REVIEW ARTICLE

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Targeting sickle cell disease root-cause pathophysiology with small molecules

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ABSTRACT

The complex, frequently devastating, multi-organ pathophysiology of sickle cell disease has a single root cause: polymerization of deoxygenated sickle hemoglobin. A logical approach to disease modification is, therefore, to interdict this root cause. Ideally, such interdiction would utilize small molecules that are practical and accessible for worldwide application. Two types of such small molecule strategies are actively being evaluated in the clinic. The first strategy intends to shift red blood cell precursor hemoglobin manufacturing away from sickle hemoglobin and towards fetal hemoglobin, which inhibits sickle hemoglobin polymerization by a number of mechanisms. The second strategy intends to chemically modify sickle hemoglobin directly in order to inhibit its polymerization. Important lessons have been learnt from the pre-clinical and clinical evaluations to date. Open questions remain, but this review summarizes the valuable experience and knowledge already gained, which can guide ongoing and future efforts for molecular mechanism-based, practical and accessible disease modification of sickle cell disease.

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Introduction

Sickle cell disease (SCD) demands practical, accessible oral therapies, since it is a problem of global scope. It afflicts millions of people worldwide, and has an especially high prevalence in pediatric populations in low-income, malaria-belt countries.[1] Such therapies are technically plausible, since despite the complex and potentially devastating multi-organ pathophysiology of SCD, this condition has a single, well-characterized root cause: polymerization of deoxygenated sickle hemoglobin (HbS). The hemoglobin molecule is an assembly of two α-like protein subunits and two β-like protein subunits ($\alpha_2\beta_2$), each with a heme moiety to transport an oxygen molecule. In SCD, the gene for the β sub-unit (*HBB*) of adult hemoglobin (HbA) contains an 'A' to 'T' mutation in the seventh codon. The β sub-units (β^S) produced by this mutated gene substitute a hydrophilic glutamate with a hydrophobic valine, predisposing deoxygenated HbS ($\alpha_2\beta^S_2$) to polymerization and gelation in red blood cells (RBC). This affects RBC viability, rheology and adhesiveness, promoting hemolysis, endothelial damage, occlusion of small blood vessels, and thromboses of large vessels. The hemolytic anemia is frequently severe, and is only partially and non-sustainably compensated by >10-fold increases in erythropoiesis.[2] The net consequence of this anemia and vaso-occlusion is decreased oxygen delivery and hypoxic injury to potentially all tissues of the body, manifest clinically as episodic pain, chronic pain, avascular necrosis of bones, infections, overt and silent strokes, renal/respiratory/cardiac/hepatic failure, and early death. In the USA >$1 billion in annual health care costs is attributed to SCD, and even so, the median life expectancy of affected individuals is shortened by two or more decades on average.[3,4] Most children with SCD in low-income countries do not even survive to adulthood.[1] By way of emphasis, all this morbidity and mortality begins with a single process, polymerization of deoxygenated HbS in RBC, and it is therefore logical to attempt to interdict this root cause. Two major small molecule drug approaches are in active clinical evaluation: (i) small molecules to shift the hemoglobin manufactured by RBC precursors from HbS to fetal hemoglobin (HbF), and (ii) small molecules to chemically modify HbS to impede its polymer-

ization. These active efforts are discussed in turn, with an emphasis on lessons learned so far and remaining open questions.

Small molecule approaches for which there are no active clinical efforts that we are aware of are not discussed here, e.g., small molecules to decrease HbS concentration by increasing RBC hydration.[5,6] Methods to interdict HbS polymerization that are not based on small molecule drugs are also not discussed here, because their application in the areas of the world most affected by SCD will be difficult for reasons of infrastructure and costs, e.g., harvesting of autologous hematopoietic stem cells, their engineering *ex vivo*, then re-infusion after myeloablative bone marrow conditioning by chemotherapy and/or radiation (gene therapy), or use of hematopoietic stem cells from immune-compatible non-SCD donors for transplant – a valuable approach in the West that has been thoroughly and recently reviewed elsewhere.[7]

Interdicting HbS polymerization by pharmacological induction of HbF

At the fetal stage of life, RBC contain fetal hemoglobin (HbF), an assembly of two α-globin subunits and two γ-globin subunits ($\alpha_2\gamma_2$), with the γ-globin subunits being encoded by duplicated γ-globin genes (*HBG2* and *HBG1*). During human development, the switch from HbF to HbA production begins late in fetal gestation (~ 7 months), and the typical adult pattern of <1% HbF and >90% HbA in RBC is established by ~12 months post-conception.[8,9] Several genetic polymorphisms or mutations in humans, some but not all identified, promote persistent, relatively high RBC HbF content beyond infancy. The phenotypes with particularly generous HbF levels (HbF >10%) are referred to as hereditary persistence of fetal hemoglobin (HPFH). SCD patients who co-inherit such genetic variants can, in the best cases, have asymptomatic, normal life-spans.[10-12] Notably, HbF has benefits even at lower dynamic ranges than seen in HPFH: HbF levels correlate continuously with fewer vaso-occlusive pain crises, less renal damage, less pulmonary hypertension, fewer strokes and longer survival.[4,13-19] In short, nature has demonstrated that HbF is a highly potent modulator of SCD.[20]

Detailed biochemical studies have demonstrated how: the intracellular concentration of HbS is a major determinant of polymerization kinetics, and HbF substitution for HbS decreases this concentration.[20-22] Moreover, HbF does not polymerize with deoxygenated HbS for reasons of molecular structure (the sophisticated biophysics underlying this have recently been reviewed in detail).[5] By contrast, HbA can polymerize with deoxygenated HbS.[20-22] In short, HbF interdicts the root-cause pathophysiology of SCD. It is logical therefore to attempt to use pharmacology to recapitulate such naturally demonstrated, powerful disease modulation.[23]

The earliest efforts at HbF induction

The earliest efforts built on the observation that HbF is enriched in RBC produced during the recovery phase of

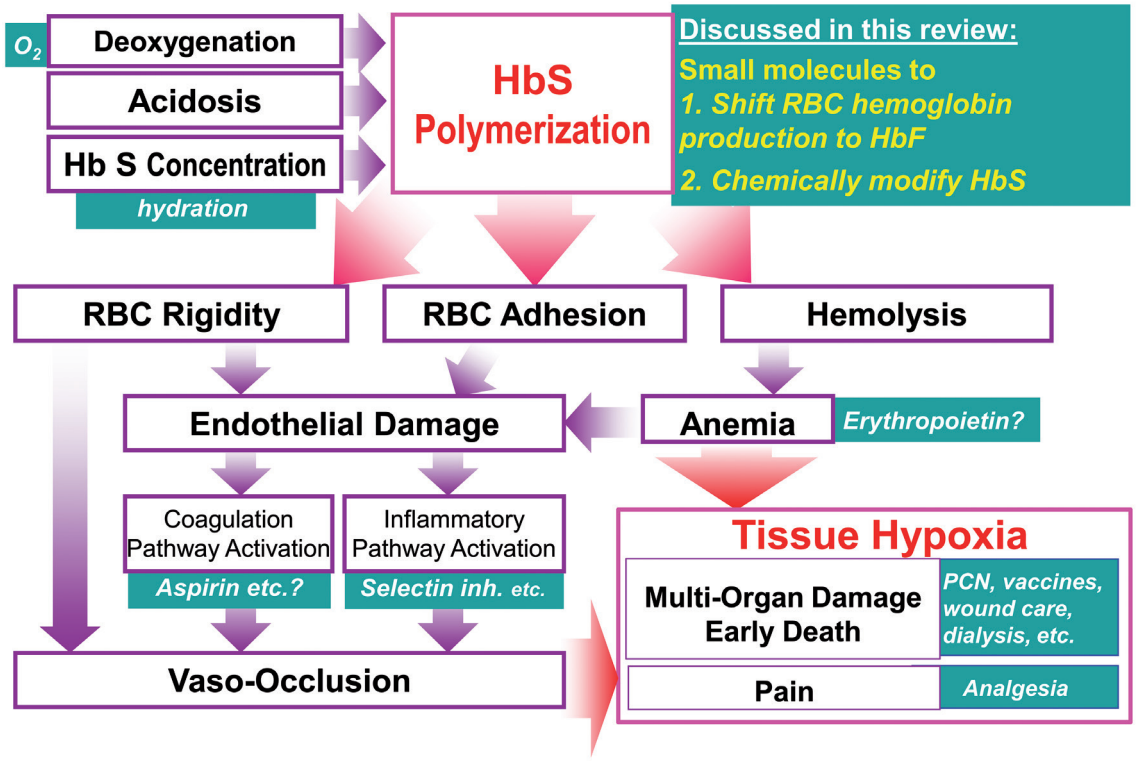

Figure 1. Polymerization of sickle hemoglobin drives the multi-organ cascade of sickle cell disease pathophysiology. This review examines the strategies to interdict the multi-organ cascade of sickle cell disease at its inception using small molecules that shift red blood cell precursor production from sickle hemoglobin (HbS) toward fetal hemoglobin (HbF), and small molecules that chemically modify HbS to decrease its polymerization. We published variations of this figure in Molokie *et al.*[95] and Lavelle *et al.*[23]

bone marrow from severe insults or stress.[24-29] One way of creating such stress is to administer cytotoxic (cell killing) drugs, leading to clinical evaluation in SCD of the oral ribonucleotide reductase inhibitor hydroxyurea.[28-30] In the pivotal trial, hydroxyurea (15-35 mg/kg) increased HbF for 2 years in ~50% of the adult SCD patients treated.[30,31] As predicted, HbF increases with hydroxyurea correlated strongly with longer RBC half-life,[32,33] fewer pain crises,[31] and better quality of life[34] (the benefits of hydroxyurea therapy in sickle cell mice also depended on HbF induction).[35] Trial patients with HbF levels >0.5 g/dL also survived longer[15] although a caveat to these analyses was that it was not known whether the higher HbF levels were intrinsic to the patients or a result of the hydroxyurea therapy.

There were, however, noteworthy limitations to the induction of HbF by hydroxyurea: (i) average HbF increases at 2 years were modest (3.6%);[28-31,36] (ii) HbF increases were particularly unlikely in patients with the lowest baseline HbF levels and thus at highest risk of morbidity and mortality,[31,33,37,38] and (iii) HbF increases diminished over time, even in the ~50% of patients with excellent initial HbF induction.[31,39]

A shared basis for these several limitations was suggested by the correlation between lower HbF increases and fewer reticulocytes (<300,000x10^9/L) and neutrophils (<7.5x10^9/L) at baseline: this correlation underscored that HbF induction by cytotoxicity requires sufficient reserves of hematopoietic precursors to mount repeated recoveries from the stress that destroys their counterparts.[29,31] Such reserves are circumscribed, subject to attrition via vaso-occlusion in the marrow and kidneys, and decline with aging.[31,33,37,38] A declining capacity to compensate for hemolytic anemia is a problem even separate from considerations of sustainable HbF induction via cytotoxicity: SCD patients require erythropoiesis at >10-fold the normal rate simply to sustain hemoglobin levels compatible with life, and dwindling compensatory reticulocytosis is a major cause of early death.[2,15,31,40] Therefore, alternative, non-cytotoxic, durable, and more potent methods of inducing HbF are needed.

Directly targeting the enzymes that silence the γ-globin gene

DNA in nuclei is packaged together with RNA and structural proteins – histones - to form chromatin. Chromatin regulates gene transcription by determining accessibility of genes to the massive machinery (~150 proteins) that transcribes genes. Reorganization ('remodeling') of chromatin, to facilitate or hinder this machinery, is signaled via post-translational modifications to histones - methylation and acetylation of lysine residues, phosphorylation of threonines and serines – and by modifications to DNA, mainly, methylation of cytosines that precede guanines (CpG). These signals determine whether ATP-dependent chromatin remodelers shift histones towards or away from gene transcription start sites, repositioning these physical barriers to either welcome or obstruct the gene transcribing basal transcription factor machinery.

Thus, induction of HbF, even when it is indirectly via bone marrow stress, implies remodeling γ-globin and β-globin gene loci, to activate one and not the other.[41] Specifically, persistent HbF expression requires: (i) decreased operation at *HBG2/HBG1* of epigenetic enzymes that create 'off' marks and that reposition histones to obstruct transcription start sites, and (ii) increased function of the epigenetic enzymes that create epigenetic 'on' marks and that reposition histones away from transcription start sites, with *vice versa* at *HBB*. Cytotoxic methods of inducing HbF achieve such chromatin remodeling crudely and indirectly, via bone marrow stress[29,41,42] (Figure 2).

So why not identify repressing epigenetic enzymes and inhibit them directly[43,44] (Figure 2)? Cells contain dozens of epigenetic enzymes mediating gene activation and repression, and not all repressing epigenetic enzymes (corepres-

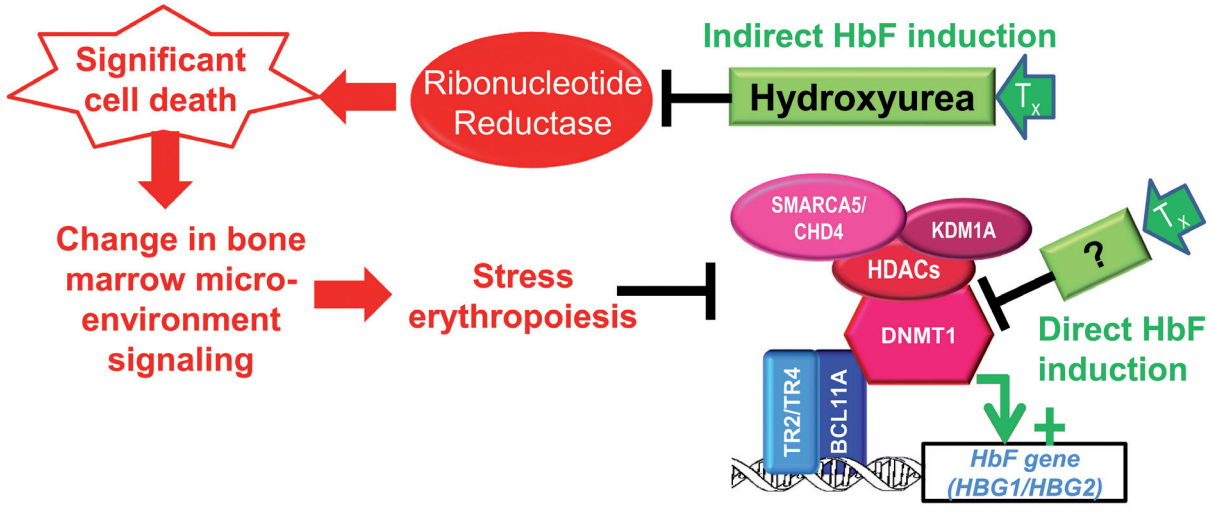

Figure 2. Induction of fetal hemoglobin (HbF) requires chromatin remodeling, including DNA hypomethylation, of the HbF gene locus. Bone marrow stress, e.g., from cytotoxic drugs such as hydroxyurea, can create chromatin remodeling during the recovery phase of surviving erythroid precursors. An alternative approach is to remodel the hemoglobin F (HbF) gene locus (*HBG*) directly, e.g., by directly inhibiting/repressing epigenetic enzymes. Enzymes shown are those known to be recruited by BCL11A, TR2 or TR4 (EHMT2 and PRMT5 are not reported participants in the BCL11A/TR2/TR4 hub). The relative efficiencies of these approaches are illustrated by the greater HbF increases produced in the same non-human primates or patients by decitabine ~0.2 mg/kg twice weekly *versus* hydroxyurea ~20 mg/kg daily. That is, the molar amount of decitabine administered per week is <1/1000th the amount of hydroxyurea administered per week. We published variations of this figure in Molokie *et al.*[95] and Lavelle *et al.*[23]

sor protein complexes) are logical molecular targets for therapy. Sequence-specific DNA-binding factors are particular in their epigenetic co-regulator usage, e.g., even distinguishing between closely similar BAF and PBAF coactivator complexes.[45-48] Logically, the epigenetic enzymes to target for HbF induction are those that have been directly implicated in silencing of *HBG2/HBG1*. Multi-protein corepressor complexes directed to the *HBG* loci by the DNA-binding factors DRED and BCL11A have been characterized in great detail.[49-53] Druggable epigenetic silencing enzymes contained in these recruited corepressor complexes include DNA methyltransferase 1 (DNMT1), various histone deacetylases (HDAC), lysine demethylase 1 (LSD1, KDM1A), and chromodomain helicase DNA binding protein 4 (CHD4) and other members of the ISWI family of ATP-dependent chromatin remodelers[52-55] (Table 1). Other types of biochemical studies have implicated euchromatic histone lysine methyltransferase 2 (EHMT2, G9a),[56] and protein arginine methyltransferase 5 (PRMT5) in the silencing of *HBG2/HBG1*.[57,58] (Table 1). Yet another approach to identifying candidate targets has been chemical screens for HbF inducers. This approach has identified histone methyltransferases EHMT1 and EHMT2 as candidates for inhibition[59,60] (Table 1). Notably not identified by studies thus far, given that there are clinically available inhibitors for these targets, are epigenetic enzymes in polycomb repressor complex 2 (e.g., EZH2).[61]

Since the natural genetic experiment of HPFH provides a fundamental rationale for pursuing pharmacological induction of HbF, by extension, can the genetic variants underlying HPFH help to identify or prioritize molecular targets for manipulation? HPFH-linked point mutations cluster in two regions 115 and 200 base-pairs upstream of the *HBG2* start site, suggesting these are sites at which key repressors of *HBG2/HBG1* bind.[62] BCL11A and ZBTB7A have been shown to bind at these locations, and HPFH mutations have been shown to abrogate such binding.[63] Moreover, some HPFH mutations occur at *BCL11A* rather than β-globin gene loci.[51,64] In short, the natural genetic experiment of HPFH also seems to support drugging of the corepressors recruited by BCL11A (and ZBTB7A and DRED).[49-53]

The candidate targets are discussed below in turn.

Histone deacetylases (HDAC)

HDAC were among the first candidate targets identified for HbF induction.[65] Moreover, a number of HDAC inhibitors have already been approved by the United States Food and Drug Administration (FDA) to treat peripheral T-cell malignancies (Table 1). Unfortunately, despite exciting pre-clinical results, clinical application of marketed HDAC inhibitors for HbF induction is limited by the pleiotropic roles of HDAC outside of chromatin. That is, clinical side-effects, arising from HDAC participation in multiple cellular and physiological functions, limit the achievement of an epigenetic pharmacodynamic effect in the target compartment, and thus of HbF induction *in vivo*.[66-71] There are efforts to develop HDAC inhibitors that are more selective to specific HDAC than the broad HDAC inhibiting activity of the currently marketed drugs (Table 1), and perhaps these more selective agents will have a more suitable safety profile for HbF induction. The caution remains that even an on-target, specific drug action can generate toxicities if the molecular target of that action has pleiotropic physiological roles.

DNA methyltransferase 1 (DNMT1)

DNMT1 is well known to maintain methylation marks on DNA through cell division. In addition, DNMT1 is a corepressor that is recruited by sequence-specific DNA-binding factors, e.g., DRED (TR2/TR4) and BCL11A, which direct epigenetic silencing of *HBG*.[43,53,72-82] The deoxycytidine analog decitabine and its pro-drug 5-azacytidine, FDA-approved to treat the myeloid malignancy myelodysplastic syndrome, can deplete DNMT1: a nitrogen substituted for a carbon in the decitabine pyrimidine

Table 1. Scientifically validated molecular targets for HbF induction and candidate drugs

Target	Recruited by BCL11A	Drugs	Stage
HDAC*	Yes	- Depsipeptide (HDAC1,2,4,6) - Belinostat (broad HDAC inhibitor) - Panobinostat (broad) - Vorinostat (broad)	- Marketed for peripheral T-cell lymphomas - Phase I in SCD (panobinostat) (*ClinicalTrials.gov identifier: NCT01245179*) - Phase II in SCD and β-thalassemia (HQK-1001) (*ClinicalTrials.gov identifiers: NCT01642758, NCT01601340*)
DNMT1	Yes	- Decitabine - 5-azacytidine (decitabine pro-drug)	- Marketed for myelodysplastic syndromes - Oral forms, including in combination with inhibitors of degradation, are in phase I/II for liquid/solid malignancies, and SCD (*ClinicalTrials.gov identifier: NCT01685515*)
KDM1A#	Yes	- ORY-1001 (related to RN-1) - GSK2879552 - 4SC-202 - INCB059872	- Phase I/II in liquid/solid malignancies - Phase I in SCD (INCB059872) (*ClinicalTrials.gov identifier: NCT03132324*) (terminated, results not publicly available)
PRMT5	Not reported	- GSK3326595	- Phase I in liquid/solid malignancies
EHMT2	Not reported	- UNC0638	- Pre-clinical *in vitro*
ISWI (CHD4, SMARCA5)	Yes	- not officially designated, patent issued	- Pre-clinical *in vitro*

*Only histone deacetylase (HDAC) inhibitors approved in the USA are listed, several other HDAC inhibitors are in clinical trials. #Only KDM1A inhibitors registered in clinical trials in the USA are listed, several other compounds are in development. SCD: sickle cell disease.

ring covalently binds to DNMT1 and causes its degradation.[83] By depleting DNMT1 protein, decitabine disrupts its scaffolding functions for other epigenetic enzymes such as KDM1A.[84,85] That is, decitabine does not just inhibit the enzyme function of DNMT1 but produces a broad corepressor disrupting effect. Because the deoxyribose moiety of decitabine is unmodified, it can incorporate into the elongating DNA strand during the S-phase without terminating chain extension or causing cytotoxicity, contrasting with most nucleoside analogs used in the clinic to treat cancer.[86,87] High concentrations of decitabine do, however, produce off-target anti-metabolite effects and cytotoxicity, in significant part via its uridine moiety degradation products that can misincorporate into DNA or inhibit thymidylate synthase.[88,89] We designed decitabine dose, schedule and route-of-administration regimens to produce non-cytotoxic depletion of DNMT1 *in vivo*.[43,90-93] These regimens increased HbF by >10% in SCD patients who had no HbF response (~0.3%) to hydroxyurea in the pivotal clinical trial.[43,81,94] That is, very small, non-cytotoxic doses of ~0.2 mg/kg twice weekly were sufficient to produce large increases in HbF and total hemoglobins, even in patients in whom hydroxyurea ~20 mg/kg/day, >1000-fold the molar amount of decitabine, did not induce HbF (Figure 2).[43,44]

Marketed decitabine, however, is a parenteral drug with trivial oral bioavailability, undermining potential for worldwide application. We have therefore combined oral decitabine with tetrahydrouridine to inhibit the enzyme that limits its oral bioavailability, cytidine deaminase.[95] This combination was well-tolerated and safe in a phase I study in patients with severe SCD. The target decitabine dose of 0.16 mg/kg produced a wide decitabine concentration-time profile (low C_{max}, long T_{max}) ideal for non-cytotoxic DNMT1 depletion[83,96-98] and decreased DNMT1 protein in peripheral blood mononuclear cells by >75% and repetitive element CpG methylation by ~10%. This increased HbF by 4-9%, doubling HbF-enriched RBC (F-cells) up to ~80% of total RBC. Total hemoglobin increased by 1.2-1.9 g/dL (P=0.01) as reticulocytes simultaneously decreased; that is, better quality and efficiency of HbF-enriched erythropoiesis elevated hemoglobin using fewer reticulocytes. Other indications of better RBC quality, biomarkers of hemolysis, thrombophilia and inflammation (lactate dehydrogenase, bilirubin, D-dimer, C-reactive protein) also improved. The side-effects were a concurrent increase in platelets and decrease in neutrophils, expected with non-cytotoxic DNMT1 depletion. In the relatively short treatment duration of 8 weeks, these blood count shifts did not cross thresholds requiring withholding or modification of treatment, that is, neutrophil counts and platelets remained in ranges observed in SCD patients receiving standard-of-care therapies.

The major limitation is the need for longer term studies to demonstrate durable safety and efficacy of the oral tetrahydrouridine/decitabine combination.

Lysine demethylase 1 (LSD1, KDM1A)

KDM1A, like DNMT1, is recruited by the *HBG2/HBG1* repressing DNA-binding factors DRED and BCL11A, and KDM1A inhibition with either of two specific inhibitors induced HbF *in vitro*, in sickle mice and in non-human primates.[99-102] Several KDM1A inhibitors are in clinical trials for cancer indications (Table 1). At least two of the compounds in trials (ORY-1001, GSK2879552) are built around a tranylcypropamine warhead that inhibits monoamine oxidases that metabolize catecholamine neurotransmitters in the brain. Although cancer clinical trials are ongoing and unpublished (*EudraCT number: 2013-002447-29*; *ClinicalTrials.gov identifiers: NCT02177812, NCT02034123*), there is concern regarding side-effects related to the potential for inhibition of monoamine oxidases other than KDM1A. Thus, there are ongoing efforts to develop and evaluate KDM1A inhibitors with other scaffolds (e.g., *ClinicalTrials.gov identifier: NCT01344707*). One registered phase I clinical trial evaluated a KDM1A inhibitor for HbF induction in SCD (*ClinicalTrials.gov identifier: NCT03132324*). This trial has been terminated but results are not publicly available at this time.

Protein arginine methyltransferase 5 (PRMT5)

PRMT5 methylation of histone H4 arginine 3 has been implicated as a signal that recruits additional chromatin-modifying enzymes and represses *HBG*.[57] There is a PRMT5 inhibitor in clinical trials (GSK3326595) for cancer indications. No trials of this molecule for HbF induction in SCD have been registered so far.

Euchromatic histone lysine methyltransferase 2 (EHMT2, G9a)

EHMT2 has been shown to be recruited to the β-globin locus by the sequence-specific DNA binding factor NFE2, and the EHMT2 inhibitor UNC0638 has been shown to induce HbF *in vitro*.[59,60] As of this time, there are no registered clinical trials evaluating EHMT2 inhibition to induce HbF.

Chromodomain helicase DNA binding protein 4 (CHD4) and SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5) (ISWI family of ATP-dependent chromatin remodelers

The culmination of chromatin remodeling for gene repression or activation is nucleosome (histone octamer) repositioning around the transcription start site. This is energetically expensive work executed by SWI/SNF or ISWI family proteins containing the HELICc-DExx ATPase domain, with SWI/SNF moving histones away to facilitate basal transcription factor machinery access and ISWI executing the opposite.[46,103,104] Since such nucleosome repositioning is the crux of chromatin remodeling, inhibition of this action should in principle offer corresponding potency. CHD4 and SMARCA5 are HELICc-DExx-containing corepressors that are recruited by BCL11A and DRED to repress *HBG2/HBG1*.[54] We have identified a first-in-class drug-like compound series that preliminarily appears to inhibit the HELICc-DExx domains of SMARCA5 and CHD4, and we are actively investigating the potential of this series to induce HbF (US20170253589A1).

Epigenetic targeting - Lessons so far and open questions

Pre-clinical and clinical experience to date provide various lessons and raise some questions regarding epigenetic targeting to induce HbF, as described below.

The consequences of inhibiting an epigenetic enzyme depend on cellular context

The baseline expression pattern of transcription factors is a key determinant of a cell's fate or function response to epigenetic enzyme inhibition, because sequence-specific

DNA-binding factors direct the function of these epigenetic enzymes and are mandatory for gene activation.[105] Stated another way, the consequences of inhibiting a particular epigenetic enzyme depend very much on cellular context.[105] A corollary of the above is that although inhibiting silencing epigenetic enzymes can produce cell fate or function shifts, these relate to what the cells were to begin with and are not drastic.[105] This is of course critical clinically, since a candidate epigenetic therapeutic for SCD will be distributed systemically.

What then about the cellular/transcription factor context of erythropoiesis enables inhibition of DNMT1 etc., to activate *HBG2/HBG1*? Several groups have found that the developmental switch from *HBG2/HBG1* to *HBB* activation is recapitulated, albeit very rapidly, during erythroid lineage maturation (a 'maturational switch' during routine erythropoiesis).[106-110] The maturational switch entails removal of activating and acquisition of repressive epigenetic marks at *HBG2/HBG1*.[59,102] with physical migration of the shared enhancer, the locus control region, from the *HBG2/HBG1* to the *HBB* locus.[54] These dynamics at *HBG2/HBG1* and *HBB* during erythropoiesis creates an opportunity for pharmacological/biochemical intervention to prevent enhancer migration, to stall the massive gene activating machinery at *HBG2/HBG1*. That is, HbF induction by inhibiting epigenetic 'off' enzymes such as DNMT1 is not predicated on returning the enhancer from *HBB* back to *HBG2/HBG1* (turning a gene that is 'off' to 'on'), but on preventing a switch from *HBG2/HBG1* to *HBB* in the first place (preventing a gene that is 'on' from being turned 'off'). Accordingly, HbF induction by an inhibitor of the silencing epigenetic enzyme EHMT2 (UNC0638) depended on the timing of its addition to cultures of synchronously maturing erythroid progenitors,[59] with similar observations in our hands with DNMT1-depleting drugs (*personal communication*).

Why are these drugs being evaluated for, or used, to treat cancer?

Some of the most recurrently mutated, deleted or amplified genes in cancers encode for chromatin remodelers. Thus, another concern with epigenetic targeting is whether it might mimic some of these genetic changes and favor activation of oncogenic programs. It is reassuring to some extent, however, that the epigenetic targets and drugs discussed above have been or are being developed to treat and/or prevent cancer. We recently reviewed the biological rationale for this,[105] and it is briefly summarized here: cancer cells, including self-replicating cancer cells (cancer or leukemia 'stem' cells), contain high amounts of the lineage master transcription factors that normally activate terminal lineage-fates, and depend on specific corepressors ('addictions') in order to avoid these terminal fates.[111] The pathway of action is activation of the terminal lineage-fates intended by cancer cell lineage master transcription factor content. The same chromatin-'relaxing' treatments that trigger terminal lineage-fates of cancer/leukemia stem cells preserve self-renewal of uncommitted tissue stem cells, since these cells express stem cell master transcription factors, not high levels of lineage-specifying transcription factors.[92,93] This therapeutic index explains why non-cytotoxic doses and schedules of decitabine can suppress malignant clones and simultaneously improve functional blood counts even in elderly patients with myeloid malignancies.[91,105,111-115] Stated simply, several corepressor components (repressing epigenetic enzymes), e.g., DNMT1, HDAC, KDM1A, have been biologically validated as molecular targets for the treatment and prevention of cancer.[111]

Teratogenic risks

Another concern is the potential for teratogenicity: this should be assumed for individual agents, unless shown otherwise by formal toxicological studies.

Drug metabolism is central to the clinical profile of activity

Drugs, being biologically active, are metabolized, and this too can contribute substantially to their *in vivo* profile of activity. For example, DNMT1-depleting decitabine is a pyrmidine nucleoside analog pro-drug that depends absolutely for its activity on the pyrimidine metabolism enzyme deoxycytidine kinase: Deoxycytidine kinase executes the initial phosphorylation of decitabine in cells, which rate-limits its conversion into the nucleotide form that actually depletes DNMT1. Serendipitously, deoxycytidine kinase is most highly expressed in the myeloid compartment, especially erythroid precursors. Thus, the clinical profile of decitabine activity is in major part dictated by its metabolically driven tropism for the myeloid compartment.

Baseline HbF levels dictate final HbF levels

There is a wide variation in baseline HbF levels in patients with SCD and even in the general population, reflecting the influence of various genetic polymorphisms on the regulation of this locus.[9] Even if a molecular targeted therapy produces similar rates of increase in HbF% (the percentage of total hemoglobin that is HbF) in all patients, the final HbF% will be dictated by the level at which HbF% began. Moreover, in clinical trials we have conducted with DNMT1-depleting decitabine, we have noticed a slightly lower slope to the rate of increase in HbF% in SCD patients with lower HbF% at baseline. Fortunately and importantly, however, HbF induced by this epigenetic strategy was well-distributed among RBC, and the rates of increase of HbF-enriched RBC (F-cells) was actually higher in patients with low F-cells at baseline.[43,81,94,95]

At some time-point after starting therapy, F-cells entering the circulation are matched by a similar number of F-cells leaving the circulation, producing plateaus in HbF% and F-cell%.

Small molecules to chemically modify HbS to impede polymerization

The scientific foundation for efforts to chemically modify HbS is the two-state allosteric Monod-Wyman-Changeux structural model which characterizes the rapidly reversible equilibrium between the quarternary structure of hemoglobin with low oxygen affinity (fully deoxygenated hemoglobin, "T' quarternary structure) and the hemoglobin quarternary structure with high affinity for oxygen (oxygenated hemoglobin, 'R' quarternary structure).[116,117] The Monod-Wyman-Changeux model demonstrated incompatibility of the R conformation with polymerization, creating a foundation to propose molecules to favor the high oxygen affinity R conformation, as a method to delay HbS polymerization.[5]

The basic concern with such an approach is that SCD is a disease of decreased oxygen delivery to tissues and,

thus, if a chemical modification produces a high oxygen affinity hemoglobin molecule, there is a necessary play off between decreased oxygen supply from increased oxygen affinity of hemoglobin *versus* increased oxygen supply from less HbS polymerization/higher total hemoglobin. This balancing act is discussed in more detail in the section 'Lessons learned so far' below. Ultimately, however, rigorous clinical evaluation is key,[5,118] and clinical evaluation has started or is underway for a number of candidate drugs that exploit these principles:

Small molecules to convert hemoglobin to methemoglobin

The earliest clinical effort in this field evaluated the conversion of hemoglobin to methemoglobin following the administration of sodium nitrite or para-amino-propriophenone (PAPP) to five patients.[119] Both agents were able to increase methemoglobin. Methemoglobin levels of >20% (but not less) produced by sodium nitrite extended RBC survival as measured by chromium-labeling. The methemoglobinemia itself was apparently well-tolerated, but there was no evidence of any clinical benefit. Instead, there were significant side-effects from the administered drugs.[119]

Interestingly, higher methemoglobin levels produced by PAPP did not extend RBC survival, possibly because PAPP was directly hemolytic.

Small molecules to convert hemoglobin to carboxyhemoglobin

Carbon monoxide can be used to convert hemoglobin to carboxyhemoglobin. Infusion of free pegylated carboxyhemoglobin (MP4CO), as a hemoglobin-based carbon monoxide carrier, was evaluated in a phase I study.[120] In an abstract description of results in 18 patients, the maximum increase in carboxyhemoglobin was to 2%, which returned to pre-dosing levels within 8 h of completion of the MP4CO infusion. There was no significant increase in total hemoglobin. No further studies have been reported.

Small molecules that delay HbS polymerization by unclear mechanisms

Niprisan (Nix-0699) and related small molecules (SCD-101) are plant-derived molecules that have been found to delay polymerization of deoxygenated HbS, but by unclear mechanisms.[121] SCD-101 has been evaluated in a phase IB clinical trial in 26 SCD patients. There were no major adverse events attributed to the drug taken for 28 days, and it appeared to decrease chronic pain and fatigue at higher doses. However, there were no laboratory data providing evidence of decreased hemolysis or increased total hemoglobin, although analysis of peripheral smears suggested improvements in RBC shape.[122]

Small molecules to increase hemoglobin oxygen affinity

Specific small molecule aldehydes have been found to form reversible Schiff base linkages with the N-terminal amino group of hemoglobin α chains to lock in the high oxygen affinity R conformation, and the polyaromatic adldehyde GBT440 (voxeletor) has been developed through to phase III clinical trial evaluation. In phase I/II randomized, double-blind, placebo-controlled evaluation in SCD patients, some of whom were receiving concurrent therapy with hydroxyurea, there were increases in total hemoglobin of ≥1 g/dL in six of 12 patients who received the drug for 90 days or more.[123] There were concurrent decreases in markers of hemolysis (lactate dehydrogenase, total bilirubin). There were no significant adverse events attributed to study drug. Oxygen delivery was evaluated by measurement of oxygen consumption during cardiopulmonary exercise testing, erythropoietin levels, resting heart rate and heart rate during peak exercise, and these parameters did not suggest decreased oxygen delivery to tissues.[123] A subsequent double-blind, randomized, placebo-controlled phase III clinical trial evaluated two different doses of the study drug (900 and 1500 mg per day) in 274 SCD patients, two-thirds of whom remained on stable doses of hydroxyurea initiated well before study enrollment.[124] A hemoglobin response, defined as an increase from baseline of >1 g/dL at week 24, occurred in 51% of the patients on the 1500 mg dose, 33% on the 900 mg dose, and 7% on placebo, in intention-to-treat analyses. There were also improvements in biomarkers of hemolysis. The frequency of vaso-occlusive crises did not differ between the treatment arms. Breakdown of vaso-occlusive crisis frequency according to whether or not the patients were taking hydroxyurea was not reported. Erythropoietin levels (as a surrogate for oxygen delivery) as well as grade 3 and serious adverse events were similar between the treatment arms.[124]

Chemical modification of HbS – lessons learned so far and open questions

Balancing acts

The clinical trial results with GBT440 thus illustrate that chemical modification of hemoglobin to increase its oxygen affinity (promote the hemoglobin R conformation) can indeed significantly decrease hemolysis and significantly increase total hemoglobin. The hope and goal is that higher hemoglobin increases oxygen supply by amounts that exceed any decrease in oxygen supply from the higher oxygen affinity of the modified hemoglobin molecule,[5,118] as per the equation:

Oxygen Supply = Blood Flow (mL blood/100 g tissue/min) *x Arterial Oxygen Saturation* (%) *x Total Hemoglobin* (g/dL).[125]

Thus, increasing total hemoglobin increases oxygen supply, but chemical modification of some of these hemoglobin molecules to increase oxygen affinity decreases effective arterial oxygen saturation and oxygen supply. Some tissues, e.g., the brain, have limited capacity to increase the 'blood flow' component in the equation, and hence, are particularly dependent on the 'arterial oxygen saturation' x 'total hemoglobin' components, as extensively modeled recently.[118,125] Underscoring this point, most silent cerebral infarctions in SCD children have been found to be caused by disruption to oxygen supply that is not caused by large vessel vasculopathy, implying anemia and/or blood oxygen saturation are critical drivers of this hypoxic damage.[126-128]

Even the 'blood flow' component of the equation is a balancing act in SCD patients: whole blood viscosity is a key determinant of blood flow; less HbS polymerization, by increasing (improving) RBC deformability, can decrease whole blood viscosity and thus increase blood flow. On the other hand, higher total hemoglobin/hematocrit can increase blood viscosity which can decrease blood flow, even with hematocrits in an anemia range, because of the contribution of baseline low RBC deformability of SCD to viscosity. This blood flow calculus needs

to be considered with small molecules aiming to chemically modify HbS, and with small molecules aiming to substitute HbS with HbF.

Ultimately, the risk/benefit calculus for any therapeutic approach requires careful clinical trial determination.

Combinatorial approaches

In oncology, combinations of drugs are almost mandatory, because the target cell population is evolving, and will select to evade the effects of drugs. Although target cells in SCD are not evolving, other biological realities compel consideration of combination therapies. One reality is that most SCD patients will already have tissue/organ damage that can undermine the potential benefits of novel small molecule therapeutics. For example, diminished bone marrow reserve from vaso-occlusive damage and/or replication-mediated exhaustion, which decreases compensatory reticulocytosis, and which contributes to early death,[2,15,31,33,37,38,40] could limit the scope of potential benefit that can be produced by HbF inducers or HbS modifiers. Another biological reality, but potentially positive, is demonstrated by the approval by the FDA of the amino acid glutamine as a treatment to reduce the frequency of vaso-occlusive crises in SCD patients.[129] Natural substances, which in most humans can be assumed to be satisfactorily maintained by a normal diet, might actually be important pharmaceuticals for SCD patients. By way of bringing such negative and potentially positive biological realities together, it is noteworthy that the natural substance nicotinamide (vitamin B3) markedly expands hematopoietic stem cells *in vitro* at concentrations that can be readily and safely produced *in vivo* with oral supplementation.[130-132] Moreover, nicotinamide is a direct precursor for the vital energy currency nicotinamide adenine dinucleotide (NAD) which is depleted in SCD RBC, increasing their susceptibility to oxidative damage. In fact, replenishing NAD is one of the rationales for glutamine administration to SCD patients.[129] In short, in considering combination therapy, there could be important, highly feasible, but unexplored opportunities around relatively non-toxic natural substances (glutamine, nicotinamide, vitamin D, etc.). Other under-evaluated natural molecules include the kidney hormone erythropoietin, since declining kidney erythropoietin production also contributes to declining compensatory reticulocytosis.[2]

Combining small molecules to inhibit more than one co-repressing enzyme in the BCL11A hub, each used at doses low enough to avoid side-effects from off-target actions, and with non-overlapping side-effects from on-target actions, might produce greater HbF induction than achieved with a single target. Such molecules should have non-cytotoxic mechanisms of action, to avoid potential injury to needed bone marrow capacity. Unfortunately, there are few non-cytotoxic small molecule drugs targeting rational epigenetic targets, and even fewer for which optimal single molecule application has been characterized (Table 1). That is, more non-cytotoxic epigenetic drugs, and more information on their profiles of side-effects from on-target and off-target actions, are needed to guide any consideration of combination therapy.

What about combining HbF inducers with HbS modifiers? This has in effect been evaluated in the clinical trials of GBT440, since this drug was added to stable doses of hydroxyurea in >60% of clinical trial participants. Hemoglobin increases of >1 g/dL occurred in ~40% of patients taking GBT440 1500 mg alone *versus* ~55% of patients taking GBT440 1500 mg + hydroxyurea in the phase III trial, but whether vaso-occlusive crisis frequency and other adverse events varied between these two groups was not described.[124] The efficacy calculus and hope is that increases in oxygen delivery from better RBC deformability and higher total hemoglobin will exceed decreases in oxygen delivery caused by greater blood viscosity and chemical modification of HbS.

Conclusions

Clinical proof-of-principle that substantial total hemoglobin increases can be produced by non-cytotoxic inhibition of specific epigenetic enzymes, to shift RBC precursor hemoglobin manufacturing from HbS to HbF, and by chemical modification of hemoglobin to promote the high oxygen affinity 'R' quarternary structure of the hemoglobin molecule, has already been generated in SCD patients. Clinical evaluation to determine the long-term safety, the impact on symptoms and multi-organ pathophysiology, and the durability of any benefits, is ongoing. There is hope that one or more of the small molecules being evaluated will pass rigorous scrutiny and culminate in practical, accessible, cost-effective, safe and potent disease-modifying therapy for SCD patients worldwide.

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