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REVIEW

Triterpenoid inducers of Nrf2 signaling as potential therapeutic agents in sickle cell disease: a review

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Abstract **Sickle cell disease (SCD) is an inherited disorder of hemoglobin in which the abnormal hemoglobin S polymerizes when deoxygenated. This polymerization of hemoglobin S not only results in hemolysis and vaso-occlusion but also precipitates inflammation, oxidative stress and chronic organ dysfunction. Oxidative stress is increasingly recognized as an important intermediate in these pathophysiological processes and is therefore an important target for therapeutic intervention. The transcription factor nuclear erythroid derived- 2 related factor 2 (Nrf2) controls the expression of anti-oxidant enzymes and is emerging as a protein whose function can be exploited with therapeutic intent. This review article is focused on triterpenoids that activate Nrf2, and their potential for reducing oxidative stress in SCD as an approach to prevent organ dysfunction associated with this disease. A brief overview of oxidative stress in the clinical context of SCD is accompanied by a discussion of several pathophysiological mechanisms contributing to oxidative stress. Finally, these mechanisms are then related to current management strategies in SCD that are either utilized currently or under evaluation. The article concludes with a perspective on the potential of the various therapeutic interventions to reduce oxidative stress and morbidity associated with SCD.**

Keywords oxidative stress; Nrf2; triterpenoids; sickle cell disease; vaso-occlusion; CDDO-Me

Introduction

Sickle cell disease (SCD) is a monogenic disorder that is inherited in an autosomal recessive fashion. The cause of SCD is a point mutation at the 6th codon of the β-globin gene causing substitution of valine for glutamic acid (β6-Glu→Val) [1–3]. This substitution results in a mutant hemoglobin molecule, hemoglobin S (Hb S), that when deoxygenated, polymerizes to produce rigid, sickle-shaped erythrocytes that are prone to oxidative stress and have a shortened life span [2].

An individual has SCD when they are either homozygous for Hb S or are compound heterozygous for Hb S and another clinically significant hemoglobin variant or β thalassemia [2,3]. The main pathophysiological mechanisms underlying the clinical manifestations of the disease are chronic hemolysis and intermittent vaso-occlusion associated with ischemia-reperfusion injury [2,4]. At the level of the microvasculature SCD is characterized by endothelial cell activation, abnormal adherence of blood cells to endothelium, inflammation, oxidant damage and nitric oxide depletion [3]. The aforementioned processes all contribute to the progressive organ damage that is associated with SCD [1–4]. An increased level of fetal hemoglobin (Hb F) ameliorates many clinical manifestations of SCD [5–7]. The presence of Hb F inhibits polymerization of sickle hemoglobin by reducing cellular concentration of Hb S and by formation of a mixed tetramer ($\alpha 2\beta^s\gamma$) that does not participate in polymerization [8]. Consequently, research efforts have focused heavily on approaches that might lead to increased levels of Hb F in patients with SCD [9]. However, there is renewed interest in the role of oxidative stress not only as a key contributor to the pathophysiology of SCD [1,4,10–12], but also as a target for therapeutic intervention. Investigations focused on oxidative stress pathways and the production of reactive oxygen species (ROS) are pointing to potential therapeutic targets that may be exploited to either prevent or reduce the

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frequency of complications of SCD [11,12]. Triterpenoids are a class of molecules with potent activities against ROS generation [13,14]. This review will focus on the role of synthetic triterpenoids that activate Nrf2 as potential therapeutic agents that prevent organ dysfunction in SCD.

The Nrf2 antioxidant pathway

Nrf2 is a basic leucine zipper transcription factor that has been identified as a regulator of stress response and redox balance [15]. The role of Nrf2 in multi-organ protection has been described [16]. The importance of targeting the Nrf2 pathway for therapeutic intervention in SCD is underscored by results from a study by Sangokoya *et al.* which showed microRNA miR-144 repressed Nrf2 levels in reticulocytes and that the highest level of miR-144 was in a subgroup of SCD patients who had the most profound anemia [17]. Under basal conditions, Nrf2 is bound to Kelch-like ECH associated protein (Keap1) in the cytoplasm and is quickly ubiquitinated. Under conditions of electrophilic stress, Nrf2 is released from Keap1, protected from degradation in the cytosol and accumulates in the nucleus, where it binds to the antioxidant response element (ARE) present on the promoters of several genes [18]. Fig. 1 provides a graphical representation of the Keap1-Nrf2 pathway. Binding of Nrf2 to the ARE leads to transcription of genes involved in cellular defense in a manner referred to as the coordinated phase II response. These Nrf2 target genes include, but are not limited to, heme oxygenase 1 (*HO-1*) (the inducible form of heme oxygenase), glutamate cysteine ligase catalytic and modifier subunits (*GCLC* and *GCLM*, respectively), glutathione S-transferase (*GST*), and NADPH quinone oxidoreductase 1 (*NQO1*). HO-1 catalyzes the rate-limiting step in the breakdown of heme to biliverdin and carbon monoxide both of which cause relaxation of vasculature. GCLC catalyzes the rate-limiting step in formation of glutathione, a powerful antioxidant, and NQO1 catalyzes the breakdown of reactive quinones. The expression of superoxide dismutase (SOD) which catalyzes the dismutation of superoxide (O_2^-) and of catalase (CAT) which breaks down hydrogen peroxide is Nrf2-dependent as well [19]. Nrf2 is therefore a key regulator of the redox balance.

What is oxidative stress?

During aerobic metabolism, free radicals such as superoxide (O_2^-) and nitric oxide (NO) are generated by NADPH oxidases, xanthine oxidase (XO) and nitric oxide synthases [4]. Acting as intermediates, these free radicals form other reactive species such as hydrogen peroxide (H_2O_2) and hydroxyl ions (OH^-). In low

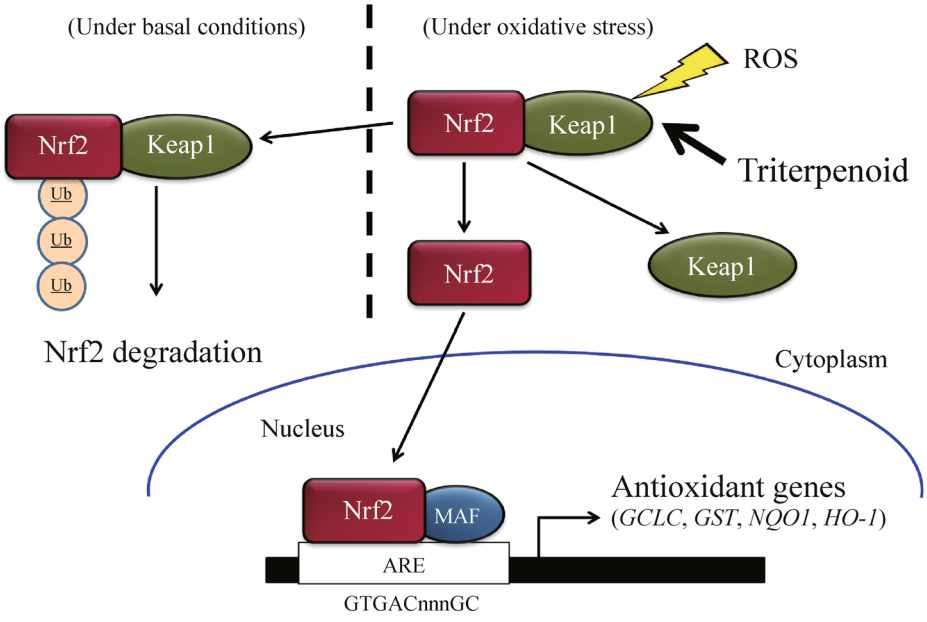


Fig. 1 The Keap1-Nrf2 antioxidant pathway. CDDO-Me is a triterpenoid inhibitor of Keap1, the cytosolic repressor that targets Nrf2 for ubiquitination. Triterpenoid binds to reactive thiol groups on cysteine 151 within the redox sensitive BTB domain of Keap1. Under basal conditions, Nrf2 is bound to Keap1 in the cytoplasm and is ubiquitinated. Under conditions of electrophilic or oxidative stress, Nrf2 dissociates from Keap1 and is protected from degradation. Nrf2 then accumulates in the nucleus where it will heterodimerize with MAF proteins and then bind to the antioxidant response element (ARE), located on the promoter of several genes involved in the phase 2 response such as *HO-1, GST, GCLC* and *NQO1*.

concentrations, these ROS molecules play important roles by modulating several signaling pathways involved in cell division or death, and they influence oxygen-sensitive processes such as erythropoiesis or ventilation, just to mention a few [20]. In high concentrations, ROS are deleterious to the cell, causing oxidation of proteins and DNA as well as lipid peroxidation. To counter or prevent the deleterious effects of ROS, a system of antioxidant molecules exists to maintain ROS levels at physiological concentrations. The antioxidant system consists of, but is not limited to, enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) catalase (CAT) and non-enzymatic molecules including glutathione, flavonoids, β-carotene, ascorbic acid (vitamin C) and vitamin E. This system of anti-oxidants scavenges ROS and restores equilibrium between pro-oxidants and anti-oxidants, a state referred to as redox homeostasis. A disturbance of this equilibrium where oxidant production predominates and exceeds the scavenging or antioxidant capacity is referred to as oxidative stress. Transient oxidative stress serves the purpose of restoring redox homeostasis while prolonged oxidative stress is associated with disease [4,20,21].

The clinical significance of oxidative stress in sickle cell disease

The existence of oxidative stress in sickle cell disease is well established in the literature with well-documented efforts to either quantify oxidative stress or measure specific biomarkers of oxidative stress [4,11,12,22,23]. Yet many questions remain regarding the significance of oxidative stress among all the interrelated pathophysiological pathways that contribute to the clinical manifestations and long-term complications of SCD. Is the oxidative stress pathway worth pursuing as a therapeutic target? Oxidative stress not only triggers hemolysis and inflammation, but it has been implicated in downstream events including the pathogenesis of acute chest syndrome [24] and the increased sickling of red blood cells within the kidney, involving the renal medulla and extending to the renal cortex of patients with SCD [25]. The interplay between multiple signaling pathways and the molecular mediators of oxidative stress in SCD make the factors controlling the expression of these mediators as attractive targets for therapeutic intervention.

Contributors to oxidative stress in sickle cell disease

The role of hemolysis

In SCD, both senescent and young erythrocytes are prone to hemolysis leading to release of significant amounts of free hemoglobin and arginase into plasma. Heme diffuses into the cell membrane where it releases its iron and generates free radicals [4]. Cell-free hemoglobin rapidly scavenges nitric oxide 1000 times faster than intra-erythrocytic hemoglobin [26], and arginase depletes L-arginine (by converting it to ornithine), a precursor of nitric oxide and a substrate for endothelial nitric oxide (eNOS) synthase. Fig. 2 depicts the pathophysiological processes in SCD that contribute to oxidative stress.

Hemolysis leads to several effects that include both the depletion of nitric oxide (NO) (which would normally inhibit expression of endothelial adhesion molecules, inflammatory response and platelet aggregation), and increased plasma arginase levels; the latter leads to increased ornithine which competes with arginine for uptake into cells to further deplete NO levels [27]. NO levels are regulated not only by availability of substrates and co-factors (tetrahydrobiopterin) for eNOS but also by inhibitors of eNOS such as asymmetric dimethyl arginine (ADMA). Schnog *et al.* demonstrated significantly elevated levels of ADMA in samples of adult SCD patients, with the highest levels of ADMA in those with the lowest hemoglobin and highest white blood cell counts [28]. ADMA competes with L-arginine for eNOS. Under conditions of reduced availability of L-arginine and/or the co-factor tetrahydrobiopterin, and in the presence of ADMA, inflammation-related eNOS tends to generate O_2^- and its by-products H_2O_2, OH^- and peroxynitrite ($ONOO^-$) instead of nitric oxide, (referred to as uncoupling of eNOS), further exacerbating oxidative stress [27,29] and leading to endothelial activation. NO depletion removes its vasodilator effect leading to unopposed action of endothelin-1 (ET-1), a powerful vasoconstrictor. NO depletion removes its inhibitory action on adhesion molecules, platelet activation and inflammation. This results in expression of adhesion molecules like vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM) [2], the thrombospondin (TSP) and the selectins. Sickle reticulocytes adhere tightly to thrombospondin through its receptor CD 36, which is expressed on the reticulocytes. L-selectin recruits neutrophils and P-selectin is involved in platelet activation. Furthermore, heme independently triggers inflammation by enhanced recruitment and adhesion of leukocytes to the endothelium, via increased expression of adhesion molecules.

The role of sickle red blood cell

There are two principally important mechanisms through which sickled erythrocytes contribute to oxidative stress: first, by generating O_2^- through auto-oxidation of hemoglobin S and second, by recruiting leukocytes in a P-selectin-dependent manner during the inflammatory response [30]. Polymerization of Hb S leads to cell membrane damage leading to loss of potassium and water

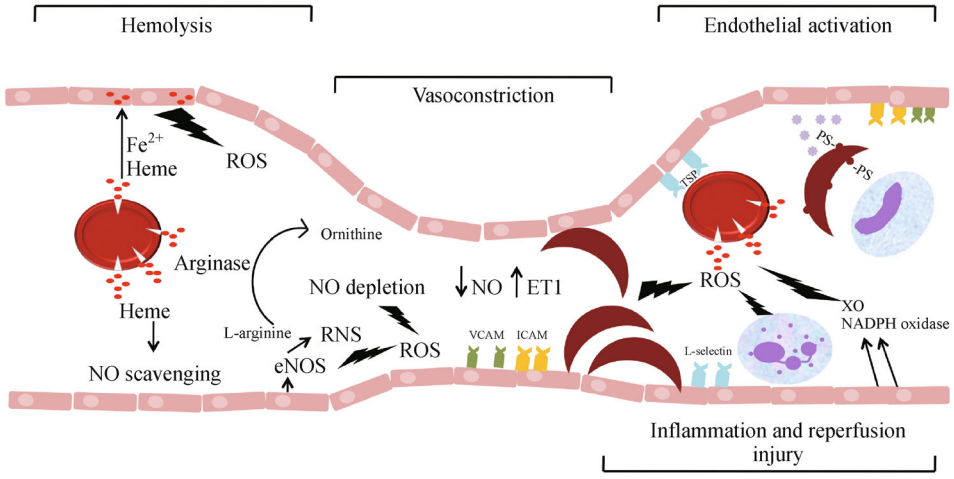


Fig. 2 Pathophysiological processes in SCD contribute to oxidative stress. A schematic diagram of how hemolysis, vasoconstriction, inflammation and ischemia reperfusion injury interact to create a state of oxidative stress is depicted. As byproducts of hemolysis, both arginase-depleting L-arginine and free heme are released from red cells and subsequently diffuse into neighboring cells, leading to iron deposits which catalyze ROS formation. Free heme also recruits neutrophils to the endothelium. Endothelial NOS becomes uncoupled under conditions of NO depletion, generating ROS and reactive nitrogen species (RNS) instead of NO. Vasoconstriction occurs when endothelin-1 (ET1), a vasoconstrictor that opposes the vasodilatory effects of NO, predominates in NO depletion. The inhibitory effect of NO on inflammation and endothelial activation is lost leading to expression of adhesion molecules like VCAM, ICAM, P-selectins and L-selectins that recruit PMN and thrombospondin (TSP) to which reticulocytes adhere via CD36. Sickle red cells expressing externalized phosphatidylserine (PS) on their membranes cause platelet (plt) activation and are phagocytosed by macrophages (M) contributing further to hemolysis. Xanthine oxidase (XO) levels are increased during reperfusion injury, increasing ROS production.

from the cell so dense erythrocytes form [31]. Dense sickle erythrocytes that have undergone repeated cycles of polymerization and de-polymerization have been shown to adhere more strongly to polymorphonuclear leukocytes (PMN) and activate the generation of ROS by these leukocytes (the respiratory burst) to a greater extent than non-dense sickle erythrocytes [32].

The role of ischemia reperfusion injury

Repeated episodes of vaso-occlusion set up cycles of ischemia and reperfusion, which lead to ROS mediated tissue damage. During reperfusion, production of XO is increased in the oxidative environment [4]. XO catalyzes conversion of hypoxanthine and xanthine to uric acid utilizing oxygen and generating O_2^- as a byproduct. O_2^- can be converted by SOD to H_2O_2 or to the highly reactive OH^- in the presence of reactive ferrous iron leading to oxidative damage. The free radical O_2^- may also uncouple eNOS to further drive oxidative stress, activating the endothelial cells locally and in organs distant from the site of ischemia reperfusion injury. The initiation of leukocyte recruitment occurs as a late event in reperfusion in response to endothelial activation and expression of selectins, leading to further generation of ROS, mediated by NADPH oxidase.

The contribution of inflammation

SCD is established as a pro inflammatory state as evidenced by high leukocyte counts, elevated levels of circulating cytokines and soluble adhesion molecules in steady-state and even higher levels of the same during acute vaso-occlusive episodes [22,33]. PMNs, which exist in an activated state in SCD patients, are recruited to the endothelium via increased endothelial expression of adhesion molecules in response to free heme and decreased NO. The PMNs then produce ROS via the activity of NADPH oxidase. ROS stimulate increased production of cytokines, which drives the formation of more ROS. Monocytes are also activated and contain increased cytoplasmic TNF-α and interleukin-1β. Akohoue *et al.* demonstrated elevated levels of inflammatory markers such as C reactive protein, interleukin-8 and 2,3-dinor-5, 6-dihydro-$15F_{2t}$-isoprostane (F2-IsoPM), a marker of oxidative stress in patients with SCD in the steady-state [22]. The NF-κB pathway is activated in SCD by ROS, leading to increased production of inflammatory cytokines [4].

The role of decreased antioxidant capacity

Reduced antioxidant capacity of the erythrocytes in SCD has been described [11,12,17,34]. SCD erythrocytes reportedly have low levels of antioxidant enzymes such

as SOD, GCLC and glutathione [17]. In a study published by Gizi *et al.*, total and reduced glutathione in erythrocytes of SCD subjects were decreased by 32%–36% together with reduced levels of vitamins A, C and E [12]. It has been proposed that the reduced antioxidant capacity in SCD is due to increased utilization of antioxidant molecules rather than inadequate production. Sangokoya *et al.* described low levels of GCLC, SOD and CAT enzymes in erythrocytes of SCD patients and low levels of the transcription factor Nrf2 in reticulocytes and developing SCD erythroid progenitors compared with those of control subjects [17].

Therapeutic strategies for reducing oxidative stress

Induction of fetal hemoglobin expression

Since polymerization of Hb S is a central inciting event for the multiple pathophysiological processes leading to oxidative stress in SCD, strategies that are currently employed to increase Hb F should theoretically decrease oxidative stress. In this regard, hydroxyurea (HU) which produces its disease modifying effects through several mechanisms most notable of which is its ability to increase Hb F [35], was shown to lower measures of oxidative stress in a cohort of sickle cell disease patients taking HU compared to those not taking it in a study conducted by Torres *et al.* [36]. In their study plasma glutathione levels were higher in the HU group, directly correlating with Hb F levels [37]. The Multicenter Study of Hydroxyurea (MSH) and other studies have established that HU significantly reduced frequency of painful vaso-occlusive crises, the rate of hospitalization, blood transfusion requirements and overall mortality in SCD [38,39]. The elucidation of epigenetic factors influencing γ globin gene expression has targeted DNA hypomethylation and histone acetylation as effective strategies to increase Hb F expression [40]. As a result, several Hb F inducers, which act as DNA hypomethylating agents and chromatin modifiers, e.g., histone deacetylase (HDAC) inhibitors, have entered into clinical trials in SCD. 5-azacytidine and its analog 5-aza-2 deoxycytidine (Decitabine) are DNA methyl transferase (DNMT) inhibitors that effectively enhance levels of Hb F in preclinical studies in baboons [41,42]. Decitabine, a safer and more effective DNMT inhibitor than its analog, increased Hb F, the percentage of F-cells (RBCs containing more than 20% Hb F) and total hemoglobin in a phase I/II study of SCD patients who had not tolerated or responded to HU [41,43]. Additionally, Decitabine decreased endothelial damage, red cell adhesion and coagulation activation parameters without evidence of myelotoxicity and was effective in 100% of SCD patients [41,43]. Currently there are two open trials of Decitabine: a phase II study of parenteral Decitabine for adults with SCD who have not responded to or are intolerant of HU (ClinicalTrials.gov Identifier NCT01375608), and a phase I study of combined oral Decitabine with Tetrahydrouridine in patients with high risk SCD (ClinicalTrials.gov identifier NCT01685515). Hyperacetylation of histone components enhances transcription of γ-globin genes by allowing transcription factor binding [40,44]. *In vitro*, HDAC inhibitors are powerful inducers of Hb F. HDAC inhibitors include Scriptaid Trichostati A, Butyrate and hydroxamic acids such as Vorinostat and Panobinostat. Vorinostat is currently in phase II trial to determine its efficacy in increasing Hb F in SCD patients who have not benefited from prior therapy (ClinicalTrials.gov identifier NCT0100155). There is an ongoing phase I study of Panobinostat in SCD patients (ClinicalTrials.gov identifier NCT01245179). Lenalidomide, an immune-modulator used in the treatment of some hematologic malignancies, reduced transfusion dependency in patients with chromosome 5q-associated myelodysplastic syndrome [45]. Based on this, Lenalidomide and Pomalidomide were studied for their effects on hemoglobin synthesis in $CD34^+$ cell derived-erythroid progenitors from SCD patients and controls [46]. Pomalidomide emerged as a more potent inducer of Hb F than Butyrate. Lenalidomide acted synergistically with HU in increasing Hb F [46]. Pomalidomide, whose mechanism of action is through acetylation of histone H3 on the γ-globin promoter [46], has been evaluated in a phase I trial in patients with SCD (ClincalTrials.gov identifier NCT01522547).

Reduction of hemolysis and its downstream effects

The increases in Hb F levels that accompany therapy with HU were associated with a small decrease in hemolysis in study of SCD subjects [47]. NO donor capabilities of HU have been described [48,49] and likely are another mechanism for the clinical benefit seen in SCD patients exposed to this therapy. Inhaled NO in transgenic mouse models of SCD was effective in decreasing vasculopathy and mortality [33]. However, a multicenter randomized double blind placebo controlled trial of inhaled NO did not show benefit compared to placebo in reaching primary or secondary endpoints of the study (ClinicalTrials.gov identifier NCT00094887) [50]. Arginine therapy increases NO in plasma in a dose dependent fashion [33]. In low doses it did not demonstrate clinical benefit [33] but in high doses it significantly decreased narcotic requirements as demonstrated in a clinical trial of arginine therapy in children hospitalized with painful vaso-occlusive episodes [51]. As mentioned earlier, ferrous iron participates in the generation of ROS in circulation and on erythrocyte membranes from heme protein deposition. Deferiprone, an

oral iron chelator which is able to permeate cells, was shown to reduce abnormal iron deposits on red cell membranes, therefore it may potentially reduce oxidative stress [33].

Reduction of the contribution of sickle erythrocytes to oxidative stress

The contribution of sickle erythrocytes to oxidative stress can be attenuated by therapies that promote hydration, prevent formation of dense sickled red cells, and those that prevent sickle erythrocytes adhesion. In a phase II trial of N-acetyl cysteine (NAC), a daily dose of 2400 mg significantly reduced the proportion of dense sickled red cells in a cohort of SCD patients [52]. In a mouse model of sickle cell disease, arginine decreased water and ion loss through Gardos channel inhibition and reduced the formation of dense red sickle erythrocytes [33]. It has been suggested that glutamine supplementation as well as heparin may be beneficial in reducing the adhesive properties of blood cells in patients with SCD [33]. Clinical trials testing the efficacy of propranolol in reducing soluble adhesion markers and β-adrenergic mediated red blood cell adhesion are in progress [33].

Repression of inflammation and ischemia-reperfusion injury

Strategies such as inhibition of NADPH oxidases and XO have been proposed to reduce the generation of ROS that leads to ischemia reperfusion injury [1]. Zinc has anti-inflammatory and antioxidant effects likely through the inhibition of NADPH oxidase. Zinc competes with iron for binding sites on cell membranes and possibly functions as a cofactor for SOD [11]. The statins have demonstrated anti-inflammatory activity mainly through NO production, prevention of endothelial activation and reducing levels of IL-6. A2A inhibitors, which decrease invariant natural killer cells, have also been used in a safety trial involving SCD patients [33,53]. Currently a multicenter phase II randomized placebo-controlled trial of Regadenoson, an A2A adenosine receptor agonist, is underway to evaluate its efficacy in reducing iNKT cells in SCD (ClinicalTrials.gov identifier NCT01788631). Inhibition of the leukotriene pathway [33] and of the nuclear factor κ light chain enhancer of activated B cells (NF-κB) signaling [1] are other strategies currently being employed to reduce inflammation in SCD. Similarly, carbon monoxide (normally generated from heme breakdown) has anti-inflammatory and vasodilator properties [11].

Enhancement of cellular antioxidant capacity

Glutamine replenishes the supply of NADPH and is a precursor of glutathione [33,54]. Glutamine has been shown to reduce energy expenditure of cells. NAC provides the precursors (cysteine) for glutathione, increasing its synthesis in cells. In the phase II double blind randomized clinical trial published by Pace *et al.,* NAC increased blood glutathione levels to near normal levels and reduced frequency of painful vaso-occlusive episodes [52]. The CURAMA investigators conducted a randomized open label trial of NAC in a cohort of SCD patients [34]. NAC at a daily dose of 1200 mg or 2400 mg increased whole blood glutathione levels, cell free hemoglobin levels and decreased expression of erythrocyte phosphatidylserine (PS) on the external surface of the erythrocytes membrane. α-lipoic acid also increases antioxidant capacity by downregulating NF-κB, increasing transcription of antioxidant genes and glutathione synthesis [33]. L-acylcarnitine which is involved in the transport of long chain fatty acids into mitochondria and decreases markers of lipid peroxidation, is another important molecule involved in antioxidant capacity of erythrocytes [33].

The natural and synthetic triterpenoids

The capacity to modulate many of the aforementioned pathways through therapeutic intervention has become a topic of increasing interest, particularly in SCD. The triterpenoids are a class of small molecules, both natural and synthetic, that has great potential for this application. The triterpenoids are derivatives of squalene and oxidosqualene containing 30 carbons that exist in natural and synthetic forms [55]. Both groups have demonstrated anti-inflammatory, anti-angiogenic and anti-proliferative properties [56]. Numerous plant-derived triterpenoids have been used for their medicinal properties in Asian traditional medicine over a long period of time [56]. They exist in nature as oleananes, lupanes, ursanes and many other forms [56].

Two natural triterpenoids, oleanolic acid and ursolic acid, were found to have anti-inflammatory activity but required very high doses to exert their effect *in vitro*, hence an effort ensued to develop more potent synthetic derivatives of these compounds [55]. Synthetic oleanane (derived from oleanolic acid) triterpenoids (SOTs) were developed by Michael Sporn, Gordon Gribble and colleagues in a quest to provide novel classes of anti-inflammatory molecules for application in various chronic inflammatory disorders including those that serve as a precursor to cancer development [56]. The SOTs were tested for anti-inflammatory properties by their ability to inhibit cytokine (IL-1, interferon-γ and TNF-α)-mediated transcription of inducible nitric oxide synthase and cyclooxygenase II during inflammation without interfering with the activity of existing iNOS itself [56,57]. In addition to their anti-inflammatory properties, SOTs exert cyto-

protective effects through induction of phase 2 responses via Nrf2 activation, leading to transcription of enzymes including NQO1 and HO1 as described earlier [58]. Specifically, SOTs including the prototype CDDO-Me act as inhibitors of Keap1 (Fig. 1). They activate the Nrf2-mediated antioxidant response through covalent binding to particular cysteine residues within the oxidative stress-sensing BTB (Broad complex, TramTrac and Bric a Brac) domain of Keap1 [59]. Evidence that this effect is mediated through the Keap1-Nrf2 pathway was provided by studies showing muted iNOS inhibition in Nrf2 null mice, and there are overlapping gene expression profiles between mice with deletion of the Keap1 gene and triterpenoid treated wild type mice [60]. A direct correlation ($r^2 = 0.91$) between the phase 2 response and anti-inflammatory ability of the SOTs has been reported [58].

Triterpenoids: potential as modulators of oxidative stress in sickle cell disease?

Targeting events downstream of hemolysis through induction of HO-1

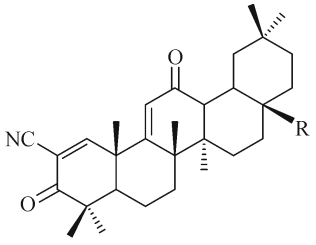
Small molecules in the SOT family have the capacity to potently induce HO-1 expression, which catalyzes breakdown of heme to biliverdin and carbon monoxide. An end result of this effect is a reduction in the amount of free heme available to deposit iron on red cell membranes or to scavenge NO. There is also a concomitant reduction in heme-driven inflammation via endothelial activation and leukocyte recruitment. Importantly, the response to molecules in the SOT family is accompanied by an added beneficial anti-inflammatory and vasodilatory action of carbon monoxide (CO) that is generated from the breakdown of heme.

Suppression of ROS generation during ischemia reperfusion injury

Activation of Nrf2 leads to transcription of phase 2 cytoprotective enzymes including the thioredoxins, peroxiredoxin I, and NQO1. These enzymes scavenge and detoxify ROS and would be of potential benefit in reducing oxidative stress in SCD erythrocytes which generate O_2^- species at twice the rate of normal erythrocytes [4]. Since oxidative stress and ROS have been implicated in chronic organ damage in SCD, the potential that triterpenoids will decrease ROS, thereby reducing or preventing organ damage in SCD, is likely more than theoretical.

Inflammation

Inflammation and oxidative stress are two phenomena that perpetuate each other in an unending cycle of ROS production. This cycle is mediated by inflammatory cells and activated endothelial cells [55]. Several hundred triterpenoid derivatives of oleanolic acid have been screened for their anti-inflammatory activity [55,58]. The most potent SOTs of this group were derivatives of 2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oic acid (CDDO) including the methyl ester (CDDO-Me or Bardoxolone methyl), the methyl amide (CDDO-MA), the imidazolide (CDDO-Im) and the trifluoroamide (CDDO-TFEA) derivatives [55]. Fig. 3 depicts the chemical structures of CDDO, CDDO-Me, CDDO-MA, CDDO-Im and CDDO-TFEA. All of these compounds demonstrated potent anti-inflammatory activity in nanomolar concentrations. It was noted that at these concentrations, these SOTs are potent activators of the Nrf2 antioxidant pathway through their interactions with Keap1 [55].



R = COO-H (CDDO)
R = COO-CH_3 (CDDO-Me)
R = CO-NH-Me (CDDO-MA)
R = CO-$C_3H_3N_2$ (CDDO-Im)
R = CO-NH-CH_2-CF_3 (CDDO-TFEA)

Fig. 3 Chemical structures of select SOTs. The structures of SOTs are depicted, including 2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oic acid (CDDO) and its analogs methyl 2-cyano-3,12-dioxooleana-1,9(11)-diene-28-oate(CDDO-Me), 2-cyano-3,12-dioxooleana-1,9-diene-28-oic acid-methylamide (CDDO-MA), 1[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]-imidazole (CDDO-Im), and CDDO trifluoroethylamide (CDDO-TFEA).

Thimmulappa *et al.* showed that neutrophils derived from the peritoneum of *nrf2*$^{-/-}$ mice exhibited lipopolysaccharide (LPS)-induced ROS generation that was 4-fold higher than those derived from wild type *nrf2*$^{+/+}$ mice [61]. They also demonstrated higher levels of inflammatory cytokines IL6 and TNF-α. Pretreatment of neutrophils with CDDO-Im prior to LPS challenge induced a phase II response, attenuating ROS generation and lowering levels of the aforementioned inflammatory cytokines in *nrf2*$^{+/+}$ but not *nrf2*$^{-/-}$ mice [61].

In human peripheral blood mononuclear cells and neutrophils, CDDO-Im and CDDO-Me demonstrated antioxidant gene induction and suppression of cytokines induced by LPS [62]. The role of CDDO-Im in increasing NO production through *HO-1* induction and reduced uncoupling of eNOS has been described by Heiss *et al.*

[63]. Cho *et al.* described the protective effects of Nrf2 from bleomycin-induced lung fibrosis in mice [64]. Clinically, CDDO-Me was the first orally administered SOT to be used in a phase III clinical trial of adult patients with diabetic nephropathy [65,66]. It significantly increased estimated glomerular filtration rate at all 3 doses tested at the study endpoint of 24 weeks, with the beneficial effect persisted for at least 52 weeks [67]. Currently, a clinical trial is open to evaluate the efficacy and safety of CDDO-Me for pulmonary arterial hypertension (ClinicalTrials.gov identifier NCT02036970).

Possible clinical application for triterpenoids in sickle cell disease

One possible benefit of the application of SOTs in SCD might be the reduction in the frequency of vaso-occlusive crises via several mechanisms, including reduction of inflammation, adhesion and ROS levels in the steady-state, scavenging of free heme through induction of heme oxygenase 1 and increasing bioavailability of NO, and through prevention of the uncoupling of eNOS. Red cell survival would be expected to increase following a reduction in ROS levels and oxidative stress.

Another possible application for SOTs would be the prevention of or delayed progression of organ dysfunction in SCD. In SCD, asthma is a frequent co-morbid condition [27,68] and the risk of chronic lung disease increases with both age and frequent episodes of acute chest syndrome. In chronic lung disease associated with SCD, lung remodeling with fibrosis is part of the pathology [27]. Activation of Nrf2 prevents fibroblasts from differentiating into myofibroblasts that cause lung scarring, so in theory SOTs may be able to ameliorate or prevent initiation and progression of chronic lung disease in SCD. Triterpenoids, partially through a reduction of oxidative stress in the kidney, may prevent onset or progression of chronic kidney disease in SCD.

With the entry of CDDO-Me into phase II clinical trials for pulmonary arterial hypertension there may emerge yet another indication in the near future for SOTs in SCD patients with pulmonary hypertension, if proven effective in this setting.

In conclusion, the pathophysiology of SCD, known for the complexity of its interrelated pathways, has long been a challenge to multiple therapeutic agents that appeared promising in preclinical studies, but failed to demonstrate significant benefit in clinical trials. It is evident that no single agent will most likely ameliorate every pathophysiological process of SCD. However, the SOTs are a unique group of multifunctional molecules whose multiple targets and ability to reduce oxidative stress make them worthy of further study for clinical application in SC. They could be used as single agents or in combination with drugs that increase production of fetal hemoglobin. Indeed, the SOTs represent perhaps the most attractive and rationally designed approach to the long-term prevention of morbidity and mortality in sickle cell disease. The proof of their efficacy in this context will depend on the development and execution of well-designed and executed clinical trials. These clinical trials must also consider carefully the effects of dosing, duration of exposure and patient age upon the administration of SOTs in order to yield the greatest clinical benefit.

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Compliance with ethics guidelines

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