

Review

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[Athena Starlard-Davenport](#) , [Chitra Palani](#) , [Xingguo Zhu](#) , [Betty S Pace](#) *

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Review

Innovations in Drug Discovery for Sickle Cell Disease Targeting Oxidative Stress and NRF2 Activation – A Short Review

Athena Starlard-Davenport ¹, Chithra D. Palani ², Xingguo Zhu ^{2,3} and Betty S. Pace ^{2,4,*}

¹ College of Medicine, Department of Genetics, Genomics and Informatics, University of Tennessee Health Science Center, Memphis, TN 38103 USA

² Department of Pediatrics, Division of Hematology/Oncology Augusta University, Augusta, GA 30912 USA

³ Georgia Cancer Center, Augusta University, Augusta, GA 30912 USA

⁴ Department of Molecular and Cell Biology, Augusta University, Augusta, GA 30912 USA

* Correspondence: bpace@augusta.edu; Tel.: 706-721-6893; Fax: 706-721-7271

Abstract: Sickle cell disease (SCD) is a monogenic blood disorder characterized by abnormal hemoglobin S production, which polymerizes under hypoxia conditions to produce chronic hemolysis, widespread organ damage, and vasculopathy. As a result of vaso-occlusion and ischemia-reperfusion injury, individuals with SCD have recurrent pain episodes, infection, pulmonary disease and fall victim to early death. Oxidative stress due to chronic hemolysis and the release of hemoglobin and free heme is a key driver of the clinical manifestations of SCD. The net result is the generation of reactive oxygen species that consume nitric oxide and overwhelm the antioxidant system due to a reduction in enzymes such as superoxide dismutase and glutathione peroxidase. The primary mechanism for handling cellular oxidative stress is the activation of antioxidant proteins by the transcription factor NRF2, a promising target for treatment development, given the significant role of oxidative stress in the clinical severity of SCD. In this review, we discuss the role of oxidative stress in health and the clinical complications of SCD, and the potential of NRF2 as a treatment target, offering hope for developing effective therapies for SCD. This task requires our collective dedication and focus.

Keywords: sickle cell disease; oxidative stress; NRF2; glutathione; heme; hemolysis; fetal hemoglobin; hemoglobin S; reactive oxygen species

1. Introduction

Sickle cell disease (SCD) is a global health problem affecting millions worldwide, mainly in sub-Saharan Africa, where 4–6 million people live with the disease [1,2]. The urgency of this problem is underscored by the fact that 90% of the 150,000 babies born annually in Nigeria will die by 5 years of age [3]. It is imperative that we change these statistics by developing robust newborn screening programs and designing safe and effective low-cost oral agents to treat children affected with SCD.

The most common genetic mutation causing SCD is the A to T transversion in the *HBB* gene on chromosome 11, leading to a substitution of valine for glutamic acid in the sixth codon [4] of the β -globin protein chain and the production of sickle hemoglobin S (HbS). Due to the process of hemoglobin switching at birth when the γ -globin to β -globin switch occurs during the first year of life [5], the manifestations of SCD are delayed. However, as fetal hemoglobin (HbF) decreases and HbS levels increase, the pathophysiology of SCD is observed clinically. Under low oxygen conditions, HbS forms polymer, thus producing sickled red blood cells (RBCs), vaso-occlusive episodes, and tissue damage due to ischemia [6]. Inheritance of the homozygous hemoglobin SS genotype produces sickle cell anemia, the most common subtype of hemoglobinopathies observed worldwide. Other

common forms of SCD, include HbSC, HbS β^0 -Thalassemia, and HbS β^+ -Thalassemia, and many others, comprise this group of disorders [7].

The clinical manifestations of SCD consist of chronic hemolytic anemia and recurrent acute vaso-occlusive pain episodes that contribute to clinical severity, morbidity, and early mortality [7]. Common complications include splenic infarcts, high infection rates, dactylitis, stroke, acute chest syndrome, and pulmonary hypertension, among others. The kidneys are particularly susceptible to vaso-occlusive episodes due to functional abnormalities produced by RBCs sickling in the hypoxic microenvironment, producing focal and segmental glomerular sclerosis [8]. As more individuals with SCD survive to adulthood, the prevalence of chronic kidney disease is increasing by 15-30%, with significant mortality due to end-stage kidney disease and a lack of tissue matched transplant donors in the US.

In addition to the formation of sickled RBCs by HbS polymerization, it also triggers RBC hemolysis and the release of hemoglobin and free heme, causing inflammation, oxidative stress, and vascular-endothelial dysfunction [9,10]. Excessive free radicals produced by the release of hemoglobin cause the activation of pro-oxidant enzymes and the release of reactive oxygen species (ROS), mediating oxidative stress [2,11]. The primary function of mitochondria is the process of oxidative phosphorylation, which controls ROS production and ATP synthesis to provide cells with energy. Although normal RBCs lose mitochondria when fully mature, that is not true for individuals with SCD. Rivers et al. [12,13] demonstrated abnormal mitochondrial retention in people and mice with SCD-mediated ROS-associated hemolysis. Subsequent work from the Rivers lab showed that stress erythropoiesis contributes to mitochondrial retention and ROS generation in SCD [14].

The transcription factor NRF2 (erythroid-derived 2)-like 2) plays a crucial role in regulating the antioxidant response to oxidative stress in cells [15,16]. This factor is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (KEAP1) and β -transducin repeat-containing protein and directed to the proteasome for degradation. However, under oxidative stress conditions NRF2 is released and translocated to the nucleus where it activates several antioxidant proteins, including heme oxygenase 1 (HMOX1), quinone oxidoreductase 1, glutamate-cysteine ligase catalytic subunit, and glutamate-cysteine ligase modifier subunit. These proteins work together to reduce cellular ROS and inflammation. Our group and others have demonstrated another role of NRF2 as a modulator of γ -globin gene transcription and HbF induction [17–19]. In the case of SCD, NRF2 provides a unique benefit through HbF induction to inhibit HbS polymerization and lowering oxidative stress to ameliorate clinical symptoms. Therefore, US FDA-approved oral activators of NRF2, such as dimethyl fumarate (DMF) [20,21] and simvastatin [22,23], are attractive targets for drug development in SCD.

Efforts to target oxidative stress using small oral-active chemical molecules to decrease the toxic side effects are widespread. However, despite these efforts, there remains a paucity of drugs approved for clinical treatment of oxidative stress in SCD. Hydroxyurea (HU) was the first US FDA-approved agent specifically for treating SCD, mediating several beneficial effects, including HbF induction which blocks HbS polymerization, decreasing inflammation and oxidative stress, and increasing nitric oxide production [24,25]. These benefits contribute to amelioration of pain episodes and other complications by HU in adults and children with SCD. However, there remain concerns over the long-term use of HU and its effects on fertility and secondary malignancy; the latter has not occurred in SCD after more than 25 years of clinical use [26,27]. Children living in Sub-Saharan Africa received HU at a fixed dose or with dose escalation. After an adequate evaluation period, the data safety monitoring board halted the trial when the number of clinical events was significantly lower among children receiving escalated dosing of HU when compared to a fixed dose [27]. They have fewer vaso-occlusive pain episodes, acute chest syndrome, transfusions, and hospitalizations. Laboratory tests confirmed similar toxicity in the two groups. However, there were no cases of severe neutropenia or thrombocytopenia.

Over the last 10 years, three additional agents were US FDA-approved for the treatment of SCD, including L-glutamine [28] and crizanlizumab [29,30] that indirectly target oxidative stress and

voxelator [31], which inhibits HbS polymerization to improve total hemoglobin levels. Recently, during the fall of 2024, there was an abrupt discontinuation of voxelator due to safety concerns. Extraordinary progress was made by two pharmaceutical companies in 2023 with the US FDA approval of the first two gene therapies for SCD, Casgevy (exagamglogene autotemcel) [32,33] and Lyfgenia (lovotibeglogene autotemcel) [34,35]. Whether these therapies will be curative requires long-term follow-up. However, there was a remarkable resolution of vaso-occlusive episodes, marker of chronic hemolysis, and oxidative stress levels, along with improved quality of life for individuals treated with both innovative drugs. Whether there will be equal accessibility to gene therapy for individuals with SCD in the US or globally is a challenging question for healthcare providers.

In this review, we will discuss progress made in the field to understand the role of oxidative stress in human health and the pathophysiology of SCD. We will also discuss current strategies for developing small molecules and NRF2-activating drugs for treating oxidative stress. Specifically, we will explore the impact of these therapies in the field, highlighting the promising role of targeting NRF2 to improve clinical outcomes and reducing the global burden of SCD.

2. Oxidative Stress

The pathological state of oxidative stress arises from an imbalance between the production of ROS and the capacity of the antioxidant defense system to neutralize them. Under physiological conditions, ROS plays an essential role in cellular signaling and homeostasis [36]. Various natural biological processes in the human body, including respiration, digestion, alcohol and drug metabolism, and the conversion of fats into energy, generate ROS as a metabolic byproduct [37]. Moderate levels of ROS are beneficial for protecting against pathogens and wound healing activities [38]. However, excessive ROS accumulation leads to cellular and tissue damage through lipid peroxidation, protein oxidation, and DNA damage, contributing to disease pathogenesis [37,39–41].

Reactive oxygen species are generated from various sources in the human body, such as the Phase I cytochrome P450 metabolizing enzymes, NADPH oxidases, and the mitochondrial electron transport chain (ETC). The P450 system utilizes molecular oxygen to form superoxide anions and other ROS [42], which participate in cell signaling or contribute to oxidative stress and tissue damage [42,43]. Membrane-bound enzymes like NADPH oxidases generate superoxide in response to cell signaling activated by inflammatory stimuli. These enzymes play a crucial role in immune defenses by producing ROS to kill pathogens; however, sustained oxidative damage causes chronic disease states [44]. Other enzymatic sources of ROS include xanthine oxidase during purine metabolism and uncoupled endothelial nitric oxide synthase generating superoxide under conditions of endothelial dysfunction [45,46]. However, the primary generator of oxidative stress is mitochondria through the ETC [47]. These mechanisms regulate ROS levels to prevent oxidative stress and tissue damage [48,49]. As a physiologic mechanism, NRF2, superoxide dismutase, catalase, and glutathione peroxidase are the primary antioxidants to neutralize oxidative stress [50].

3. Electron Transport Chain

The ETC in mitochondria is crucial for energy production in eukaryotic cells. It primarily generates ATP while being a significant source of ROS generation. Mitochondria utilizes about 90% of the body's oxygen for ATP production through oxidative phosphorylation, leading to ROS generation as a byproduct [51,52]. Under normal conditions, the NRF2-mediated antioxidant defense system neutralizes excess ROS, but when oxidative stress ensues the production exceeds its capacity to neutralize, causing cellular damage [48,49].

Glucose is a primary energy source, undergoing glycolysis in the cytoplasm to yield pyruvate, which is then oxidized to acetyl-CoA or carboxylated to oxaloacetate before entering the tricarboxylic acid cycle, generating NADH and FADH₂ for the ETC [53]. The ETC in the inner mitochondrial membrane consists of four protein complexes, with ubiquinone and cytochrome c as mobile electron

carriers. A series of electron transfers through Complexes I-IV reduces molecular oxygen to water to create a proton gradient utilized for ATP synthesis [54,55].

Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are vital for mitigating oxidative stress by oxidative phosphorylation [56]. Superoxide dismutase converts superoxide radicals into oxygen and hydrogen peroxide to protect cellular structures. Mitochondrial superoxide dismutase 2 is crucial for detoxifying mitochondrial ROS; deficiencies can worsen oxidative damage linked to disease manifestations [57–59]. Catalase metabolizes hydrogen peroxide into water and oxygen and is critical for protecting cells from oxidative injury in ROS-producing tissues such as the liver [60]. Glutathione peroxidase reduces hydrogen peroxide using glutathione to maintain cellular homeostasis and host defenses against oxidative damage in diseases characterized by high oxidant stress [61,62].

4. Oxidative Stress in Sick Cell Disease

Thus far, we have reviewed oxidative stress mainly in healthy people, but now we will turn our attention to explore its detrimental effects in SCD. Under hypoxic conditions, HbS polymerizes to produce unstable sickled RBCs, which undergo autooxidation, producing excessive ROS by NADPH oxidase, creating a milieu of oxidative stress [63–66]. Moreover, oxidation of HbS contributes to cell membrane and protein damage and activates lipid peroxides to accelerate intravascular hemolysis [11,67]. This characteristic sequence of events causes the release of hemoglobin, the formation of microparticles, and damage-associated molecular patterns such as Hsp-70, interleukin 33, and ATP; ultimately, from the breakdown of hemoglobin, free heme is released into plasma, causing the accumulation of ROS. Both microparticles and damage-associated molecular patterns increase blood viscosity and stimulate the formation of inflammasomes by interacting with macrophages through TLR-4 binding and activation of the NF- κ B and NLRP3 signaling pathways. These activated immune cells and sickled RBCs recruit endothelial cells to expand the inflammatory niche initiating vaso-occlusion, vasculopathy, and end organ ischemic-reperfusion injury [18,68]. This toxic process contributes to cardiac dysfunction, leading to cardiomyopathy, heart failure, and pulmonary hypertension because of repeated injury to cells [69–72].

4.1. Environmental Factors

Parallel with endogenous ROS production sources, environmental stressors, including infections and aging, contribute to oxidative stress in SCD. For example, pneumonia causes an inflammatory response and triggers immune activation, generating ROS by neutrophils and macrophages. These cells produce oxidative bursts and secrete effector proteins or toxins that interfere with the translocation of the NADPH oxidase complex or signaling pathways, exacerbating endothelial dysfunction and hemolysis [73,74]. The immune response to pathogens is associated with ROS production, as immune cells such as neutrophils and macrophages utilize ROS to eliminate invading microorganisms [75]. Additionally, aging is associated with mitochondrial dysfunction, reduced antioxidant enzyme activity, and cumulative oxidative damage [76], all of which worsen the oxidative burden in SCD [77], underscoring the need for targeted antioxidant therapies for clinical management [74].

The process of vaso-occlusion and oxidative stress affects every body organ system, which is especially detrimental in SCD, where chronic oxidative stress exists. For example, cardiopulmonary complications are the leading cause of death due to diastolic heart failure and pulmonary hypertension [78]. Chronic hemolysis produces excessive pro-oxidant enzymes and HbS and free heme, which promotes free hydroxy radicals accumulation via the Fenton reaction [2], retained mitochondrial ETC activity, and RBC auto-oxidation [79–81].

Splenic sequestration crisis is another major complication of SCD mediated by oxidative stress that occurs when sickled RBC obstructs the draining vein in the red pulp of the spleen. This leads to enlargement of this organ [82] and disordered architecture of the white pulp. Over time, the number

of splenic follicles are reduced and replaced by fibrotic tissue from RBC congestion in the red pulp [83], and eventual ischemic necrosis and infarction i.e., autosplenectomy [84,85].

The scale of liver disease in SCD ranges from hepatocyte damage to severe liver failure associated with multiple organ failure syndrome [86,87], accounting for 3-11% of deaths in SCD [88–90]. Oxidative stress related to iron overload markedly increases malondialdehyde, a marker of lipid peroxidation [91], and high levels of myeloperoxidase released by activated leukocytes and neutrophils, causing endothelial dysfunction and liver injury [92]. Lastly, acute RBC intrahepatic cholestasis can occur due to sickling in the hepatic circulation, leading to ischemia and liver failure [93].

Oxidative stress-mediated endothelial dysfunction and ischemia-reperfusion injury play a key role in developing proliferative retinopathy [94], which causes vision loss and blindness in SCD. This complication affects mainly adults with SCD due to retinal ischemia with secondary neovascularization and hemorrhage, mediating retinal artery infarction and retinal detachment [95]. Reactive oxygen species inflict endothelial cell damage through oxidative reactions with membrane lipids, peptides, and nucleic acids [96,97], exposing subendothelial structures and proteins, including tissue factors, causing a hypercoagulable state [98]. Previous studies demonstrated improvement in retinal pigment epithelial cells after treatment with the oral antioxidant monomethylfumarate in SCD mice [99].

Lastly, sickle nephropathy is a significant complication of SCD, with up to 18% of affected individuals developing end-stage renal disease [100–102]. Oxidative stress occurs in the kidney due to high heme content and lipid peroxidation [103–105] producing malondialdehyde and damage to the cell membrane that plays a key role in the pathophysiology of sickle nephropathy [68]. Oxidative stress damages the endothelial lining of kidney blood vessels, leading to proteinuria, impaired glomerular filtration, hypertension, and the development of chronic kidney disease [105–107].

5. Murine Studies Linking Oxidative Stress to SCD Clinical Severity

Several published murine studies in the Townes SCD mouse show the involvement of oxidative stress in SCD phenotype severity through drug development. Simvastatin (Zocor) is a statin drug developed to decrease cholesterol levels in humans by inhibiting the function of hydroxymethylglutaryl-coenzyme A reductase [108,109]. This agent modulates ROS levels, activates NRF2 expression, and activates PI3K/AKT signaling. Simvastatin controls iron metabolism through *HMOX1* activation [110] and glutathione reduction in the liver [111], lungs [112], and spleen. Our group recently published data on Townes mice treated with simvastatin (Table 1) showed the ability of simvastatin to mitigate HbS sickling and ROS stress levels in SCD transgenic mice. [23]. A Phase I/II clinical trial with short-term simvastatin treatment in SCD patients showed increased nitric oxide levels and decreased C-reactive protein and interleukin-6 expression. However, no effects on vascular endothelial growth factor, vascular cell adhesion molecule-1, or tissue factor protein levels were observed [22].

Our group also conducted studies in SCD mice treated with another NRF2 activator DMF, which inhibits the cytoplasmic sequestration by KEAP1 to release NRF2 and activation of its downstream target *HMOX1* among others [21]. DMF also reduces inflammation and organ failure and improves the SCD phenotype [18,113], supported by decreased proinflammatory cytokine such as interleukin-6 and interleukin-1 β , adhesion molecule, heme and oxidative stress levels. Moreover, our group showed that DMF induces HbF expression and reduced the percentage of sickle RBCs under hypoxia conditions [21]. Chronic DMF administration induced the expression of NRF2-dependent antioxidant genes to detoxify heme and suppress inflammation. On the contrary, in an SCD NRF2 knockout mouse, we showed that *HMOX1* and γ -globin gene expression were silenced [114], and disease severity increased significantly [113]. These findings support the rationale of NRF2 induction as a therapeutic approach to induce HbF expression, reduce oxidative stress, and improve organ function [114].

Various other compounds have been evaluated in SCD mice as NRF2 activators or their ability to reduce ROS and/or inflammatory cytokines. For example, the NRF2 activator 1-(2-cyano-3,12,28-trioxooleana-1,9(11)-dien-28-yl)-1H-imidazole (CDDO-Im) reduces inflammation and improves organ function in SCD mice [114]. Similarly, when SCD mice were treated with 3H-1,2-dithiole-3-thione, a small chemical inducer of NRF2, a decrease in acute chest syndrome and increased survival was observed [115]. In SCD mice, activated neutrophils release myeloperoxidase, which generates oxidants that impair endothelial cell function. Zhang et al. showed that inhibiting myeloperoxidase decreases myeloperoxidase-dependent oxidative stress and restores endothelial cell function [92]. The inhibition of NADPH oxidase decreases oxidative stress to improve vascular functions in SCD [116]. Lastly, ongoing hemolysis in SCD, due to hemoglobin and free heme, activates the toll-like receptor 4 that mediates ROS generation. Of note, treatment of BERK SCD mice microglial cells with toll-like receptor-4 inhibitors reversed this effect [117].

6. Targeted Therapy to Reduce Oxidative Stress in SCD

N-acetyl cysteine (NAC) provides cysteine for glutathione de novo biosynthesis and has been investigated for its effects on cellular oxidative damage, hemolysis, coagulation, and endothelial activation. We were the first to show in an early phase I trial that NAC inhibits dense RBC and irreversible sickle cell formation and restores glutathione levels toward normal in adolescents and young adults with SCD [118]. In addition, we observed a trend toward the resolution of vaso-occlusive episodes in this population after a 6-month treatment period; subsequently, other groups confirmed our findings. Nur et al. demonstrated that 6 weeks of NAC treatment increases whole blood glutathione levels and decreases plasma advanced glycation end-products and cell-free hemoglobin levels; moreover, none of the SCD patients experienced painful episodes or other significant NAC-related complications while on treatment [119]. In addition, Ozpolat et al. demonstrated that NAC increases total and free-thiol concentrations of cysteine and glutathione in blood after intravenous administration, thus relieving oxidative stresses and reducing intra-chain disulfide bonds in von Willebrand factor; however, only two SCD patients were recruited for their study [120].

Previous clinical trials demonstrated the beneficial effects of glutamine in preventing SCD vaso-occlusive crises [28]. SCD patients treated with L-glutamine showed significant increases in NADH and NAD redox potential. Furthermore, L-glutamine decreased endothelial adhesion of sickle RBCs in vitro, suggesting that clinical improvement most likely occurred through oxidative stress mechanisms. The ability of NRF2 to regulate glutamine metabolism is another reason to support the development of small molecule activators for treating SCD.

6.1. Hemopexin

Hemopexin is a crucial plasma protein that binds and transports heme for scavenging. The hemopexin levels are significantly reduced in individuals with SCD compared to healthy controls. Hemopexin was shown to prevent heme-iron loading in the cardiovascular system, thus limiting the production of ROS and the activation of adhesion molecules; on the contrary, hemopexin promoted heme recovery and detoxification by the liver mainly through the activation of *HMOX1* [121,122]. Three times a week administration of hemopexin for three months produced a dose-dependent reduction in heme exposure and pulmonary hypertension while improving cardiac pressure-volume relationships and exercise tolerance in SCD mice. In addition, hemopexin administration attenuated pulmonary fibrosis and oxidative modifications in the lung and myocardium of the right ventricle [123]. More widely, hemopexin reduces endothelial exposure of P-selectin and von Willebrand factor, stimulating hepatic *HMOX1* to decrease vascular inflammation and vaso-occlusion [124]. These studies demonstrate therapeutic proof-of-concept for the development of hemopexin for treating SCD.

Recently, plasma-derived human hemopexin (CSL889) was investigated in a phase 1 study to evaluate its safety, tolerability, and pharmacokinetics in SCD patients [125]. The results showed that CSL889 has an excellent safety and tolerability profile when administered as a single dose of up to 200 mg/kg in subjects in steady-state SCD status and at 60 mg/kg in subjects in an acute vaso-occlusive episode (Table 2). These results provide a strong foundation for future trials to evaluate the efficacy of hemopexin in SCD [125].

6.2. Alpha-Lipoic Acid

Alpha-lipoic acid (ALA) is another potent antioxidant drug that has been investigated in the treatment of several diseases. Recently, Simonia et al. reported that ALA reduced platelet activation and thrombus formation in SCD mice [126]. High dietary intake of ALA reduced sickle RBCs, liver fibrosis, and adhesion molecule expression, including (vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and von Willebrand Factor) in multiple organs in SCD mice. Martins et al. recently evaluated the effects of ALA in sickle trait and SCD individuals. Unfortunately, ALA (200 mg) decreased glutathione peroxidase 4 activity in both groups while increasing catalase activity and reducing lipid and protein damage only in sickle trait subjects [127]. These results indicate that more studies are required to optimize the dose of ALA to produce therapeutic efficacy in SCD.

6.3. δ -Aminolevulinate

Previously, we investigated the ability of δ -aminolevulinate, the heme precursor, to activate γ -globin gene expression and its effects on cellular functions in erythroid cell systems. We demonstrated that this small molecule induced γ -globin expression at both the transcriptional and protein levels in KU812 erythroid cells [128]. Using inhibitors of the heme biosynthesis pathway, we demonstrated that heme participated in δ -aminolevulinate-mediated γ -globin transcription via NRF2 activation. These data support future studies to explore the potential of stimulating intracellular heme biosynthesis by δ -aminolevulinate or similar compounds as a novel therapeutic strategy for NRF2 activation for treating SCD and β -thalassemia.

6.4. Arginine

Arginine levels are acutely deficient in SCD patients due to consumption during vaso-occlusive pain episodes. When arginine levels are low, superoxide is produced instead of nitric oxide, reducing its bioavailability, and generating oxidative stress. Arginine recently emerged as a new treatment option for SCD since intravenous arginine was shown to improve mitochondrial function and reduce oxidative stress in children and young adults with SCD during vaso-occlusive pain episodes [129]. An ongoing phase 3 randomized controlled trial, Sickle Cell Disease Treatment with Arginine Therapy (STArT), is testing the efficacy of arginine treatment in 360 children, adolescents, and young adults in an acute vaso-occlusive pain episode [130].

7. Role of NRF2 in Globin Gene Regulation

Reactivation of HbF expression is a key therapeutic option in developing novel therapies for SCD. It is well established that HbF protein inhibits the polymerization of HbS to reverse the pathophysiology of SCD and disease severity. The FDA-approved drug HU and other small molecule compounds, such as butyrate and decitabine, were shown to induce HbF expression and inhibit HbS polymerization under hypoxic conditions to ameliorate the clinical severity of SCD [131–134]. Indeed, multiple transcription factors participate in the regulation of the five functional globin genes located in the *HBB* locus on chromosome 11. Interestingly, HU has been shown to remodel the γ -globin gene promoter in association with GATA1, GATA2, NFY, and BCL11A to facilitate an open chromatin structure [135], while butyrate and decitabine affect γ -globin gene transcription through

modifications of the proximal promoter histone acetylation and DNA methylation levels respectively [132–134].

Additionally, CRISPR strategies showed significantly higher γ -globin expression in erythroid cells carrying mutations producing hereditary persistence of HbF reverse the phenotypes of SCD and β -thalassemia major [136–138]. An important finding by insertions and deletions of binding sites for the γ -globin repressors BCL11A and Leukemia/lymphoma-related factor (LRF) in the proximal promoter resulted in γ -globin gene activation and reversal of the sickle RBC phenotype [139] [140]. The corresponding mechanistic studies support the notion that HbF induction is associated with epigenetic modifications and changes in chromatin contacts within the *HBB* locus.

The essential transcription factor NRF2 binds to the *HBB* locus to alter chromatin structure and globin gene expression. NRF2 was originally identified as a DNA-binding protein in the *HBB* locus and later was characterized as the major regulator of oxidative stress [141]. Previous studies conducted by Lowrey and colleagues demonstrated enhanced NRF2 binding in the γ -globin promoter and HbF induction after tert-butylhydroquinone and simvastatin treatment [17]. Deletion of a critical region 100 bp upstream of the γ -globin gene transcription-start site, 50-GACAAGGC-30, abolished HbF induction by these agents. Likewise, our group investigated the ability of DMF to activate γ -globin expression in primary human erythroid progenitors through NRF2 binding in the γ -globin antioxidant response element [142]. Using JASPAR61 software, we identified 23 NRF2 consensus motifs 5'-TGAnnnnGC-3' in the *HBB* locus. Subsequent studies demonstrated in vivo binding in the locus control region and proximal γ -globin promoter, supporting long-range chromatin looping to regulate globin gene expression during drug treatment [19]. Protein-protein interaction studies demonstrated that NRF2 dimerizes with small MAF proteins to bind DNA and activate gene transcription. Moreover, NRF2 competes with the transcription factor BACH1 (tBTB Domain and CNC Homolog 1), a cap 'n' collar protein family member, which binds the antioxidant response elements of target genes to regulate cellular oxidative stress levels [143].

7.1. BACH 1 Inhibitors

Heme binds to the transcription factor BACH1 and represses NRF2-mediated gene transcription. ASP8731 is a selective small molecule inhibitor of BACH1 [144]. In HepG2 liver cells, ASP8731 increased *HMOX1* transcription, and in Townes-SCD mice, it inhibited heme-mediated microvascular stasis, activated *HMOX1* and decreased hepatic NF- κ B phospho-p65 protein expression. ASP8731 increased γ -globin transcription and F-cells percentages. In human erythroid progenitors generated from CD34⁺ stem cells, ASP8731 increased γ -globin mRNA levels and the percentage of F-cells 2-fold. These data indicate that BACH1 inhibitors may offer a new therapeutic target to treat SCD.

Another novel BACH-1 inhibitor HPP-D was developed by Attucks and colleagues at vTv Therapeutics. They showed that HPP-D promotes the nuclear export of BACH-1 and the translocation of NRF2 into the nucleus to activate *HMOX1* gene expression [145]. In a subsequent study, in collaboration with vTv, we showed the ability of HPP-D to attenuate BACH-1 and enhance NRF2 protein levels while binding in the γ -globin antioxidant response element and *HBB* locus control hypersensitive site 2 by ChIP assay in KU812 cells; moreover, HPP-D activated γ -globin gene transcription and HbF protein synthesis [146]. To validate the potential of HPP-D for treating SCD, an anti-sickling study was conducted in primary erythroid cells generated from individuals with SCD. Cells grown under hypoxia conditions, demonstrated a 50% reduction of the number of sickled RBC observed, supporting the ability of HPP-D to induce HbF and produce anti-sickling effects.

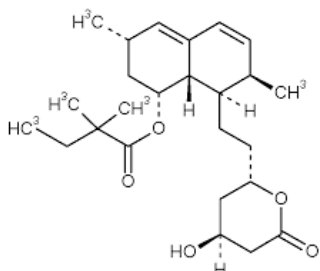
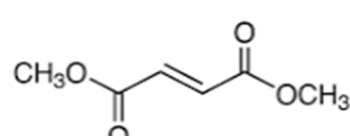
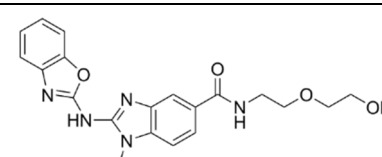
Subsequently, we completed preclinical studies in the Townes SCD transgenic mice to evaluate the capability of oral HPP-D to induce HbF (unpublished data). The mice were treated with HPP-D by oral gavage 5 days a week for 4 weeks, and blood was analyzed at weeks 0, 2, and 4. The drug was well-tolerated with normal behavior, weight gain, and no toxic effects on peripheral blood cell counts. From week 0 to week 4, we observed a maximal 3-fold increase in F-cells (HbF-positive RBCs) and

activation of γ -globin gene transcription measured by mRNA levels; by contrast β -globin mRNA levels were not changed significantly by HPP-D. To assess the antioxidant effects of HPP-D, we measured ROS and sickle RBCs levels under hypoxic conditions, which both showed an antioxidant effect with significantly decreased levels of both parameters. These data support the possible clinical development of the BACH 1 inhibitors as another class of drugs with a dual impact of HbF induction and antioxidant properties.

7.2. Regulation by MicroRNA Genes

Recently, miR-144 and miR-451 (miR-144/451) were demonstrated to play a significant role in erythroid differentiation and fine-tuning gene expression during erythropoiesis [147,148]. Interestingly, in adults with SCD, higher miR-144 levels decreased NRF2 and glutathione levels and was associated with a more severe anemia [149]. Moreover, low NRF2 expression was associated with a lack of the antioxidant proteins glutamate cysteine ligase C/M and superoxide dismutase 1 [150]. To further investigate this observation, we conducted genome-wide microarray analysis to discover miRNA genes associated with HbF expression in individuals with SCD. We observed the upregulation of miR-144 in subjects with lower HbF levels than those with higher HbF levels [149]. Functional studies in adult erythroid progenitors generated from CD34⁺ stem cells showed NRF2 silencing by miR-144 and concomitant repression of γ -globin transcription; by contrast, treatment with antagomir that inhibits miR-144 function reversed its silencing effects on NRF2. Additional studies have shown miR-28 inhibits NRF2 through a KEAP1-independent mechanism and conversely miR-153, miR-27a, and miR-142-5p down-regulate NRF2 expression [151–153]. These studies support miRNA-mediated mechanisms of NRF2 regulation might be fruitful mechanism to explore their role in γ -globin gene regulation.

Table 1. Summary of drugs that target NRF2 as a mechanism of γ -globin gene activation.

Drug	Chemical structure/DNA sequence	Mechanism of action	Clinical trials/Preclinical studies	References
Simvastatin		Competitively 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor.	NCT00508027 NCT01702246 NCT03599609	[155,156]
Dimethyl fumarate		A small-molecule NRF2 agonist	In vitro, preclinical SCD mice studies	[20,21,114,142]
ASP8731		A selective small molecule inhibitor of BACH1	In vitro and preclinical SCD mice studies.	[144]

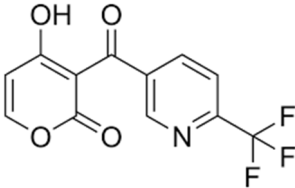
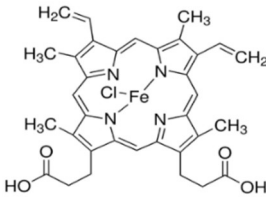
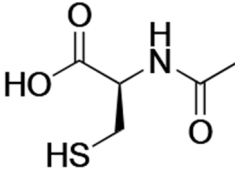
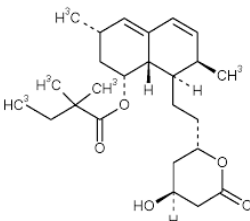
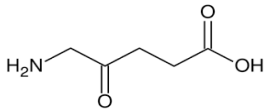
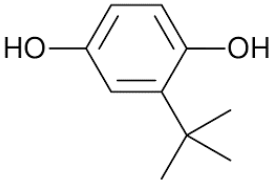
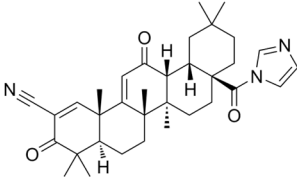
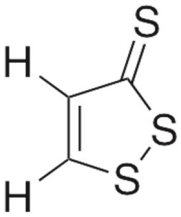
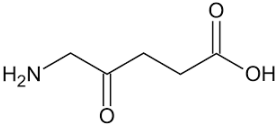
HPP-D		Bach 1 inhibitor blocks the activity of the protein BACH1.	In vitro and preclinical SCD mice	[145,146]
miR-144	<pre>-u - u G A -A gc ggg gccc ggcugG AUAUCAUC UAUACUGUA Guuu g ccc cggg cugaUC UGUAGUAG AUAUGACAU caga a cc a c A - ca gu</pre>	Inhibits NRF2 through potential miR-144 target sites in the 3'UTR of NRF2	In vitro	[147–149].

Table 2. Summary of drugs that decrease oxidative stress by different mechanisms as treatments of sickle cell disease.

Drug	Chemical structure	Mechanism of action	Clinical Trial/in vitro studies	References
Hemopexin		Scavenger of ROS by binding to free heme molecules released from damaged red blood cells	NCT06699849	[154]
N-acetyl cysteine		Precursor for glutathione biosynthesis that serves as an antioxidant through scavenging ROS and reducing disulfide bonds	NCT01800526 NCT01849016	[118–120]
Simvastatin		Competitive HMG-CoA reductase inhibitor	NCT00508027 NCT01702246 NCT03599609	[155,156]
Alpha-lipoic acid		NRF2 agonist that mediates glutathione production	NCT03161028	[127]

tert-butylhydroquinone		NRF2 agonist that mediates glutathione production	In vitro	[142]
1-(2-cyano-3,12,28-trioxoleana-1,9(11)-dien-28-yl)-1H-imidazole		NRF2 agonist that mediates glutathione production	In vitro	[114]
3H-1,2 dithole-3-thione		NRF2 agonist that mediates glutathione production	In vivo	[115]
δ-aminolaevulinic acid		Metabolic precursor of protoporphyrin IX in heme biosynthesis	In vitro	[128]

8. Future directions

Targeting oxidative stress through NRF2 activation represents a promising future direction for drug discovery in treating SCD since this transcription factor plays a pivotal role in cellular defenses against oxidative stress by activating the transcription of antioxidant genes and enzymes. As the hypoxia-reperfusion injury and inflammatory characteristic of SCD lead to increased oxidative damage, enhancing NRF2 activity should mitigate these effects and improve clinical outcomes. However, the translation of NRF2-targeted therapies from preclinical studies to clinical application remains limited. This gap is mainly due to funding limitations to conduct clinical trials and the number of investigators with the expertise to lead these endeavors to rigorously evaluate the safety and efficacy of NRF2 activators in SCD.

Inter-individual variability poses another clinical challenge in harnessing NRF2 activators and antioxidant small molecules as a treatment strategy for SCD. Genetic factors, such as polymorphisms in NRF2 or related antioxidant genes, can influence steady-state oxidative stress levels and the response to activators. Additionally, environmental exposures, dietary habits, and lifestyle choices significantly modulate oxidative stress responses, leading to variability in treatment outcomes. Understanding these influences is crucial to tailor NRF2-targeted therapies and developing personalized treatment plans. Future studies should focus on characterizing these factors within

diverse patient populations to maximize the efficacy of NRF2 interventions in managing oxidative stress associated with SCD.

9. Conclusions

Sickle cell disease is a common genetic disorder caused by a single-point mutation in the *HBB* gene, leading to HbS synthesis, which polymerizes under hypoxic conditions. The net result is a chronic intravascular hemolytic anemia, producing a severe oxidative stress state due to the release of hemoglobin and free heme into the plasma. Coupled with RBCs sickling, vaso-occlusion, and ischemia-reperfusion damage to internal organs, individuals with SCD suffer high morbidity and mortality. Therefore, reducing oxidative stress and enhancing HbF levels is an efficacious strategy to develop novel therapies for SCD.

As we move into the era where we have multiple FDA-approved drugs, we can start to develop combination regimens and take a multi-pronged treatment approach, especially with novel small molecules that act by different molecular and cellular mechanisms. We reviewed the most promising agents that either lower ROS and/or induce HbF to produce a synergistic effect on ameliorating the pathophysiology of SCD. Thus far, the most efficacious clinically tested combination has been HU and voxelator, but the latter was recently removed from the market. Fortunately, we know that NRF2 is the major regulator of oxidative stress and serves as a trans-activator of the γ -globin gene promoter with the potential as a single agent capable of increasing HbF and dampening oxidative stress. Several drugs are ripe for the picking that merit clinical safety testing in individuals with SCD, such as the FDA-approved small molecule dimethyl fumarate, among others, in the pipeline.

Agents with anti-inflammatory properties are also desirable to inhibit the release of cytokines that cause tissue damage. Hydroxyurea is an effective therapy for SCD; however, some individuals do not respond, and there are concerns over long-term safety and adverse effects. Hydroxyurea has been used for over 25 years and has improved survival and the quality of life for individuals with SCD. Although drug development has progressed, there remains a need for expanded preclinical testing in SCD mice to grow the repertoire of drugs available for clinical trials in SCD. Lastly, from the perspective of a global lens, we must develop drugs that are safe, efficacious, and low-cost, making them affordable to individuals around the world suffering with the bulk of SCD in African, India, and South America.

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