA methylation, an epigenetic process adding a methyl group to the C5 position of cytosine, thus forming 5-methylcytosine,[79] was one of the first epigenetic effects found to be associated with gene silencing, while DNA demethylation was associated with gene activation. The fetal (γ, HBG) globin genes, which encode the non-alpha subunits of HbF ($\alpha_2\gamma_2$), were found to be associated with methylation in specific promoter regions in adult erythroid cells, associated with repression. In contrast, the beta-globin genes, which encode the subunits of HbA, are demethylated and activated after birth.[80,81] Therefore, modulating the globin genes' DNA methylation status was investigated to increase HbF expression.

The first studies of DNA hypomethylation to increase HbF expression were in anemic baboons with 5-azacytidine, a nucleoside analog that inhibits DNA methyltransferases that catalyze DNA methylation and in combination with the HDAC inhibitor, butyrate.[80,81] 5-azacytidine was then studied in a patient with β^+ thalassemia; fetal globin mRNA increased dramatically and total Hb increased by 2 g/dl, and proved beneficial in severe beta thalassemia patients.[82,83] Although 5-azacytidine requires parenteral administration, which was inconvenient for long-term use and had cytotoxic and mutagenic effects, these trials demonstrated proof-of-concept and feasibility of pharmacologically reactivating fetal globin gene expression to a clinically beneficial degree.

Decitabine, an analog of 5-azacytidine that inhibits DNMT1 expression and is not incorporated into DNA, was found to be more potent and less toxic than 5-azacytidine. It induced HbF expression in patients with SCD and β-thalassemia, consequent reducing factors promoting vaso-occlusion-adhesion, thrombosis, inflammation.[84-87] Intermittent dosing regimens treating 2-3 times per week were developed which avoided neutropenia and thrombocytopenia and still maintained high levels of fetal and total Hb in high-risk patients with SCD, who had with HbF levels <7.5% and did not respond adequately to HU.[84-87]

A pharmaceutic limitation of 5-azacytidine and decitabine for long-term use is low oral bioavailability due to rapid metabolism in the GI tract by cytidine deaminase. They require preparation shortly before administration due to instability, which is inconvenient for long-term or home therapy. To overcome these issues, oral decitabine formulations have been developed, employing an innovative combination using tetrahydrouridine (THU), an inhibitor of cytosine deaminase.[88] A Phase I trial (*NCT01685515*) administered THU an hour before decitabine, followed by decitabine. This inhibited DNMT1 protein and increased HbF levels and F- cells to 80%.[88,89] A trial was recently conducted in six patients with SCD who were treated with a single formulation in which THU is released first, followed by decitabine (*NCT04055818*). In 5 of 6 high-risk patients with low HbF levels at baseline and negative prediction markers for response to HU, this formulation, administered only once day/week, produced sustained increases in HbF and F-cell proportions, reduced multiple cellular abnormalities and the frequency of painful events over 12 months of therapy compared to the year prior to the trial, without any dose-limiting toxicities.[90] Advantages of decitabine include that high HbF responses occur in a majority of subjects and occur rapidly, within two weeks. Currently, an industry-sponsored trial of a single therapeutic containing the two agents together is in prorgess, which may lead to registrational approval of a potent agent to induce HbF which has efficacy in patients who do not tolerate HU.

Histone deacetylase inhibitors and short-chain fatty acid derivatives

Histone deacetylases (HDAC) are regulatory molecules involved in the epigenetic silencing of embryonic and human γ-globin genes.[40,81,91-95] A combination of 5-azacytidine and the pan-HDAC inhibitor sodium butyrate, a short-chain fatty acid (SCFAD), induced embryonic globin expression in chickens.[81] In a search for physiologic factors associated with a delay in globin switching, an analogue of butyrate, alpha amino-n-butyric acid, was reported to be higher in the plasma of infants of diabetic mothers than in normal infants.[19] In follow-on investigations, infusions of an analogue, the short chain fatty acid and HDAC-inhibitor, butyrate, delayed the fetal to adult globin gene switch in fetal sheep and increased fetal globin

mRNA, protein, and F-cells in erythroid progenitor cells from patients with SCD.[19,91]

Subsequently, in clinical trials in patients with beta-thalassemia or SCD, intravenous arginine butyrate therapy increased HbF levels and total Hb by 2 to 5 g/dl above baseline.[38,39,96,97] However, continued daily dosing resulted in a subsequent decline in total Hb levels, hypothesized to result from HDAC inhibitor-induced erythroid cell growth inhibition in G1. This is potentially limiting, particularly in thalassemia, where erythrokinetics and erythroid expansion are important, and apoptosis of thalassemia progenitors occurs at the polychromatophilic normoblast stage.[7,98-101] Pulsed, intermittent dosing for four days once/month was therefore evaluated, which proved effective in increasing HbF levels by 3-fold in 9 of 11 SCD patients, from a mean of 7% to 21%, and mean total Hb levels by 1 g/dl.[39] This regimen maintained higher HbF and total Hb when administered for several years; hospitalization rates for vaso-occlusive events were reduced by 4-fold. In β-thalassemia intermedia and major subjects, pulse dosing twice a month increased fetal and total Hb and maintained previously transfusion-dependent patients at hemoglobin levels that did *not* require transfusions. Pulse dosing was adapted to home therapy by an innovative hematology nurse who taught home administration to patients with allo-antibodies who required, but could not receive, regular transfusions, in three countries and maintained them transfusion-independent.[39,101]

The level of responses to arginine butyrate varied in patients with different molecular mutations and by baseline EPO levels, which were higher in subjects with β^0 thalassemia mutations than in those with β^+ thalassemia mutations. Accelerated apoptosis of erythroid progenitors characterizes beta-thalassemia, and likely makes erythroid progenitors susceptible to the cell growth arrest associated with some HDAC inhibitors.[7,27,98,100-102] A pilot study using the combination of butyrate and recombinant hu-EPO demonstrated that patients with β^+-thalassemia, who had relatively lower baseline levels of endogenous EPO (<145 mU/mL) experienced additive responses. Higher baseline HbF levels (>60%) and EPO levels (>160 mU/mL) were observed in patients with at least one β^0-globin mutation, and these patients responded to butyrate alone without added benefit from exogenous EPO.[101] This pilot trial demonstrated proof-of-concept that enhancing fetal globin expression could be induced to a degree that provides hematologic and clinical benefit with intermittent regimens to avoid anti-proliferative effects, and that combining an erythroid stimulant with a globin gene inducing agent had additive activity for selected patients. However, parenteral administration was cumbersome for broad use. Currently, a clinical trial of an oral HDAC inhibitor, panobinostat (LBH589), has begun in SCD *(NCT01245179)*.

Searches for orally bioavailable butyrate analogs have been conducted using structure-function and pharmacophore modeling, which identified several generations of small molecules that induce HbF expression.[103-105] One candidate developed into clinical trials is sodium 2,2 dimethylbutyrate (SDMB, HQK-1001), an orally bioavailable short-chain fatty acid derivative with a structure predicted to have a prolonged half-life compared to butyrate. SDMB was found to inhibit HDAC-2, rather than having pan-HDAC-inhibition, but still induced γ-globin expression experimentally in promoter-reporter assays, erythroid progenitors cultured from patients, and in anemic baboons.[131] SDMB also had an additional effect of stimulating erythropoiesis through the STAT-5 signaling pathway utilized by EPO and other growth factors and increased expression of the survival factors Bcl-2 and Mcl-2. [95] Because EPO combined with HU or the butyrate had shown additive effects in some subjects, this candidate was evaluated in preclinical nonhuman primate studies, found to have 90% oral bioavailability with high plasma levels achieved at low doses, a long biologic half-life of many hours, and pharmacodynamic effects in increasing fetal globin mRNA, F-reticulocytes, HbF, and total hemoglobin despite ongoing phlebotomy which exchanged the blood volume every two weeks.[131] While hemoglobin levels was stable on this phlebotomy regimen with placebo or other agents, the total Hb increased by 2 g/dl with SDMB therapy. In a five-sixth nephrectomized rat model, which removes most EPO-producing renal tissue, SDMB therapy increased total Hb by 1 g/dl compared to placebo. These studies confirmed *in vivo* erythropoietic activity of SDMB, suggesting that its dual activity may offer additional benefit, as was observed with HU + EPO.

Accordingly, preclinical toxicology and formulation development was undertaken. Clinical studies were performed first in normal subjects, in whom multiple doses were found to stimulate reticulocytosis within two weeks of treatment and were well-tolerated.[106] In a short-term, open-label, randomized dose-escalation study in patients with HbSS or S/β^0 thalassemia, modest increases in HbF up to 4% and in total Hb of 0.8 g/dl were observed with brief (eight weeks) treatment.[107] HbF responses by a mean increase of 5% or 10.9% above baseline (range 3 to 20%) were observed in patients with

β+-thalassemia intermedia (NTDT) or HbE/β0 thalassemia, respectively.[108,109] Higher HbF responses to 20% were observed in the HbE/β0 thalassemia patients; 9 of 10 had the genetic modifier SNP designated Xmn-I, which confers higher baseline HbF levels.[42]

Dosing at 20-30 mg/kg appeared optimal, while a later trial with 50 mg/kg doses, which reached millimolar levels in patients, levels which suppressed erythropoiesis in erythroid progenitors *in vitro*, had reduced activity in patients with SCD. Responses to some inducing agents may require prolonged courses; some subjects did not begin to respond until after 20 weeks, notably in those with markedly enlarged spleens. These trials collectively demonstrated that lower doses and intermittent dosing often conferred higher responses than high doses and continuous therapy.

Isobutyramide, another SCFAD with oral bioavailability, has been studied in subjects with non-transfusion-dependent thalassemia (NTDT) and TDT. While less active than butyrate, it increased HbF and F-cells in NTDT and reduced transfusion requirements in TDT.[110,111] Exploration of different dosing regimens may be indicated with this therapeutic.

Subsequent generations of HbF-inducing candidates were identified through molecular modeling and activity was confirmed in patients' erythroid progenitors and *in vivo* in anemic baboons.[101,103,104] Interesting new molecular mechanisms were identified with these candidates.[112] One involved EKLF (erythroid Kruppel-like factor) which was previously associated with activating the β-globin gene in erythroid cells specifically. A next generation molecule identified in molecular modeling, RB7, was found to have a novel action of recruiting EKLF and a remodeling complex BRG1 from the β to the γ-globin gene promoter, inducing high level γ globin expression.[113,114] HDAC inhibitors were also found to inhibit DNMT-1, the target of decitabine, through ERK signaling.[115] Thus, multiple therapeutic candidates alter multiple molecular targets and interact through diverse signaling pathways.[112]

Targeted Inhibitors of Multiple Repressors

Expression of the γ globin genes requires accessibility to their promoters for looping of the locus control region (LCR) and transcriptional machinery to the γ globin gene promoter rather than the β globin gene promoter. After infancy, the fetal globin genes are usually blocked by complexes of multiple proteins, including histones and transcription factors that recruit and interact with others, as recently described in detail.[13] Pharmacologic candidates have been identified for specific components of these large repressor complexes.[13] Following the identification of LSD-1 as an enzymatic repressor of g-globin expression, LSD-1 inhibitors, including RN-1and ORY-1001 were found to induce HbF in baboon models and in erythroid progenitors.[43,116-120] RN-1 acts in a synergistic manner with decitabine.[121] An inhibitor of the methyltransferase (G9a) that mediates the looping of the LCR to the γ globin gene promoters, an appealing target, has been identified, but not developed into clinical trials.[122]

Benserazide, a repurposed candidate, has recently entered clinical trials *(NCT04432623)*. It has been used for a different activity in combination with L-dopa to enhance its half-life for the treatment of Parkinson's disease for > 40 years in Europe and Canada.[123,124] It was discovered in a high throughput screen of clinical-stage drugs to have high inducing activity compared to other active and non-cytotoxic candidates.[104,105] Benserazide profoundly suppressed or displaced several repressors of the fetal globin gene promoter in patients' erythroid progenitors and induced γ globin mRNA and protein, F-reticulocytes, F-cells, and total Hb in nonhuman primates and transgenic mice.[104,105,112,124] Repressors that are suppressed or displaced from binding to the fetal globin promoter included *BCL-11A, LSD-1, KLF-1*, and *HDAC*3.[112] These results were intriguing, as haploinsufficiency of *BCL-11A,KLF-1,* and *ZBTB7A* are associated with HPFH; KLF activates *BCL-11A* and ZBTB7A.[29-34,54,131]

Accordingly, a dose-finding Phase 1b trial has been initiated in patients with β-thalassemia intermedia (NTDT) and subsequently in SCD. Initial findings have shown high-level induction of F-reticulocytes (3 to 12-fold), F-cells (2 to 7-fold), HbF/cell (2.7 to 12-fold) and increases in HbF (up to 14% above baseline), in small cohorts in both populations with single oral daily doses given three times per week. High additive activity with HU, previously demonstrated in patients' erythroid progenitors, was observed in patients with SCD who were taking HU at their maximum tolerated dose. Further increases in HbF by 5-13.8% to total F-cells > 80% were observed in SCD patients above levels on HU alone.[123,125] This therapeutic is undergoing further schedule optimization.

Imids and Protein Degraders

Pomalidomide, a thalidomide analog, was studied in patients with sickle cell disease following an unexpected observation of an increase in HbF in

two patients treated for anti-inflammatory effects. Pomalidomide has the desirable cellular actions of promoting erythropoiesis, so it may be particularly useful in β-thalassemias and with HU, when optimal doses are not tolerated.[44]

Small molecules (dWIZ-1 and dWIZ-2) which promote degradation through ubiquitination of a zinc finger motif repressor protein (WIZ) were recently shown to induce HbF expression in erythroid progenitors and in humanized mice. Targeting protein degradation of repressor proteins with agents that act as "molecular glue" is an area of great interest in pharmacologic development.

A Pipeline of HbF-Inducing Therapeutics to Optimize Molecular and Cellular Actions

Pharmacologic inducers of HbF have shown proof-of-principle for treatment of SCD and β-thalassemia in producing hematologic and clinical benefit in patients. Hydroxyurea maintains high levels of HbF when begun in infancy, and even modest increments have proven benefit on multiple organ systems and longer survival in patients who respond. A limitation of HU is that optimal doses are not tolerated by older patients with less "marrow reserve,"so therapies which have additive effects with HU are desirable. Extensive investigation from the Hemoglobin Switching Field has identified many repressors and interactions that can be modulated to increase expression of HbF, including by candidates that inhibit, suppress, displace, or degrade protein repressors that bind the γ-globin promoter or inhibit looping of the LCR to the γ-globin gene promoter. More than 70 agents enhance HbF expression through differing molecular mechanisms and targets.[48,126,127] Combinations of these agents, rationally administered together, should have potent effects.[128,129]

Dose regimens to produce the benefits of high HbF levels and F-cell proportions and avoid potential undesirable cellular effects have been achieved with intermittent/ interval or metronomic dosing strategies. Combinations of therapies with complementary actions produce higher efficacy in patients' erythroid progenitors and are being evaluated in clinical trials in SCD, in patients with and without HU. These and new candidates offer a rational basis to significantly benefit the diverse patient populations with these "first molecular diseases".[130]

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Conflicts of Interest Statement

Y. Sauntharajah: Equity (*Epidestiny*), *Patents*
S. Perrine: *Patents (Phoenicia BioSciences, Boston University)*
K. Kuo: *Consultancy or DSMB representation (Novo Nordisk, Vertex, Sangamo)*
The other authors have no relevant conflicts of interest.

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