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Sulfated non-anticoagulant heparin derivative modifies intracellular hemoglobin, inhibits cell sickling *in vitro*, and prolongs survival of sickle cell mice under hypoxia

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Conflicts of interest

SAM holds a U.S. patent on S-NACH (28) and other related US patents. All other authors have no conflicts of interest.

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Abstract

Sickle cell disease (SCD) is an autosomal recessive genetic disease caused by a single point mutation, resulting in abnormal sickle hemoglobin (HbS). During hypoxia or dehydration, HbS polymerizes to form insoluble aggregates and induces sickling of red blood cells (RBCs). RBC sickling increases adhesiveness of RBCs to alter the rheological properties of the blood and triggers inflammatory responses, leading to hemolysis and vaso-occlusive crisis sequelae. Unfractionated heparin (UFH) and low-molecular weight heparins (LMWH) have been suggested as treatments to relieve coagulation complications in SCD. However, they are associated with bleeding complications after repeated dosing. An alternative sulfated non-anticoagulant heparin derivative (S-NACH) was previously reported to have none to low systemic anticoagulant activity and no bleeding side effects, and it interfered with P-selectin-dependent binding of sickle cells to endothelial cells, with concomitant decrease in the levels of adhesion biomarkers in SCD mice. S-NACH has been further engineered and structurally enhanced to bind with and modify HbS to directly inhibit sickling, thus employing a multimodal approach. Here, we show that S-NACH can (i) directly engage in Schiff-base reactions with HbS to decrease RBC sickling under both normoxia and hypoxia in vitro, ii) prolong the survival of SCD mice under hypoxia, and (iii) regulate the altered steady state levels of pro- and anti-inflammatory cytokines. Thus, our proof of concept in vitro and in vivo preclinical studies demonstrate that the multimodal S-NACH is a highly promising candidate for development into an improved and optimized alternative to LMWHs for the treatment of patients with SCD.
Introduction

Sickle cell disease (SCD) is a hemoglobinopathy resulting from a mutation replacing the glutamic acid amino acid with the less polar valine amino acid at the sixth position of the β chain, converting normal adult hemoglobin (HbA) to sickle hemoglobin (HbS) (1). Deoxygenated HbS polymerizes into long, rigid fibers, causing sickling of red blood cells (RBCs) (2). These characteristic sickled RBCs (sRBCs) impair blood flow through microvasculature, leading to hemolysis, episodes of vaso-occlusive crisis (VOCs), and multi-organ damage (3-7). The loss of membrane phospholipid asymmetry on sRBCs exposes phosphatidylserines (8) that increase sRBCs’ adhesion to neutrophils, monocytes, platelets, and endothelial cells to activate the coagulation and inflammatory pathways (9-12), culminating in a ‘hypercoagulable’ state (13).

Currently, four drugs have been approved by the FDA for the treatment of SCD in the USA. L-glutamine (Endari), approved in 2017, increases the amount of the reduced form of NADH in erythrocytes, which allows sickle RBCs to more appropriately maintain homeostasis during oxidative stress, ultimately resulting in fewer painful crises and adverse events (14). Crizanlizumab (Adakveo) (15) and Voxelotor (16) (Oxbryta, GBT440) were approved in 2019. Crizanlizumab is a monoclonal antibody that targets P-selectin to prevent pathological endothelial adhesion of sickle erythrocytes and leukocytes, leading to a reduction in the frequency of painful (VOCs) (17, 18). The anti-sickling agent Voxelotor is the first of a new class of aromatic aldehydes that target HbS polymerization by increasing Hb O2 affinity (19-21). Finally, hydroxyurea (HU), which works by inducing the expression of fetal Hb (HbF), is the most proven therapeutic approach for SCD (22, 23), as evidenced by its sustained clinical use for over two decades. However, a reported lack of response to HU in up to 30% of patients, and supposed poor compliance, tend to limit its use (22). The reported limitations of HU led to investigatgion of other modes of therapy, including the three more recently approved drugs. However, their true benefits will only manifest over time. Additionally, the inherently complex nature of SCD dictates the urgent need for a multimodal form of therapy.

Antiplatelet molecules, anticoagulants, and heparin have been investigated to mitigate VOC episodes (24). Although heparin is beneficial, the associated risks of internal bleeding
preclude its utility as a drug (25), therefore the need for alternatives remains critical. We developed a sulfated non-anticoagulant heparin (S-NACH) with none to low systemic anticoagulant activity that can be safely administered in mice (at doses > 300 mg/kg daily for 10 days, unpublished data) without causing internal bleeding (26, 27). S-NACH lacks antithrombin binding and thus does not inhibit systemic antithrombin-dependent coagulation factors (FXa and FIIa). Sulfation on S-NACH increases its affinity to endothelium to cause the release of endothelial tissue factor pathway inhibitor (TFPI) (26, 28). Further, S-NACH interferes with P-selectin-dependent binding of cancer cells (29) and RBCs (30) to the endothelial cells and regulates plasma levels of adhesion biomarkers in SCD mice (30). Finally, S-NACH was further optimized to directly interact with HbS to exert desirable therapeutic benefits.

This study tested our hypothesis that S-NACH can bind to HbS and directly prevent sickling and decrease inflammation in SCD due to the bidirectional relationship between inflammation and coagulation (31) and investigated S-NACH’s effects on RBC morphology.

Methods

Reagents

S-NACH (avg. mol. wt. 4,000 Da) was synthesized by Suzhou Ronnsi Pharma Co Ltd. (Jiangsu Province, China). 5-HMF (5-hydroxymethyl-2-furfural) and other common reagents were purchased from Sigma (St. Louis, MO, USA).

Sickle blood samples

Leftover blood samples from individuals with homozygous SS (SCD) were obtained and used based on an approved IRB protocol at the Children’s Hospital of Philadelphia, with informed consent. None of the subjects had been transfused within 4 months prior to using their blood samples, and 4 of the 5 donors were on hydroxyurea therapy.

Anti-sickling, oxygen equilibrium (OEC) and hemoglobin modification studies using human sickle blood
The morphology of hypoxic sickled RBCs was evaluated using a previously reported method (32, 33). Blood samples from individual donors with SCD (n=5) were diluted using HEMOX buffer supplemented with glucose (10 mM) and BSA (0.2 %) to adjust hematocrit of the suspensions to ~ 20%. We used standardized hematocrit for anti-sickling assays to normalize the ratio of RBCs to drug for assay consistency and reproducibility. The suspensions were pre-incubated under air in the absence or presence of three concentrations (0.5, 1, and 2 mM) of S-NACH at 37°C for 1 h. The suspensions were subsequently incubated under a 2.5% O2/97.5% N2 gas mixture at 37°C for 2 h. Aliquots (5–20 μL) of each sample were collected without exposure to air into 2% glutaraldehyde solution for immediate fixation. Fixed cell suspensions were thereafter introduced into glass microslides (Fiber Optic Center, New Bedford, MA, USA) (34) and subjected to microscopic morphological analysis of bright field images (at 40x magnification) of single layer cells on an Olympus BX40 microscope fitted with an Infinity 2 camera (Olympus, Waltham, MA, USA), with the coupled Image Capture software. The percentage of sickled cells for each condition was obtained using blood with a computer-assisted image analysis system, as described previously (33, 35). Untreated samples, as well as samples treated with GBT440/Voxelotor, were used as controls. The residual samples were washed in PBS and hemolyzed in hypotonic lysis buffer for subsequent analyses.

For the OEC study, approximately 100 uL aliquot samples from clarified lysates obtained from the anti-sickling studies were mixed with 3 mL of 0.1M potassium phosphate buffer, pH 7.0, in cuvettes, and subjected to hemoxyimetry analysis using Hemox™ Analyzer (TCS Scientific Corp., New Hope, PA, USA) to assess P50 shifts (36-38). Degree of P50 shift (ΔP50) was expressed as percentage fractions of control DMSO-treated samples.

Finally, for the Hb adduct formation study, clarified lysates, also from the above anti-sickling study, were subjected to cation-exchange HPLC (Hitachi D-7000 Series, Hitachi Instruments, Inc., San Jose, CA), using a weak cation-exchange column (Poly CAT A: 30 mm x 4.6 mm, Poly LC, Inc., Columbia, MD). Hemoglobin isotype peaks are eluted with a linear gradient of mobile phase B from 0% to 80% at A410nm (mobile phase A: 40 mM Bis-Tris, 5 mM EDTA, pH 6.5; mobile phase B: 40 mM Bis-Tris, 5 mM EDTA, 0.15 M sodium chloride, pH 7.5) (33, 36). A commercial standard consisting of approximately equal amounts of composite Hb F, A, S, and C (Helena Laboratories, Beaumont, TX), was used as reference isotypes. The areas of new peaks, representing HbS adducts,
were obtained, calculated as percentage fractions of total Hb area, and reported as levels of modified Hb. All assays were conducted in five biological replicates on different days.

**Animal studies**

C57/B mice aged 5–6 weeks were purchased from Harlan Laboratories (Indianapolis, IN, USA) and acclimatized for 5 days before initiating TFPI measurements after S-NACH administration. Townes model for SCD mice (stock# 013071) was purchased from The Jackson Laboratory (Bar Harbor, ME, USA), bred, genotyped, and experimented with between 10–12 weeks of age. Animal studies were conducted at the animal research facility, Albany College of Pharmacy and Health Sciences (ACPHS), Albany, NY, in accordance with and approved by the ACPHS IACUC following institutional guidelines for humane animal treatment. Animals were maintained under standard climatic and light conditions with *ad libitum* access to food and water.

For TFPI analysis, plasma was obtained from 3 groups of 4 C57/B mice each, via retro-orbital bleeding 2 h after subcutaneous (SC) injections of PBS or S-NACH (100 mg/kg or 300 mg/kg). For normoxic studies, SCD mice were grouped into 6 groups of 6 mice each. Blood smears were made from tail snips before and after SC injection of S-NACH at various time points. Total plasma was harvested for cytokine analysis. Blood smears and plasma were obtained after 2 h from 6 untreated and 5-HMF-treated animals. For survival studies, SCD mice were treated with physiological PBS (n=6) or S-NACH (n=8) by SC injection and subjected to hypoxia (5% O₂) after 30 min of treatments and observed for 1.5 h.

**TFPI and cytokine assay**

Plasma-TFPI was measured using a kit from Neoscientific (Woburn, MA, USA). Cytokines in blood plasma were measured using commercial Bio-Plex beads in a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed using Bio-Plex manager software.

**Morphological analysis**

Total blood was harvested from SCD animals in the presence of EDTA, treated with PBS, 5-HMF, or S-NACH and incubated either under normoxia or hypoxia (2% O₂) at 37°C for
an hour. Blood smear from each sample was Leishman stained and analyzed under an oil immersion light microscope (39).

**Statistical analyses**

Results are presented as the means ± standard deviation comparing experimental and control groups. T-test was used for statistical analyses, and results were considered statistically significant if $P<0.05$.

**Results**

**S-NACH modified intracellular HbS and reduced sickling of SS cells**

S-NACH was engineered to have an aldehyde moiety, which confers anti-sickling properties primarily due to specific interactions with HbS to increase its affinity for oxygen. We therefore tested S-NACH’s ability to modify HbS, increase oxygen affinity of HbS, and prevent RBC sickling.

Our *in vitro* sickling assay under hypoxic conditions demonstrated that S-NACH, in a dose-dependent manner, significantly modified intracellular Hb (Figure 1A) and reduced the sickling of SS cells, with the maximum effect at 2 mM concentration comparable to that of 1 mM GBT440 (Figure 1B; Table 1). This supports our hypothesis considering that two molecules were designed to target both N-terminal valine residues of the alpha globin in a tetrameric Hb molecule.

**Levels of modified intracellular HbS translated into a dose-dependent increase in Hb oxygen affinity**

When aliquots of HbS-complex solution from the same studies were investigated in the OEC assay, we observed a similar concentration-dependent effect on increasing HbS affinity for oxygen, with a reduction in p50 values of ~65% at the highest concentration (65.7±3.2 at 2 mM, Figure 2A; Table 1). These findings correlated linearly with the anti-sickling effects and degrees of HbS modifications, thus confirming this targeted mechanism of action (Figure 2B; Table 1).
S-NACH decreases *in vivo* RBC sickling and regulates inflammatory cytokines under normoxia

When administered to C57/B mice, S-NACH showed ~3-fold increase in plasma-TFPI after 2 h of treatment (Figure 3A) at both doses tested. To determine the effect of S-NACH on RBC sickling, total blood from SCD mice was incubated at normoxia with S-NACH. Based on the lower effective dose with respect to TFPI release, S-NACH dose for animal studies was set at 10 mg/kg. 5-HMF (10 mg/kg) was used as positive control because it decreases RBC sickling (33). Both S-NACH and 5-HMF moderately decreased the sickling of RBCs by 35-50% (data not shown). Townes SCD animals treated with S-NACH showed a significant (*P< 0.05) decrease in the percentage of circulating sRBCs for up to 4 h, with a maximum decrease at 2 h after administration (50% to 35%, Figure 3B and 3C) (n=6). Thus, S-NACH can decrease sickling of RBCs under normoxia.

Plasma samples obtained (untreated, 5-HMF, 2 and 6 h after S-NACH treatment) were quantitatively analyzed for various pro-inflammatory mediators (interleukin 1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α)), anti-inflammatory mediators (IL-10, interferon γ (IFN-γ), monocyte chemoattractant protein 1 (MCP-1)), and growth factors (M-CSF, VEGF) (Figure 4). Plasma levels of IL-1β, IL-6, IFN-γ, MCP-1, TNF-α, M-CSF, and VEGF were increased in SCD untreated samples in contrast to a significant decrease (*P< 0.0005) in S-NACH-treated samples, at both 2 and 6 h. In addition, S-NACH was able to increase the decreased levels of 1L-10. The regulatory effect of S-NACH was most effective at 2 h, similar to its effectiveness on sRBC morphology.

S-NACH decreases RBC sickling and prolongs survival time of SCD mice under hypoxia

Hypoxia in the Townes SCD mouse model increases RBC sickling, causing death within 15 min due to pulmonary sequestration by sRBCs (26). We investigated the effect of S-NACH on RBC sickling and survival under hypoxia. Ex vivo deoxygenation was associated with increasing RBC sickling up to 70%, and in the presence of S-NACH it was significantly (*P< 0.05) decreased to 30% (Figure 5).
In the survival study, while all the untreated mice died within the first 15 min under hypoxia (5% O₂), 50% of the S-NACH-treated mice survived at 30 min, which was 1 h after S-NACH administration (Figure 6), a typical duration tested for survival (33, 40, 41). Because S-NACH exhibited maximal effectiveness at 2 h, mice under hypoxia were observed for an additional 1 h, during which 37.5% of S-NACH-treated animals survived. Thus, S-NACH increased the survival of SCD animals under hypoxia for up to 3 h.

**Discussion**

We designed S-NACH, a modified low molecular weight heparin, to be devoid of anticoagulant properties, while acquiring new direct anti-sickling properties. *In vitro*, S-NACH directly modified intracellular HbS, increased oxygen affinity, and inhibited sickling of RBCs under hypoxia. Additionally, S-NACH reduced the levels of circulating sickled cells in Townes SCD mice. We confirmed the *in vitro* release of endothelial-TFPI by S-NACH (30) in C57/B6 mice, as demonstrated by a significant increase in plasma TFPI. Based on this, we speculate that S-NACH might exert local antithrombotic activity by increasing the concentration of endothelial-TFPI in the vascular area. An *in vivo* increase in plasma TFPI levels after S-NACH administration confirms our earlier reported findings (26). According to Kemme et al., the TFPI release was increased by 3-fold (from 62.9 ng/mL to 237 ng/mL) after heparin infusion (42). Kouta et al. reported the marked increase in TFPI release (~2.5-fold) within 20 min following intravenous administration of different types of heparins (bovine, ovine, and porcine) to non-human primates (43). Additionally, our observation with different species, including mice, rats, and rabbits (unpublished data) are consistent with these results.

We demonstrated *in vitro* that S-NACH permeated RBC membrane to modify HbS and exert a significant anti-sickling effect by maintaining normal RBC morphology versus typical changes in RBC morphology of untreated samples from individuals with SCD. Although there is no prior evidence to indicate a relationship between RBC morphology and inflammatory mediators, the effect of both on decreased sickling and blood viscosity cannot be ruled out (6). Based on our previous studies, the observed reduction in the levels of pro-inflammatory cytokines is not unexpected. For example, in one previous study, S-NACH in an asthma-induced mouse model showed a robust reduction in airway eosinophilia, mucus production, and airway hyperresponsiveness even after chronic repeated challenges with allergen (ovalbumin) (44).
These effects were linked to suppression of Th2 cytokines IL-4/IL-5/IL-13/GM-CSF and upregulation of IL-10. The levels of these inflammatory cytokines increased around 2-8 fold (in both serum and bronchoalvelar lavage fluid) after induction with the allergen (ovalbumin) and decreased again to baseline after treatment with S-NACH. Similar observations were noticed with total white blood cells count, eosinophils, macrophages, and lymphocytes count. The total white blood cell count, eosinophils, macrophage, neutrophils, and lymphocytes were markedly elevated in the asthma-induced mouse model (6, 4, 1.5, 1.5, 4 fold, respectively) after exposure to an allergen. S-NACH also reduced lung fibrosis in mice that were chronically exposed to the allergen. As we showed in that study, these protective effects of S-NACH were attributed to modulation of the IL-4/JAK1 signal transduction pathway through an inhibition of STAT6 phosphorylation and a subsequent inhibition of GATA-3 and inducible NO synthase expression. The protective effects of S-NACH treatment were associated with reduction of the basal expression of the two isoforms of arginase, ARG1 and ARG2, in lung epithelial cells (44).

In another previous study, we measured the different biomarkers of inflammation in patients with SCD (35 patients with painful crisis and 30 patients with steady state) in comparison to 35 healthy donors. Plasma levels of several chemokines and cytokines including TNF-α, IL-1b, IL-6, IL-8, MCP-1, macrophage inflammatory protein 1a (MIP1a), and IFN-g in patients with SCD showed a distinct and statistically significant rise either during painful crisis or at steady state in comparison to healthy donors (2-10 fold increase) (45).

The observed anti-sickling properties are in concordance with our expectations when compared to GBT440 (Voxelotor, Oxybryta), which was recently approved by the FDA. There are some concerns that oxygen affinity-shifting strategies may have unequal cerebrovascular risk (46), though this was adequately addressed by Estepp (47). Nonetheless, the definitive reports from long-term use will conclusively address this concern. With this in mind, our multimodal approach also incorporates polyanionic glycosaminoglycans such as heparins, which can be introduced into sickle RBC HbS by synthetic lipid vesicle. Once introduced, they would block sickling and also modulate ATPase activity and membrane charge of RBCs in hypoxia (48). Because SCD occurs due to replacement of acidic, hydrophilic amino acid (glutamic acid) in position 6 of the Beta chain of Hb with non-polar, hydrophobic valine amino acid, this change
causes disturbance in Hb structure. We therefore speculate that S-NACH (polyanionic glycosaminoglycan) may reverse the polarity to make the HbS more soluble. This mechanism remains under investigation.

We further speculate that S-NACH antagonizes hepcidin and might also provide additional benefits in SCD by improving iron hemostasis, as suggested in a recent report (49). Additionally, our findings demonstrate that S-NACH plays a role similar to the non-anticoagulant heparin fractions from enoxaparin, which was shown to have an effect on inflammatory mediators (26, 50). Furthermore, our TEG assay results with S-NACH did not show any changes in platelets functions (data not shown). Similarly, S-NACH retains all multi-modal actions of the LMWH tinzaparin but without systemic anticoagulation and its associated bleeding side effects. Tinzaparin demonstrated significant effects on the resolution of acute pain crisis in patients with SCD in double-blind, randomized clinical trials (51).

Overall, the effects of S-NACH on RBC sickling morphology, RBC adhesion, and regulation of inflammation resulted in increased survival of SCD mice under hypoxia. This study serves as a proof-of-concept that S-NACH is safe with respect to bleeding tendencies and argues for further detailed safety and efficacy studies in preclinical models’ toxicity investigations, the results of which would help guide and inform future human studies.

Further, S-NACH increased the levels of TFPI in plasma, decreased RBC sickling under normoxia or hypoxia, and reduced pro-inflammatory mediators IL-1, IL-6, IFN-γ, MCP-1, TNF-α, M-CSF, and VEGF while increasing anti-inflammatory factors such as IL-10, further establishing a promising bonafide multimodal candidate drug worthy of additional investigations for acute and chronic disease management in SCD patients.

In summary, our data demonstrated direct and support pleiotropic effects of the sulfated non-anticoagulant heparin derivative S-NACH in ameliorating the complex pathophysiological mechanisms involved in SCD. Development into an effective drug would lead to improved outcome in patients globally with SCD, considering the current limited therapeutic options,
especially for the vast majority of patients with SCD who reside in the underdeveloped world (52).
References


Table 1: Hemoglobin adduct formation, oxygen equilibrium, and anti-sickling studies using homozygous sickle red blood cells with S-NACH. All studies were conducted with SS cells suspensions (20% hematocrit) incubated with 0.5, 1, and 2 mM of S-NACH. Results are mean values ± SD for 5 separate experiments (biological replicates).

<table>
<thead>
<tr>
<th>S-NACH</th>
<th>Modified Hb (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔP&lt;sub&gt;50&lt;/sub&gt; (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sickling Inhibition (%)&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>0.5 mM</td>
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<td>33.1±5.3</td>
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<td>1.0 mM</td>
<td>44.5±13.0</td>
<td>57.6±9.0</td>
<td>58.6±14.3</td>
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<tr>
<td>2.0 mM</td>
<td>69.7.5±5.5</td>
<td>65.7±3.2</td>
<td>85.8±4.7</td>
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<tr>
<td>1.0 mM GBT440</td>
<td>ND</td>
<td>ND</td>
<td>92.7±4.7</td>
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<sup>a</sup> HbS adduct values obtained from HPLC elution patterns of hemolysate after incubation of compounds with SS cells.  
<sup>b</sup> P<sub>50</sub> is the oxygen pressure at which hemolysates are 50% saturated with oxygen. ΔP<sub>50</sub> (%) was determined as:

\[ \Delta P_{50} (%) = \frac{P_{50} \text{ of lysates from untreated cells} - P_{50} \text{ of lysates from treated cells}}{P_{50} \text{ of lysates from untreated cells}} \times 100 \]

<sup>c</sup> Anti-sickling studies with SS cells were conducted under hypoxia (2.5% O<sub>2</sub>/97.5% N<sub>2</sub> gas mixture).
Figure Legends

**Fig. 1. S-NACH binds to intracellular HbS (and HbF) and inhibits sickling of SS RBCs under hypoxia.** In this experiment, SS RBCs were incubated with or without S-NACH and subjected to hypoxia. (A) Cation-exchange HPLC analyses of aliquot samples demonstrated a concentration-dependent modification of intracellular HbS to the high-affinity adduct form. (B) Fixed SS RBCs aliquots were subjected to microscopic image analysis and demonstrated a corresponding dose-dependent inhibition of sickling.

**Fig. 2. Effect of S-NACH on HbS oxygen binding affinity.** (A) S-NACH increases Hb oxygen affinity. Aliquot sample hemolysates from the sickling assay were subjected to p50 analyses using the Hemox Analyzer. B. Representative curves show a dose-dependent left shift, indicating an increase in oxygen affinity. Summarized data for biological replicates (n=5) are indicated in the graph. The findings confirm the primary direct anti-sickling mechanism of S-NACH.

**Fig. 3. Effects of S-NACH under normoxia.** (A) S-NACH increases the release of endogenous TFPI levels. C57/B mice were treated with 100 and 300 mg/kg of free S-NACH, and plasma was obtained after 2 h. TFPI in plasma was measured in duplicate. TFPI levels were compared between S-NACH-treated samples and PBS-treated control samples, (n=4) (*P< 0.05). (B) S-NACH treatment decreases sickling of RBCs in Townes SCD mice. Blood smears were made from tail snips before and after SC injection of S-NACH (10 mg/kg) at time points shown. 5-HMF was used as positive control (*P< 0.05). (C) Morphology of the RBCs from Townes SCD mice was examined from stained blood smears and expressed in percentage. RBCs from 4 different fields or 120 cells were analyzed to calculate the percentage of sRBCs. Blood cells
from untreated samples contain higher percentage of sickled and distorted RBCs (shown by arrows). S-NACH treatment decreased the presence of sRBCs up to 4 h with most decrease seen at 2 h (n=6), *P< 0.05).

**Fig. 4. S-NACH treatment regulates the levels of inflammatory mediators.** Total plasma from untreated SCD mice or treated with S-NACH (10 mg/kg) or 5-HMF was harvested and frozen. Cytokines in blood plasma were measured in triplicate. S-NACH treatment significantly changed the plasma levels of the analytes (*P< 0.0005). For most analytes, the effects of 5-HMF are comparable to the effects of S-NACH at 6 h.

**Fig. 5. S-NACH treatment decreases sickling of RBCs, in *ex vivo* under hypoxia.** Total blood harvested from SCD mice (n=8) was mixed with S-NACH (1, 5, or 10 µg/mL) and incubated under 2% O₂ at 37°C for 1 h. Blood smears were made, stained, and the morphology of RBCs was analyzed. Hypoxia increased the percentage of sickled RBCs. S-NACH treatment decreased the sickling of RBCs in a dose-dependent manner. PBS was used as negative control and 5-HMF was used as positive control. *P< 0.05.

**Fig. 6. S-NACH treatment increases the survival of SCD mice under hypoxia.** SCD mice were treated with PBS (n=6) or 10 mg/kg S-NACH (n=8). After 30 min, mice were incubated in a hypoxia chamber (5% O₂), and the survival of animals was observed for 1.5 h. Surviving mice were euthanized, as per the guidelines. S-NACH treatment was associated with increased survival of mice.
Figure 1

A

HbS

Control: 0% Modified Hb

HbS\text{mod}

0.5 mM S-NACH: ~20% Modified Hb

HbF

1 mM S-NACH: ~40% Modified Hb

HbE\text{mod}

2 mM S-NACH: ~70% Modified Hb

\( A_{15\,\text{nm}} \) (Arbitrary Units)

Time (min)

B

0 mM

0.5 mM

1 mM

2 mM

S-NACH

Images of cell cultures at different S-NACH concentrations:

- 0 mM
- 0.5 mM
- 1 mM
- 2 mM
Figure 3

A

Plasma TFPI levels (pg/mL) ± STDEV

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>PBS</th>
<th>S-NACH 100 mg/kg</th>
<th>S-NACH 300 mg/kg</th>
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B

Percentage of sickle cells (%) ± STDEV

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<tr>
<th></th>
<th>Pre-treatment</th>
<th>0.5 h</th>
<th>2 h</th>
<th>4 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>5-HMF 2 h</th>
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C

Images showing cellular morphology before and after treatment.