Review Article

Fetal Hemoglobin Induction to Treat β-Hemoglobinopathies: From Bench to Bedside

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Abstract

The developmental regulation of the γ- to β-globin switch has motivated research efforts to establish therapeutic modalities for individuals affected with β-hemoglobinopathies. Fetal Hemoglobin (HbF) synthesis is high at birth but declines to adult levels by one year of ages; since HbF blocks hemoglobin S polymerization in Sickle Cell Disease (SCD) and compensated for anemia in β-Thalassemia, reactivating expression is of great research interest. Naturally occurring mutations in the β-globin locus on chromosome 11, produce elevated HbF expression after birth which ameliorates symptoms in SCD. Genome-wide studies discovered three gene loci including BCL11A that account for the majority of inherited HbF variance but considerable work is required to advance this protein as a therapeutic target. Therefore, efforts to develop chemical inducers of HbF are desirable. Hydroxyurea is a potent HbF inducer with clinical efficacy in adults and children with SCD but it has been underutilized in the clinical setting. Numerous pharmacological agents that reactivate γ-gene transcription have been discovered over the last three decades but few have been translated into clinical therapeutics. We will review the progress made in understanding molecular mechanisms of γ-globin regulation and current efforts to expand the number of chemical HbF inducers available for treatment of patients with β-hemoglobinopathies.

ABBREVIATIONS

BRE: Butyrate Response Element; DNMT: DNA Methyl Transferase; HbF: Fetal Hemoglobin; HDAC: Histone Deacetylase; HPFH: Hereditary Persistence of Fetal Hemoglobin; HU: Hydroxyurea; LCR: Locus Control Region; miRNA: MicroRNA; MSH: Multicenter Study of Hydroxyurea; SCD: Sickle Cell Disease; SCFA: Short-Chain Fatty Acids; SNP: Single Nucleotide Polymorphisms; TCD: Transcranial Doppler

INTRODUCTION

In all species that express β-like globin genes, a switch in the type of globin gene expressed coincides with the change in the site of erythropoiesis and hemoglobin composition. The five functional globin genes in the human β-locus on chromosome 11 undergo two developmental switches in expression. Hemoglobin is an oxygen transport tetrameric protein, composed of two α-like and two β-like globin polypeptide chains. After conception, primitive erythroblasts in the yolk sac produce two types of embryonic hemoglobin ($\zeta_2\varepsilon_2$ and $\alpha_2\varepsilon_2$) [1]. At 6 weeks of development, the site of erythropoiesis shifts from the yolk sac to the fetal liver [2]. The ε-globin gene is silenced and is replaced by γ-globin expression during fetal development where fetal hemoglobin (HbF; $\alpha_2\gamma_2$) is produced; after birth the second hemoglobin switch occurs when γ-globin is silenced and the β-globin gene is activated. HbF expression declines to adult levels of <1% [3] and is restricted to erythrocytes called F cells [4]. The bone marrow becomes the main site of hematopoiesis where hemoglobin A($\alpha_2\beta_2$) is the predominate form produced throughout adult life [1]. Understanding mechanisms of HbF regulation is of fundamental importance to achieve γ-globin reactivation in adult erythroid cells to benefit individuals affected by β-hemoglobinopathies.

Hereditary Persistence of Fetal Hemoglobin (HPFH) is a condition in which significant HbF production continues well into adulthood due to either point mutations or deletion in the β-locus that prevent γ-globin silencing [1]. The percentage of HbF ranges from 10-15% to 100% in heterozygotes and homozygotes respectively [1]. Individuals with HPFH are

asymptomatic, and commonly diagnosed when screening for other hemoglobinopathies. Of note, HPFH alleviates the severity of β-hemoglobinopathies and is selected for in populations where malaria is endemic. In persons with Sickle Cell Disease (SCD) who co-inherits an HPFH gene, high HbF levels ameliorates the clinical severity of the disease by inhibiting hemoglobin S (β^S-globin) polymerization. Therefore recapitulating naturally occurring HPFH mutations using gene-based techniques or the development of pharmacologic agents to re-activate γ-globin are viable strategies to treat the β-hemoglobinopathies.

Control of hemoglobin expression during development

Over the last three decades details of the molecular mechanisms controlling globin gene switching have been elucidated. In humans, the α-locus located on chromosome 16 encodes the embryonic ζ- and the two adult, α$_1$-, and α$_2$-globin genes. The β-locus located on chromosome 11 encodes five functional genes, ε-, ^{G}γ-, ^{A}γ, δ- and β-globin which are expressed from 5' to 3' sequentially during development [1]. The discovery of the Locus Control Region (LCR) enhancer region located 20 kb upstream of ε-globin moved the field forward significantly [5]. The LCR is composed of five DNase1 Hypersensitive Sites (HS) of which, HS1-HS4 are erythroid specific [6,7]. The HSs are devoid of nucleosomes and particularly accessible to interactions with transcription factors; each contain core binding motifs for GATA1 and NF-E2 [8-10]. The LCR undergoes direct interaction with the individual globin gene promoters through DNA looping to orchestrate switching which is accomplished through expression of stage specific transcription factors that bind the globin gene proximal promoter regulatory elements [11-13].

β-Locus Haplotypes: Effort to understand variations in HbF levels in sickle cell patients led to the description of clusters of Single Nucleotide Polymorphisms (SNPs) distinguishable by restriction fragment length polymorphism analysis of the β-locus. This approach was used to define inherited distinct patterns of SNPs in discrete chromosomal regions i.e. β-haplotypes. Five common haplotypes including Senegal, Benin, Central African Republic (Bantu), Cameroon, and Asian (Indian/Saudi-Arabian) determine the ancestral origin of the β^S-globin mutation in individuals with SCD [1]. HbF levels vary greatly in individuals with different β-locus haplotypes and within haplotype groups therefore a consistent correlation between the two parameters has not been established. However, patients with the Senegal haplotype generally have higher HbF levels and milder disease [14] compared to individuals with the Benin haplotype, with lower HbF levels and severe disease [15]. More recent studies by Liu et al. [16] using genomic techniques established the complexity of the β-locus that support hybrid haplotypes in African Americans making prediction models and therapeutic strategies based on DNA polymorphisms challenging.

Genome-wide studies: After completion of the Human Genome Project, genome-wide approaches became available to discover genetic modifiers of HbF expression. A number of family genetic studies and genome-wide association studies defined SNPs that contribute to high HbF levels or F cell levels in normal, SCD, and thalassemia populations using quantitative trait loci studies [17-19]. Three major loci in the human genome including -158 Xmn1-*HBG2*, the *HBS1L-MYB* region on chromosome 6q23, and *BCL11A* at 2p15 contribute ~50% of inherited HbF variance in multiple populations (reviewed in [20]).

The -158Xmn1-*HBG2* (C/T) SNP in the Gγ-globin promoter is associated with elevated Hb levels in sickle cell and thalassemia patients [21] which was confirmed in two independent studies [22,23]; this SNP accounts for 13% of variance in F cells levels [24]. A second SNPs located in the intergenic region 5' of the HBS1L and MYB genes is associated with high F cells [18]. Over expression of MYB in K562 cells decreases γ-globin, and MYB expression levels are lower in primary erythroid progenitors grown from individuals with HPFH [25]. Another study demonstrated that increased microRNA (miRNA) miR-15a and miR-16-1 levels which directly silence MYB expression [26], was associated with high HbF in people with trisomy 13. More recently using family studies another inherited mechanisms for HPFH due to haplo-insufficiency of KLF1 in European and African populations was discovered [27-29]. KLF1 trans-activates the BCL11A promoter to increase its levels which repressed γ-globin expression; furthermore, KLF1 knockdown mediates HbF reactivation [30]. These transcription factors represent potential targets for developing gene-based approaches to treat the β-hemoglobinopathies.

Molecular control of γ-globin gene expression

Reactivation of γ-globin expression is of great interest, because of the ameliorating effects of high HbF on the severity of the clinical manifestations of SCD and β-thalassemia. Gene duplication is responsible for the two fetal genes ^{G}γ- and ^{A}γ-globin. The coding region of each γ-globin gene are identical except at amino acid residue 136 where a glycine is present in ^{G}γ- globin and an alanine in ^{A}γ-globin [31]. The promoter of each γ-globin gene contains a TATA box, two CAAT Boxes, and one CACCC box [1]. Many transcription factors and regulatory proteins influence γ-globin transcription either by directly binding within the promoter (Figure 1) or other regulatory regions.

γ-Globin negative regulators: Genome-wide association studies mapped variation in HbF levels to the second intron of the gene encoding BCL11A [19], a zinc finger transcription factor [32,33]. Knockdown of BCL11A expression increases γ-globin mRNA and HbF protein levels in human erythroid progenitors [34]. BCL11A has been reported to bind the β-locus at HS3 of the LCR and the inter-genic region between the Aγ- and δ-globin genes [35]. In addition, BCL11A binding was demonstrated in the γ-globin promoter at nucleotide -56 where it recruits GATA1 and COUP-TFII into a repressor complex [36]. BCL11A interacts with several other gene repressor complexes such as LSD1/CoREST which are components of a histone demethylase complex [35] and GATA1, DNMT1, and the NuRD complex [34]. BCL11A also represses γ-globin transcription indirectly through long range interactions with SOX6 [37]. Exciting new studies support the existence of an erythroid specific enhancer within the second intron of BCL11A [38]. Removal of the enhancer sequence by TALEN technology decreased BCL11A expression and increased expression of the mouse embryonic β-like globin genes [38]; whether the same effect can be achieved on γ-globin expression in human erythroid progenitors remains to be demonstrated.

Other transcription factors are required to achieve γ-globin silencing by BCL11A. SOX6, a Sry-related high-mobility group box transcription factor silences ^{ε}γ-globin in adult mice [39]. SOX6 interacts with BCL11A to mediate binding at the γ-globin
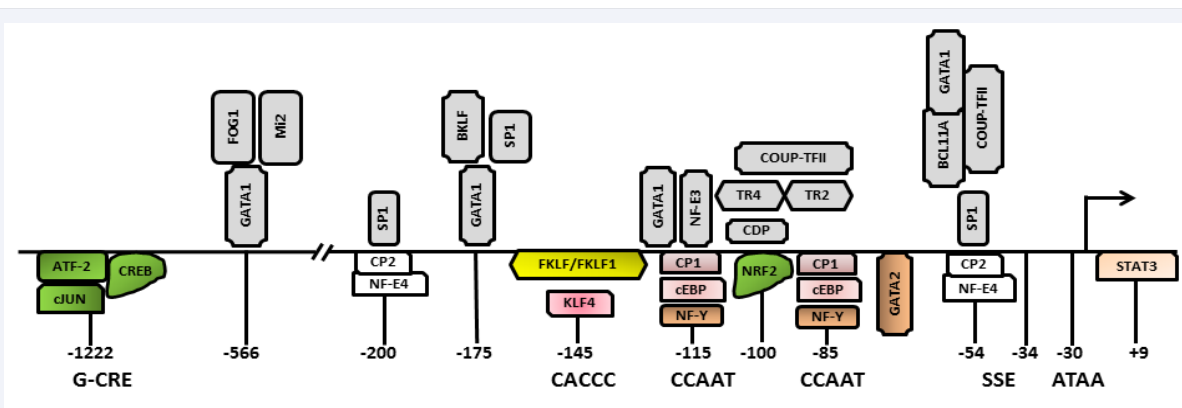
Figure 1 Transcription factor binding in the γ-globin gene promoter.
Shown are the DNA binding proteins demonstrated to bind the γ-globin promoter. Ubiquitous and hematopoietic specific transcription factors are involved in γ-globin regulation during development. The factors shown in gray above the vertical line are negative regulators. The promoter is not drawn to scale.
Abbreviations: ATF-2: Activating Transcription Factor-2; BCL11A: B-cell Lymphoma/leukemia 11A; CREB1: cAMP Response Element Binding Protein; cEBP: CCAAT Enhancer Binding Protein; CP1: CCAAT-Binding protein; CDP: CAAT Displacement Protein; FKLF: Fetal Kruppel-like Factor; KLF4: Kruppel-Like Factor 4; FOG: Friend of GATA; NF-E4: Nuclear Factor-erythroid 4; BKLF: Basic Kruppel-Like Factor; NRF2: Nuclear Factor (erythroid-derived 2)-like 2; SP1: Specificity Protein 1; STAT3: Signal Transducer and Activator of Transcription 3; TR4: Testicular Receptor 4.

promoter [37] and HS3 among other sites. Other negative regulators such as GATA1 and FOG1 bind the γ-globin promoter in the -566 region to repress expression (Figure 1); furthermore these factors associate with Mi2, a member of the NuRD chromatin remodeling complex [40]. Data also exist that the γ-globin distal CCAAT box is occupied by GATA1 and NF-E2 to silence γ-globin [41,42]. These studies suggest the repression of γ-globin expression is achieved through the association of GATA1, BCL11A, FOG1, and NuRD in a repressor complex [34].

Other negative regulatory proteins have been identified such as the orphan hormone nuclear receptors TR2 and TR4 which heterodimerize and bind to the direct repeat element in the γ-globin promoter (Figure 1). Over expression of TR2/TR4 in erythroid cells represses murine βγ-globin expression [43]. Pull down of protein complexes containing TR2/TR4 demonstrated interaction with the epigenetic repressors DNMT1, NuRD, LSD1/CoREST, and Histone Deacetylase (HDAC) 3 [44]. By contrast, enforced TR2/TR4 expression in sickle cell transgenic mice increased HbF and ameliorate disease phenotype [45]. Moreover, the orphan hormone nuclear receptor, COUP-TFII, binds the γ-globin CCAAT box [46]. Stem cell factor treatment and siRNA knockdown of COUP-TFII in adult erythroid progenitors increased γ-globin expression [47] supporting a negative role of this factor in HbF expression.

A new class of gene regulatory molecules has emerged as regulators of γ-globin expression. miRNAs negatively regulate gene expression mainly through binding in the 3'untranslated region [48] however any location in the gene can be targeted. Recent studies reported that miR-96 directly targets γ-globin mRNA in the open reading frame and inhibition of miR-96 produced a 20% increase in γ-globin expression in erythroid progenitors [49]. By contrast, over expression of Lin28 represses the let-7 family of miRNAs which mediates HbF induction in adult erythroblasts [50] through BCL11A inhibition [50]. Recently, miR-486-3p was also shown to target BCL11A to activate γ-globin

expression [51] and its expression is elevated in erythroid cells isolated from β-thalassemia patients with high HbF levels [51] suggesting a physiological role of this miRNA.

γ-Globin positive regulators: Several DNA-binding proteins are known to positively regulate γ-globin expression such as GATA2; over expression of this factor activates ε- and γ-globin expression in K562 cells [52]. Another factor, NF-Y binds the CCAAT motifs and recruits GATA2 and CCAAT-enhancer binding protein to activate γ-globin expression [36]. The most extensively characterized positive regulatory elements is the Stage Selector Element (SSE) located between nucleotide -53 and -34 of the γ-globin promoter (Figure 1); this element has been implicated in the γ- to β-globin switch *in vitro* [53]. The SSE is bound by SP1 [53] or the stage selector protein composed of CP2 and NF-E4 [54]. When NF-E4 is acetylated, its interaction with HDAC1 is reduced which results in γ-globin activation [55]. By contrast, SP1 binding to the SSE increases with methylation of the cytosine at -50 to mediate γ-globin repression [56].

The family of Kruppel-like factors has been linked to γ-globin regulation [57-59]. Binding of KLF4 at the γ-globin CACCC box activates HbF expression in human erythroid progenitors [60]. After treatment with butyrate and trichostatin A, p38 MAPK mediates ATF-2 and CREB-1 activation followed by binding to the cyclic AMP response element at -1222 in the Gγ-globin promoter to activate HbF expression [61]. Additional studies from the Pace lab demonstrated that cJun co-localizes with ATF-2 and CREB-1 to activate γ-globin expression independent of drug induction [62]; steady state γ-globin transcription requires CREB1 binding to the -1222 region [63]. Protein purification studies of the ATF-2 complex validated that cJun, and CREB1 are present along with novel binding partners such as Brg1, hnRNPC1/C2, and HDAC1/2 [64].

Recent studies support the NRF2/antioxidant signaling pathway in drug-mediated HbF induction. Agents such as tert-butyl hydroquinone activate NRF2 which binds the antioxidant

response element in the γ-globin promoter at nucleotide -100bp [65]. It was subsequently demonstrated that simvastatin induces HbF expression by a dual mechanism involving NRF2 activation and KLF1 and BCL11A repression [66].

Although miRNA in general mediate a direct gene repressor effect, by indirect mechanisms γ-globin gene activation can occur. For example, miR-210 is elevated in a patient with HPFH and β-thalassemia [67]. It was demonstrated that miR-15a and miR-16-1 repress MYB to induce HbF [26]. In another study, miR-26b and miR-151-3p were associated with a higher HbF response in patients treated with hydroxyl urea [68].

Research efforts to develop novel HbF inducers

Research efforts focused on developing different classes of pharmacological agents that reactivate γ-globin expression are ongoing. Insights into mechanisms of γ-gene activation by different chemical compounds have contributed to success in the field. Past and current efforts to develop different classes of drugs as HbF inducers for clinical use will be discussed below.

Cytotoxic agents: Cytotoxic compounds terminate actively cycling progenitors and perturb cellular growth to trigger rapid erythroid-regeneration and the formation of F cells. S stage cytotoxic drugs such as cytosine arabinoside [69], busulfan [70], vinblastine [71], and hydroxyurea [72,73] induce HbF production in primates and humans through this mechanism. Rapid erythroid regeneration allows progenitors with an active HbF program to be selectively recruited for maturation.

DNA methyl transferase (DNMT) inhibitors: The γ-globin genes are silenced in part due to CpG methylation in the promoter of adult erythroid cells [74]. The cytosine analogues, 5-azacytidine [75] and 5-aza-2′-deoxycytidine (decitabine) inhibit DNMTs [76] to reactive γ-gene expression. In baboons, the γ-globin genes are structurally and functionally similar to the human genes and are developmentally silenced by methylation of CpG residues [77]. 5-Azacytidine was shown to increase HbF levels in baboons [78] and sickle cell patients [79] but concerns over the carcinogenic potential [80] hindered 5-azacytidine development for human treatment until the safer derivative decitabine was designed.

Decitabine does not incorporate into RNA and is a better inhibitor of DNMT than 5-azacytidine [81]. Decitabine produces alteration in lineage differentiation kinetics to favor megakaryocytic/erythroid commitment at the expense of the granulocyte/monocyte lineages [82]. The hypomethylation of DNA leads to chromosome instability and reactivation of many genes, which has the potential to increase cancer risk after long-term decitabine treatment [82]. Effective oral administration of decitabine requires either high doses of drug or co-administration of the cytidinedeaminase inhibitor tetrahydouridine [83,84]; a Phase 1 clinical trial is underway to test the efficacy of a combination drug.

HDAC inhibitors: Gene expression is controlled by alterations in chromatin structure produced by acetylation or deacetylation of histone tails, resulting in gene activation or repression, respectively [85,86]. Several HDAC inhibitors, such as sodium butyrate, trichostatin A, adicipin, and scriptaid induce HbF synthesis in vitro [87,88] and in vivo in the β-YAC transgenic mouse model [89].

The first drug to be studied extensively as an HbF inducer was sodium butyrate. In human erythroleukemia cells, butyrate promotes cell differentiation and enhances gene expression by HDAC inhibition [90]. Infants of diabetic mothers have a delay in the γ- to β-globin switch after birth associated with elevated α-amino butyric acid levels [91,92]. This observation was recapitulated in sheep fetuses using butyrate infusions in utero [93]; later pre-clinical studies in baboons demonstrated HbF induction by butyrate in vivo [94]. Subsequent clinical studies with intravenous arginine butyrate in sickle cell and β-thalassemia patients confirmed its therapeutic efficacy as a potent HbF inducer [95]. To prevent bone marrow toxicity, intermittent pulsed regimens were designed that produced a sustained increase in HbF [96]. Further development of arginine butyrate was hampered by the need to administer the drug intravenously due to rapid metabolism when given orally.

Mechanistically, the HDAC inhibitors bind to a central zinc atom in HDACs to block deacetylation of histone H3 and H4. Specific DNA Butyrate Response Elements (BREs) have been demonstrated in several genes stimulated by butyrate [97]. The Pace lab proposed a dual mechanism model whereby HDAC inhibitors mediate γ-gene reactivation [98]. By inhibiting the function of HDACs, an open chromatin domain is created through histone hyperacetylation allowing increased DNA accessibility to effectors molecules activated by the chemical inducer, which bind *cis*-acting elements in the γ-promoter.

There are several BREs in the γ-globin promoter including the distal CCAAT box [99,100] and the SSE [101]. In vivo studies in transgenic mice have demonstrated an additional BRE at −822 in the γ-promoter, which was later shown to bind a transcriptional complex regulated by butyrate [102]. Subsequently, a post-transcriptional mechanism for γ-gene activation by butyrate was proposed, involving increased γ-globin mRNA stability and translation [103].

Short-chain fatty acids (SCFAs): Several SCFAs and their derivatives induce HbF synthesis in humans and baboons. Valproic acid and phenylbutyrate induce HbF when given to humans [104,105]. HbF induction by propionate was demonstrated in transgenic mice and baboons [106] and phenylacetic and phenylalkyl acids induce HbFin cultures and primates [107]. Using computer modeling, Perrine and associates [108] tested several SCFA derivatives to deduce the functional groups required for γ-globin reactivation. Compounds such as phenoxyacetic acid and 2,2 dimethylbutyrate induce Stat-5 cell signaling to achieve HbF induction [109] independent of HDAC inhibition. Studies by Pace and Perrine demonstrated HbF induction by 2,2 dimethylbutyrate and other SCFAs in β-YAC transgenic mice and baboons, demonstrating in vivo efficacy [102]. This agent stimulates hemoglobin synthesis and erythropoiesis in animal models [102,110].

The orally bio-available butyrate derivative 2,2-dimethylbutyrate sodium salt (HQK-1001) was tested further in Phase 1/2 clinical trials (Table 1)[111]. In a proof of concept study Inati and colleagues demonstrated oral HQK-1001 was well tolerated and significantly increases HbF [112]. Subsequently, three trials demonstrated that HQK-1001 increases HbF in β-thalassaemia patients [113] but was less

effective in SCD, therefore the trial was discontinued in sickle cell patients. It remains to be determined whether the magnitude of increase in HbF is sufficient to reduce long-term complications of ineffective erythropoiesis, anemia, and transfusion requirements in β-thalassemia patients.

Other novel agents under development: Skarpidi and colleagues [114] developed a rapid and efficient method for detecting HbF inducers, based on a recombinant DNA construct in which the coding sequences of the firefly and renilla luciferase genes, were substituted for the human γ-globin and β-globin genes, respectively. Several novel HDAC inhibitors were identified in this system and confirmed in human erythroid progenitor cultures [114]. Using the same reporter system Makala and Pace established a stable reporter system in human KU812 cells [115]. Chemical analogues of the HDAC inhibitor FK228 were shown to be novel HbF inducers in primary erythroid progenitors. To discover other agents, systems for high throughput screening has been developed over the last two decades. Using this approach selective HDAC 1/2 inhibitors such as ACY-957 that induce HbF were discovered [116]; studies with ACY-957 demonstrated increased HbF expression in human erythroid progenitors [117].

Developing a new agent from bench to bedside requires over 20 years on average thus repurposing FDA approved agents is a strategy encouraged by drug companies and government funding agents to expand the agents available to treat β-hemoglobinopathies. One such agent is the FDA approved agent Tecfidera (dimethyl fumarate), recently approved for

treatment of multiple sclerosis where it mediates immune-modulatory actions [118] with limited side effects. Dimethyl fumarate activates NRF2 signaling which is involved in drug-mediated HbF induction [66,119]. Makala and colleagues [120] investigated the ability of dimethyl fumarate to induce γ-globin expression in KU812 and primary erythroid cells generated from sickle cell patients supporting re-purposing of Tecfidera for SCD.

Another FDA approved agent pomalidomide is under development for SCD. This agent stimulates proliferation of erythroid progenitors and HbF induction [17]. In a subsequent pre-clinical study [121] pomalidomide was tested in the SCD knock-out transgenic mouse produced by Townes and colleagues [122] and was shown to induce HbF similar to hydroxylurea without myelo suppression. This study led to a Phase 1 clinical trial completed recently which is discussed below.

Recently, pre-clinical studies to support the repurpose of the monoamine oxidase inhibitor tranylcypromine for SCD were completed. The nuclear receptors TR2 and TR4 repress γ-globin expression by association with the co-repressors DNMT1 and lysine-specific demethylase 1 (LSD1) [43,123]. LSD1 removes methyl groups from mono- and dimethyl histone H3 lysine 4 producing an activating epigenetic signature [124]. Shi and colleagues examined the role of LSD1 in globin gene regulation in human erythroid cells [125] and demonstrated LSD1 inhibition by tranylcypromine mediated enhanced HbF expression. Subsequent pre-clinical studies using β-YAC transgenic mice [125] treated with tranylcypromine produced HbF induction

Table 1: Pharmacological agents that induce HbF in human clinical trials* .

Drug	Mechanism of Action	Trial Type	Route	Number Subjects	Age (Years)	Condition	NTC Number
5-Azacytidine	DNMT inhibitor	Phase II	SC	8	≥ 18	Thalassemia Intermedia	00661726
Decitabine	DNMT inhibitor	Phase II	SC	40	≥ 18	HbSS, HbSβ⁰, HbSC	01375608
Decitabine &Tetrahydrouridine	DNMT inhibitor	Phase I	Oral	25	≥ 18	HbSS, HbSβ⁰, HbSC	01685515
Hydroxyurea (MSH)	Ribonucleotide reductase inhibitor	Phase III	Oral	299	≥18	HbSS, HbSβ⁰	01425307
Hydroxyurea (BABY HUG)	Ribonucleotide reductase inhibitor	Phase III	Oral	193	9-18 months	HbSS, HbSβ⁰	00006400
Hydroxyurea (TWiTCH)	Ribonucleotide reductase inhibitor	Phase III	Oral	148	4-15	HbSS, HbSβ⁰, HbSOArab	01425307
Valproate	HDAC inhibitor	Phase I	IV	84	≥ 18	Thalassemia	00079378
Phenylbutyrate	HDAC inhibitor	Phase II	Oral	24	≥ 3	Thalassemia	00005934
Arginine butyrate	HDAC inhibitor	Phase I	IV	6	3-27	Thalassemia intermedia; HbSS	00006136
Arginine butyrate	HDAC inhibitor	Phase I	IV	15	16-60	HbSS, HbSβ⁰	Not Found
Vorinostat	HDAC inhibitor	Phase II	Oral	15	≥ 18	SCD	01000155
Panobinostat	HDAC inhibitor	Phase I	Oral	27	≥ 18	HbSS, HbSβ⁰	01245179
HQK-1001	SCFA derivative	Phase IIa	Oral	52	12-60	HbSS, HbSβ⁰	00842088
HQK-1001	SCFA derivative	Phase II	Oral	10	16-50	Thalassemia Intermedia	01642758
HQK-1001	SCFA derivative	Phase II	Oral	77	12-60	HbSS, HbSβ⁰	01601340
Pomalidomide	Thalidomide derivative	Phase I	Oral	12	18-60	HbSS, HbSβ⁰	01522547

*Clinicaltrials.gov;

Abbreviations: Hb: Hemoglobin; DNMT: DNA Methylase transferase; HDAC: Histone Deacetylase; SCD: Sickle Cell Disease; SCFA: Short Chain Fatty Acid

serving as the impetus for a clinical trial to test the ability of this agent to induce Hb Fin β-hemoglobinopathy patients.

Clinical development of HbF Inducer

The most successful HbF inducer in the pharmacologic armamentarium of drugs tested is Hydroxurea (HU). The first description of HU treatment in patients with SCD was in 1984 [126]. Since then HU was shown to increase HbF levels and reduce the clinical complications of SCD and in 1998 it became the only FDA approved drug for SCD. In the Multicenter Study of Hydroxyurea (MSH), adult subjects with SCD were randomized to receive HU therapy or placebo [127] and individuals in the HU group had ~50% reduction in vaso-occlusive rates. Patients with maximal change in HbF levels had the greatest reduction in white blood cell counts, indicating a hematologic response was associated with the clinical response [128]. The MSH data also established that HU therapy was not associated with significant toxicities [127] and was effective at reducing health care costs associated with SCD complications [129]. Long-term follow up of MSH patients up to 17.5 years [130] demonstrated a reduction in mortality. Similar results were observed in the 17-year open-label Laikon Study of HU in Sickle Cell Syndromes [131]; the probability of 10-year survival was 86% and 65% for HU and non-HU patients, respectively.

HU in children: Given the efficacy and safety in adult trials, investigations of HU were undertaken in children. An early pilot study of HU (HUSOFT) was conducted in 28 children with SCD where HU therapy was demonstrated to be feasible, well tolerated, and efficacious in young children. In addition, based on liver-spleen scans, HU could possibly delay functional asplenia [132]. Long-term follow-up data of patients in the HUSOFT extension study showed a reduction in acute SCD complications, improved splenic function, and enhanced growth rates in children taking HU therapy [133]. Based on these findings BABY-HUG [134], a Phase III randomized controlled trial, was conducted in children with SCD (mean age 13.6 months). While the primary endpoints of improved splenic function and renal glomerular filtration rates were not achieved, HU was effective at reducing the acute complications of SCD including pain episodes, dactylitis, acute chest syndrome, and red blood transfusions [134].

Interest has recently been given to the use of HU to prevent the long-term organ damage of SCD or to avoid chronic transfusions for children with severe phenotypes. Evidence of organ protection from HU in the current literature [135-137] led to investigations of HU in this setting. The Stroke With Transfusions Changing to Hydroxyurea (SWiTCH) study was a Phase III multicenter randomized trial designed to compare HU or phlebotomy with transfusions and chelation for the prevention of secondary stroke and reduction of transfusion iron overload [138,139]. There were no subsequent strokes on the transfusions/chelation arm but 7 (10%) on the HU/phlebotomy arm. Liver iron content was found to be equal between the groups therefore the study was closed early. The investigators concluded that transfusions and chelation remain the standard of management of children with SCD and stroke complicated by iron overload [138]. Taken collectively studies in children have yet to demonstrate a role of HU in the prevention or management of chronic complications.

Subsequently, a Phase III trial called the Switch study (NCT01425307) was initiated with the goal of comparing 24 months of HU to transfusions for subjects age 4-16 years with SCD and high (≥200 cm/sec) Transcranial Doppler (TCD) velocities. Children must have high TCD velocity with at least 12 months of erythrocyte transfusions and hemoglobin S less than 45% for 6 months. The primary outcome measure is maximum TCD time-averaged mean velocity on the index side (the side with the higher of the maximum TCD time-averaged velocity). This study completed recruitment recently (Table1).

Perhaps to prevent the long-term complications of SCD, HU therapy should be initiated at an earlier age. Among healthcare providers there remains a lack of consensus about when the safe and effective initiation of HU therapy should occur due to concerns over limited treatment experience. Therefore additional clinical evidence is needed to substantiate the safety of HU and its ability to prevent the γ- to β-globin switch if started in the first few months of life to maintain elevated HhF levels and prevent the long-term complications of SCD.

In response to growing evidence that HU is effective therapy for SCD the National Heart Lung and Blood Institute and Office of Medical Applications of Research wrote a consensus paper concluding that there exists high-grade evidence that HU increases HbF levels and decreases pain crisis and hospitalizations in pediatric patients [140]. Despite the success of HU in clinical trials however, national data indicates it is under-utilized [141].

Current clinical trials to identify novel HbF inducers (Table 1)

There are ongoing clinical trials to expand the number of drugs that induce HbF as possible treatment for SCD. A search on clinicaltrials.gov identified active studies involving decitabine, vorinostat, panobinostat and pomalidomide. Decitabine is a DNMT inhibitor being used in over 149 clinical trials mainly involving cancer therapy. Of interest is the Phase II study in sickle cell subjects >18 years who failed HU with an HbF <5% (NCT01375608). The primary outcome measure is the absolute change in HbF percent over three months. The secondary outcome measures are painful crisis frequency, safety and efficacy. Decitabine combined with tetrahydrouridine is being evaluated in a Phase I study (NCT01685515) to test whether SCD patients treated with the combination drug will have equal rates of non-hematologic toxicities compared to the placebo group.

The HDAC inhibitor panobinostat, is currently in a Phase I study for patients with HbSS or HbSβ⁰ thalassemia >18 years old (NCT01245179). The primary outcome is to determine the safety and dose limiting toxicities of oral panobinostat. Secondary outcomes are to determine the effect of escalating doses of panobinostat on HbF percent, F cells, and change in total hemoglobin among others. Another HDAC inhibitor, vorinostat was used in the treatment of cancer [142] when HbF levels were note to be increased during therapy which led to further investigations in SCD. Another Phase II study (NCT01000155) will determine the effectiveness and safety of oral vorinostat in severe sickle cell patients who failed HU therapy. The primary outcomes are to determine the efficacy of vorinostat in inducing a 4% absolute increase or a 100% increase in HbF levels and to characterize the safety and tolerability of vorinostat.

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Pomalidomide is a thalidomide derivative with immune-modulatory and antineoplastic activity. A Phase I study (NCT01522547) was conducted to evaluate the maximal tolerated dose, efficacy, and safety in SCD patients >18 years old refractory to HU therapy. The primary outcome measure is maximal tolerated dose with secondary measures of adverse events, absolute HbF change to 5%, total hemoglobin percent, and inflammation markers. The study was recently completed which showed HbF induction in a subset of subjects [143] supporting further development of the drug.

DISCUSSION AND CONCLUSION

Agents with the ability to enhance HbF synthesis represent a rational approach for the treatment of SCD and β-Thalassemia. For over three decades numerous chemical agents have been tested in tissue culture as HbF inducers but few have progressed to clinical trials or to the bedside. One challenge is the lack of suitable pre-clinical models to test agents in vivo. Baboons studies led to the clinical development of HU, butyrate, decitabine and HQK-1001 in SCD and β-thalassemia [73-76,78,79,82-84,112,113]. With the establishment of SCD mouse models newer agents such as pomalidomide have progressed to clinical trials [121] however the limited availability of the knock-out SCD mouse model has hampered progress in this area. Subsequently Townes and colleagues designed a second knock-in SCD mouse model [144] with γ-globin and β-globin constructs however HbF induction has not been effectively achieved with HU therapy (Pace, unpublished data). Therefore the knock-in mouse may not be ideal for future pre-clinical drug screens. An alternative model the β-YAC mouse [145] was used to confirm in vivo HbF induction by 5-azacytidine [125], scriptaid [89] and transcyclomide [125] demonstrating the utility of this mouse for pre-clinical drug screening. Currently few agents are in clinical trials that target HbF induction however drugs aimed at other complications of SCD such as nitric oxide deficiency, endothelium antagonist, anti-platelet agents, anti-sickle agents, and so forth are under development (clinicaltrials.gov). These agents can be combined with HU or other HbF inducers to produce an additive or synergistic clinic benefit.

Despite the success of HU in clinical trials in adults [127-130] and children [132-135] and its proven safety and efficacy, it remains underutilized in SCD [141]. Possible reasons include: 1) limited access to comprehensive sickle cell medical care, 2) lack of coordination between subspecialists and community-based clinicians, 3) concern over potential genotoxicity and 4) lack of patient adherence with the medication regimen. Future studies are needed to address early initiation of HU, the role of HU therapy in prevention of chronic complications, and improved methods for HU therapy delivery to all patients with SCD. There also remains the clinical question about the optimal age to initiate HU therapy. The Baby HUG trial established safety in young children but additional clinical safety data are needed for children <6 months at highest risk of infection, and spleen and kidney dysfunction. Whether early HU therapy administration will prevent the γ to β-globin switch has not been demonstrated.

Completion of the Human Genome Project greatly improved efforts to develop gene-based treatments for β-hemoglobinopathies alongside chemical inducers. Genome-wide association studies identified major genetic modifiers of γ-globin including -158XmnI *HBG2*, *HBS1-MYB* and *BCL11A* that account for ~50% of inherited HbF variance [17-20]. Orkin and colleagues advanced the field significantly to define mechanisms by which BCL11A repressed γ-globin expression which hold promise for the development of gene-based therapy in the future. The occurrence of inherited mutations in KLF1 that produce HPFH suggest this factor is a viable target for gene therapy that might be accomplished by RNAi technology to create a haplo-insufficiency state. Promising molecular targets such as KLF1 and BCL11A for therapeutic efforts aimed at HbF induction have been discovered, however additional pre-clinical data are needed before manipulation of transcription factors can be translated into therapeutic options at the bedside.

Curative therapy for SCD and β-thalassemia include hematopoietic stem cell transplantation [146,147], however, this option is limited by the availability of suitable donors in <20% of children with SCD. Ongoing clinical trials exploring alternative approaches such as matched unrelated donors [148] and the development of new regimens using haplo-identical donors in the future will increase the transplant option for the majority of SCD patients. A second approach to cure β-hemoglobinopathies is efforts to develop gene therapy [149,150] that was recently translated to the bedside with the successful treatment of two β-thalassemia patients with a modified β-globin lentivirus-based vector. This progress holds promise for expansion to SCD patients.

Landmark studies have created excitement that fully differentiated somatic cells can be reprogrammed to make induced pluripotent stem cells [151]. Subsequent studies demonstrated correction of a mouse model of SCD using this innovative approach thus opening the way to use of these cells to cure β-hemoglobinopathies [152]. One limitation is the inability to restore all hematopoietic lineages [153] with induced pluripotent stem cells which precludes use human therapy. Therefore until transplant and gene therapy is more widely available chemical inducers of HbF remains the most effective way to treat SCD.

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