Ferrata Storti Foundation

Haematologica 2022
Volume 107(2):532-540

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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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, which increases the adhesiveness of the cells, thereby altering the rheological properties of the blood, and triggers inflammatory responses, leading to hemolysis and vaso-occlusive crises. Unfractionated heparin and low-molecular weight heparins 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 no 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 inhibit sickling directly, thus employing a multimodal approach. Here, we show that S-NACH can: (i) directly engage in Schiff-base reactions with HbS to decrease red blood cell 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 low-molecular weight heparins for the treatment of patients with SCD.

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Received: September 15, 2020.

Accepted: January 25, 2021.

Pre-published: February 11, 2021.

https://doi.org/10.3324/haematol.2020.272393

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 (RBC).[2] These characteristic sickled RBC impair blood flow through the microvasculature, leading to hemolysis, episodes of vaso-occlusion, and multi-organ damage.[3-7] The loss of membrane phospholipid asymmetry on sickled RBC exposes phosphatidylserines[8] that increase the adhesion of sickled RBC to neutrophils, monocytes, platelets, and endothelial cells to activate coagulation and inflammatory pathways,[9-12] culminating in a 'hypercoagulable' state.[13]

Currently, four drugs have been approved by the Food and Drug Administration

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 RBC to maintain homeostasis more appropriately during oxidative stress, ultimately resulting in fewer painful vaso-occlusive 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 vaso-occlusive crises.[17,18] The anti-sickling agent voxelotor is the first of a new class of aromatic aldehydes that target HbS polymerization by increasing Hb O_2 affinity.[19-21] Finally, hydroxyurea, 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 hydroxyurea in up to 30% of patients, and supposed poor compliance, tend to limit its use.[22] The reported limitations of hydroxyurea led to investigation 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 vaso-occlusive crises.[24] Although heparin is beneficial, the associated risks of internal bleeding preclude its utility as a drug[25] and the need for alternatives remains critical. We developed a sulfated non-anticoagulant heparin (S-NACH) with no 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 does not bind antithrombin and thus does not inhibit systemic antithrombin-dependent clotting factors (activated factors X and II). Sulfation on S-NACH increases the drug's affinity for endothelium to cause the release of endothelial tissue factor pathway inhibitor (TFPI).[26,28] Furthermore, S-NACH interferes with P-selectin-dependent binding of cancer cells[29] and RBC[30] to endothelial cells and regulates plasma levels of adhesion biomarkers in SCD mice.[30] Finally, S-NACH was further optimized to interact directly with HbS to exert desirable therapeutic benefits.

In this study we 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 the effects of S-NACH on RBC morphology.

Methods

Reagents

S-NACH (average molecular weight 4,000 Da) was synthesized by Suzhou Ronnsi Pharma Co. Ltd. (Jiangsu Province, China). 5-hydroxymethyl-2-furfural (5-MF) 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 Institutional Review Board protocol at the Children's Hospital of Philadelphia, with informed consent. None of the subjects had been transfused within 4 months prior to their blood samples being used, and four of the five donors were on hydroxyurea therapy.

Anti-sickling, oxygen equilibrium and hemoglobin modification studies using human sickle blood

The morphology of hypoxic sickled RBC was evaluated using a previously reported method.[22,23] Blood samples from individual donors with SCD (n=5) were diluted using HEMOX buffer supplemented with glucose (10 mM) and bovine serum albumin (0.2%) to adjust the hematocrit of the suspensions to about 20%. We used standardized hematocrit for anti-sickling assays to normalize the ratio of RBC 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% O_2/97.5% N_2 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 determined 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 phosphate-buffered saline (PBS) and hemolysed in hypotonic lysis buffer for subsequent analyses.

For the oxygen equilibrium study, approximately 100 μL aliquot samples from clarified lysates obtained from the anti-sickling studies were mixed with 3 mL of 0.1 M potassium phosphate buffer, pH 7.0, in cuvettes, and subjected to hemoximetry analysis using a 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 dimethylsulfoxide-treated samples.

Finally, for the Hb adduct formation study, clarified lysates, also from the above anti-sickling study, were subjected to cation-exchange high performance liquid chromatography (Hitachi D-7000 Series, Hitachi Instruments, Inc., San Jose, CA, USA), using a weak cation-exchange column (Poly CAT A: 30 mm x 4.6 mm, Poly LC, Inc., Columbia, MD, USA). Hemoglobin isotype peaks were eluted with a linear gradient of mobile phase B from 0% to 80% at A_{410nm} (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 HbF, HbA, HbS, and HbC (Helena Laboratories, Beaumont, TX, USA), was used as the 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 administration of S-NACH. Townes SCD mice (stock # 013071) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), bred, genotyped, and used in experiments between 10 and 12 weeks of age. Animal studies were conducted at the animal research facility, Albany

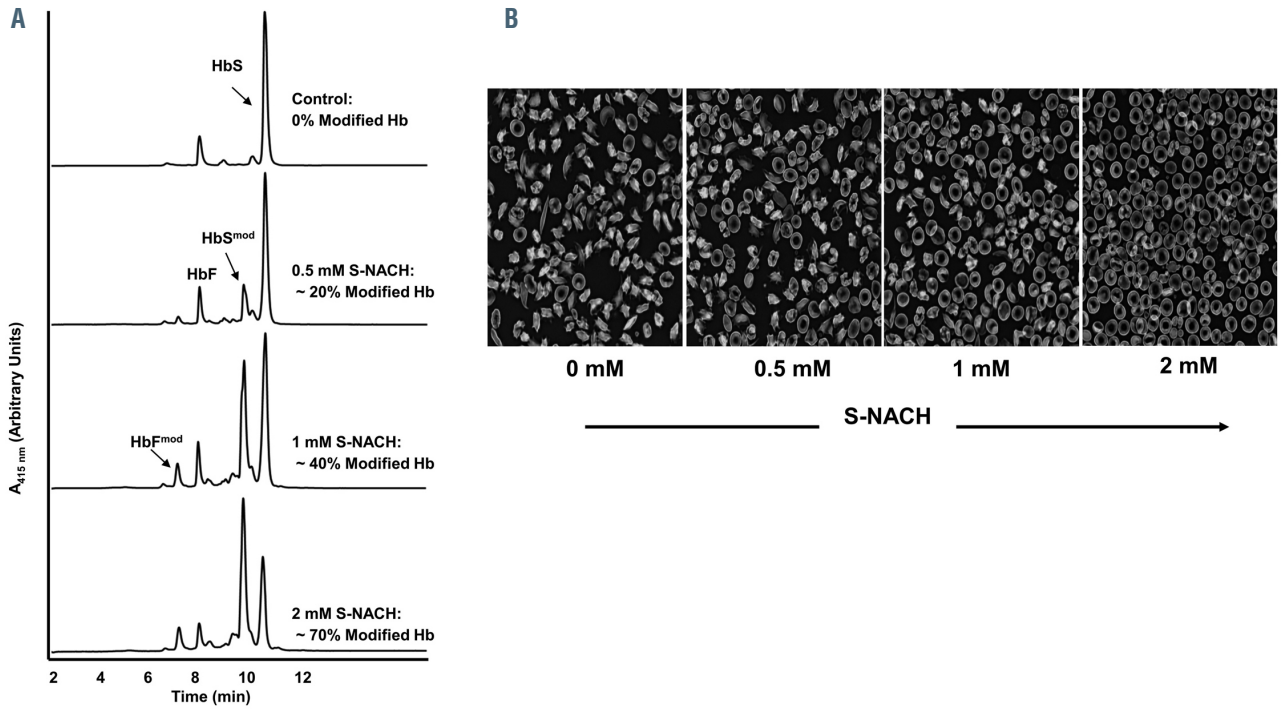

Figure 1. S-NACH binds to intracellular HbS (and HbF) and inhibits sickling of SS red blood cells under hypoxia. In this experiment, SS red blood cells (RBC) were incubated with or without sulfated non-anticoagulant heparin derivative S-NACH and subjected to hypoxia. (A) Cation-exchange high performance liquid chromatography analyses of aliquot samples demonstrated a concentration-dependent modification of intracellular HbS to the high-affinity adduct form. (B) Fixed SS RBC aliquots were subjected to microscopic image analysis and demonstrated a corresponding dose-dependent inhibition of sickling.

Table 1. Hemoglobin adduct formation, oxygen equilibrium, and anti-sickling studies using homozygous sickle red blood cells with a sulfated non-anticoagulant heparin derivative.

S-NACH	Modified Hb (%)[a]	ΔP_{50} (%)[b]	Sickling inhibition (%)[c]
0.5 mM	20.5±8.2	21.2±10.6	33.1±5.3
1.0 mM	44.5±13.0	57.6±9.0	58.6±14.3
2.0 mM	69.7.5±5.5	65.7±3.2	85.8±4.7
1.0 mM GBT440	ND	ND	92.7±4.7

All studies were conducted with SS cell suspensions (20% hematocrit) incubated with 0.5, 1, of 2 mM of sulfated non-anticoagulant heparin derivative (S-NACH). The results are mean values ± standard deviation for five separate experiments (biological replicates). [a]HbS adduct values obtained from high performance liquid chromatography elution patterns of hemolysate after incubation of compounds with SS cells. [b]P_{50} is the oxygen pressure at which hemolysates are 50% saturated with oxygen. ΔP_{50} (%) was determined as:

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

[c]Anti-sickling studies with SS cells were conducted under hypoxia (2.5% O_2/97.5% N_2 gas mixture).

College of Pharmacy and Health Sciences (ACPHS; Albany, NY, USA) in accordance with and approved by the ACPHS Institutional Animal Care and Use Committee 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 three groups of four C57/B mice each, via retro-orbital bleeding 2 h after subcutaneous injections of PBS or S-NACH (100 mg/kg or 300 mg/kg). For normoxic studies, SCD mice were grouped into six groups of six mice each. Blood smears were made from tail snips before and after subcutaneous 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 six untreated and 5-HMF-treated animals. For survival studies, SCD mice were treated with physiological PBS (n=6) or S-NACH (n=8) by subcutaneous injection and subjected to hypoxia (5% O_2) 30 min after the treatments and observed for 1.5 h.

Tissue factor pathway inhibitor and cytokine assays

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 under either normoxia or hypoxia (2% O2) at 37°C for 1 h. A blood smear from each sample was stained with Leishman stain and analyzed under an oil immersion light microscope.[39]

Statistical analyses

Results are presented as the means ± standard deviation comparing experimental and control groups. A *t*-test was used for sta-

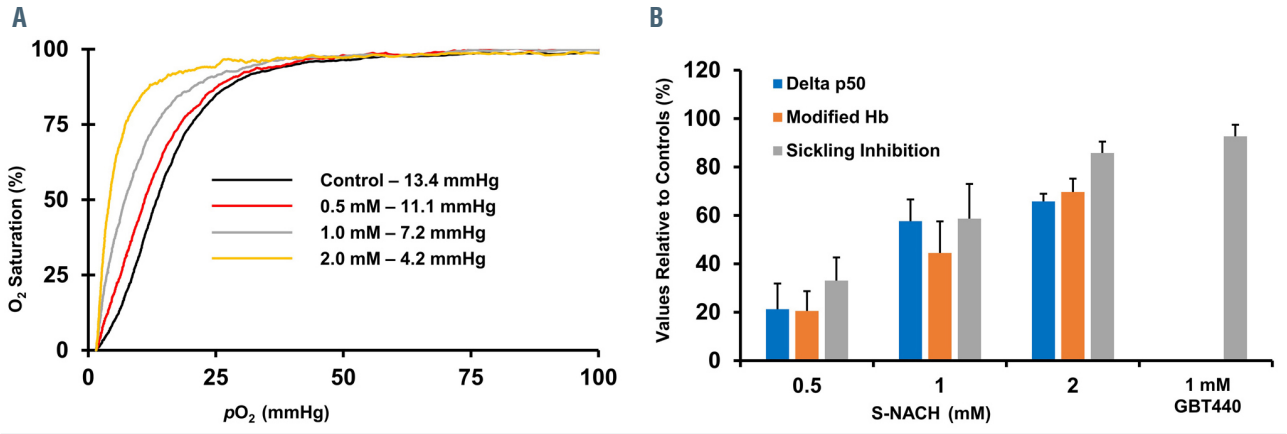

Figure 2. Effect of S-NACH on HbS oxygen binding affinity. (A) The sulfated non-anticoagulant heparin derivative (S-NACH) increases hemoglobin oxygen affinity. Aliquots of hemolysates from the sickling assay were subjected to p50 analyses using the Hemox Analyzer. (B) Representative curves show a dose-dependent 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.

tistical analyses, and results are 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 the ability of S-NACH 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 the concentration of 2 mM, 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 α 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 oxygen equilibrium assay, we observed a similar concentration-dependent effect on increasing HbS affinity for oxygen, with a reduction in P50 values of about 55% 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* red blood cell sickling and regulates inflammatory cytokines under normoxia

When administered to C57/B mice, S-NACH caused an approximately 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, the S-NACH dose for animal studies was set at 10 mg/kg. 5-HMF (10 mg/kg) was used as a positive control because it decreases RBC sickling.[33] Both S-NACH and 5-HMF moderately decreased the sickling of RBC by 35-50% (*data not shown*). Townes SCD mice treated with S-NACH showed a significant (*$P<0.05$) decrease in the percentage of circulating sickled RBC for up to 4 h, with a maximum decrease at 2 h after administration (50% to 35%) (Figure 3B, C) (n=6). Thus, S-NACH can decrease sickling of RBC under normoxia. Plasma samples (untreated, 5-HMF, 2 and 6 h after S-NACH treatment) were quantitatively analyzed for various pro-inflammatory mediators (interleukin [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 (monocyte colony-stimulating factor [M-CSF], vascular endothelial growth factor [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, whereas they were significantly decreased ($P<0.0005$) in S-NACH-treated samples, at both 2 and 6 h. Furthermore, 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 sickled RBC morphology.

S-NACH decreases red blood cell sickling and prolongs survival of sickle cell disease mice under hypoxia

In the Townes SCD mouse model, hypoxia increases RBC sickling, causing death within 15 min due to pulmonary sequestration of sickled RBC.[26] We investigated the effect of S-NACH on RBC sickling and survival under hypoxia. *Ex vivo* deoxygenation was associated with increasing RBC sickling of up to 70%. In the presence of S-NACH, sickling 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% O_2), 50% of the S-NACH-treated mice were alive at 30 min, which was 1 h after S-NACH administration (Figure 6), a typical timespan used for investigating 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.

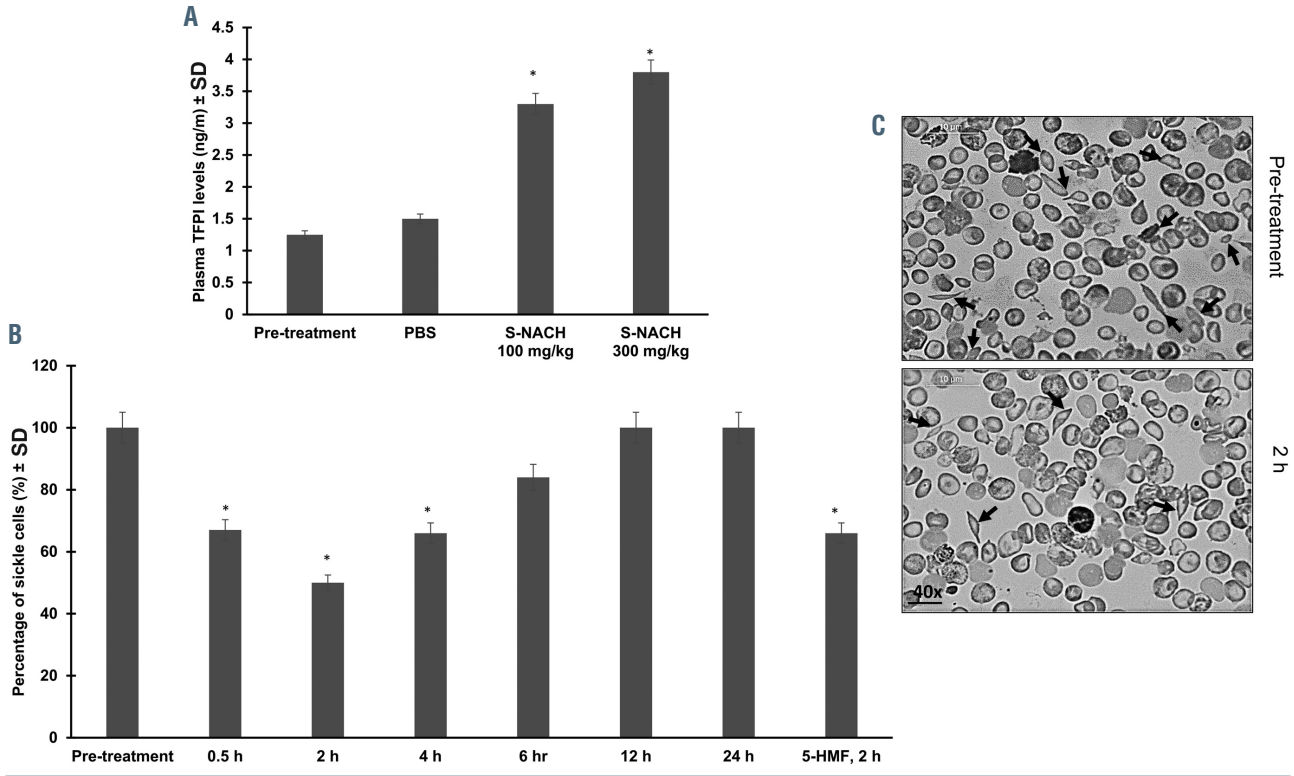

Figure 3. Effects of S-NACH under normoxia. (A) A sulfated non-anticoagulant heparin derivative (S-NACH) increases the release of endogenous tissue factor pathway inhibitor (TFPI). C57/B mice were treated with 100 and 300 mg/kg of free S-NACH, and plasma was obtained after 2 h. TFPI in plasma was measured in duplicate. TFPI levels were compared between S-NACH-treated samples and phosphate-buffered saline (PBS)-treated control samples, (n=4) (*P<0.05). (B) S-NACH treatment decreases sickling of red blood cells (RBC) in Townes sickle cell disease (SCD) mice. Blood smears were made from tail snips before and after subcutaneous injection of S-NACH (10 mg/kg) at the time points shown. 5-hydroxymethyl-2-furfural (5-MF) was used as a positive control (*P<0.05). (C) Morphology of the RBC from Townes SCD mice was examined in stained blood smears and expressed in percentage. RBC from four different fields or 120 cells were analyzed to calculate the percentage of sickled RBC. Blood from untreated samples contained higher percentages of sickled and distorted RBC (shown by arrows). S-NACH treatment decreased the presence of sickled RBC for up to 4 h with the greatest decrease seen at 2 h (n=6) (*P<0.05). SD: standard deviation.

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 RBC 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/B 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.*, TFPI release increased by 3-fold (from 62.9 ng/mL to 237 ng/mL) after infusion of heparin.[42] Kouta *et al.* reported a marked increase of TFPI release (~2.5-fold) within 20 min after 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 membranes to modify HbS and exert a significant anti-sickling effect by maintaining normal RBC morphology, protecting against the typical changes in RBC morphology that occur in untreated samples from individuals with SCD. Although there was 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 was not unexpected. For example, in one previous study in an asthma-induced mouse model, S-NACH caused 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- to 8-fold (in both serum and bronchoalveolar lavage fluid) after induction with the allergen and decreased again to baseline after treatment with S-NACH. Similar observations were made for total white blood cell count, as well as eosinophil, macrophage, and lymphocyte counts, which were markedly elevated in the asthma-induced mouse model (6-, 4-, 1.5-, 1.5-, and 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, the protective effects of S-NACH were attributable to modulation of the IL-4/JAK1 signal transduction pathway through inhibition of STAT6 phosphorylation and subsequent inhibition of GATA-3 and inducible nitric oxide syn-

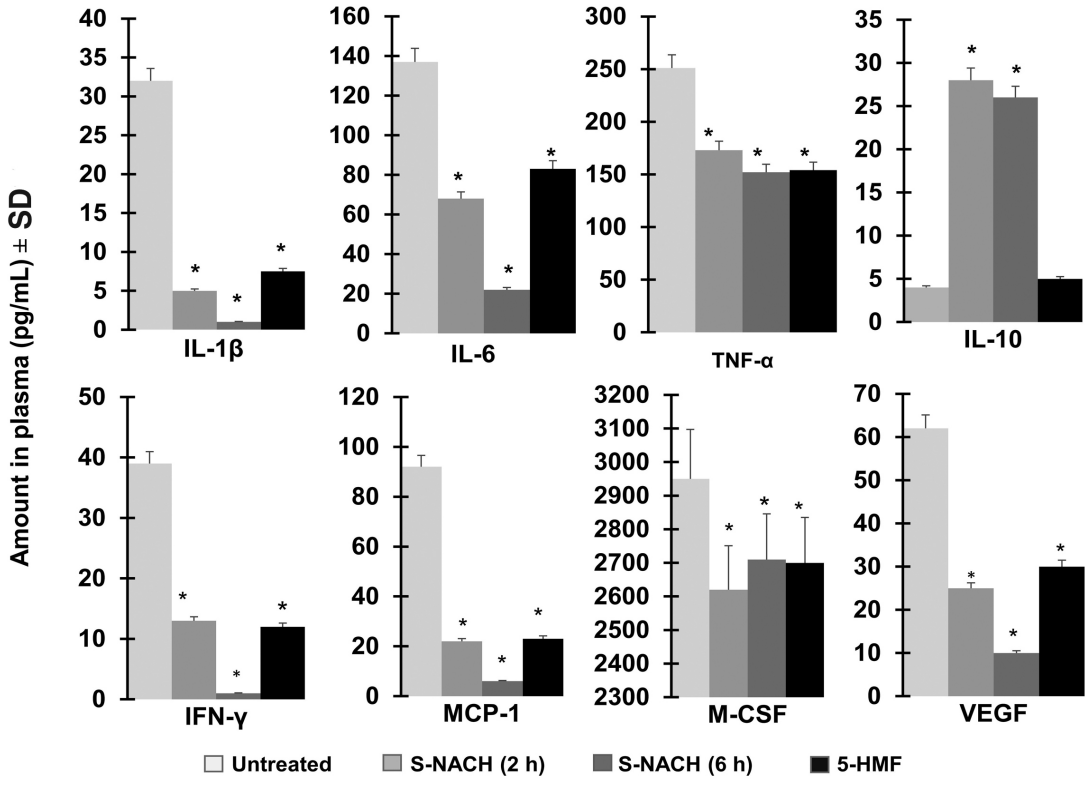

Figure 4. S-NACH treatment regulates the levels of inflammatory mediators. Total plasma from sickle cell disease (SCD) mice that were untreated, treated with sulfated non-anticoagulant heparin derivative (S-NACH; 10 mg/kg) or treated with 5-hydroxymethyl-2-furfural (5-MF) was harvested and frozen. Cytokines 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 were comparable to those of S-NACH at 6 h. SD: standard deviation; IL: interleukin; TNF: tumor necrosis factor; IFN: interferon; MCP-1: monocyte chemoattractant protein 1; M-CSF: monocyte colony-stimulating factor; VEGF: vascular endothelial growth factor

thase expression. The protective effects of S-NACH treatment were associated with reductions 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 crises and 30 patients in steady state) in and 35 healthy donors. Plasma levels of several chemokines and cytokines including TNF-α, IL-1β, IL-6, IL-8, MCP-1, macrophage inflammatory protein 1α (MIP1α), and IFN-γ in patients with SCD were distinctly and statistically significantly higher during painful crises and at steady state than in healthy donors (2- to 10-fold increases).[45]

The observed anti-sickling properties are in concordance with our expectations when compared to those produced by GBT440 (voxelotor, Oxybryta), which was recently approved for use by the Food and Drug Administration. There are some concerns that oxygen affinity-shifting strategies may be associated with different cerebrovascular risks,[46] although this was adequately addressed by Estepp.[47] Nonetheless, definitive reports on long-term use will provide conclusive information on this issue. 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 vesicles. Once introduced, they would block sickling and also modulate ATPase activity and the charge of the RBC membrane in hypoxia.[48] SCD occurs due to the replacement of an acidic, hydrophilic amino acid (glutamic acid) in position 6 of the β chain of Hb with a non-polar, hydrophobic valine amino acid and this change causes a disturbance in Hb structure. We therefore speculate that S-NACH (polyanionic glycosaminoglycan) may reverse the polarity to make the HbS more soluble. This mechanism of action remains under investigation.

We further speculate that S-NACH antagonizes hepcidin and might 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 that of the non-anticoagulant heparin fractions from enoxaparin, which were shown to have an effect on inflammatory mediators.[26,50] Furthermore, our thromboelastography assay results with S-NACH did not show any changes in platelet functions (*data not shown*). Indeed, S-NACH retains all the multimodal actions of the low molecular weight heparin tinzaparin but without systemic anticoagulation and its associated bleeding side effects. Tinzaparin demonstrated significant effects on the resolution of acute pain crises 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 of toxicity, the results of which would help guide and inform future human studies.

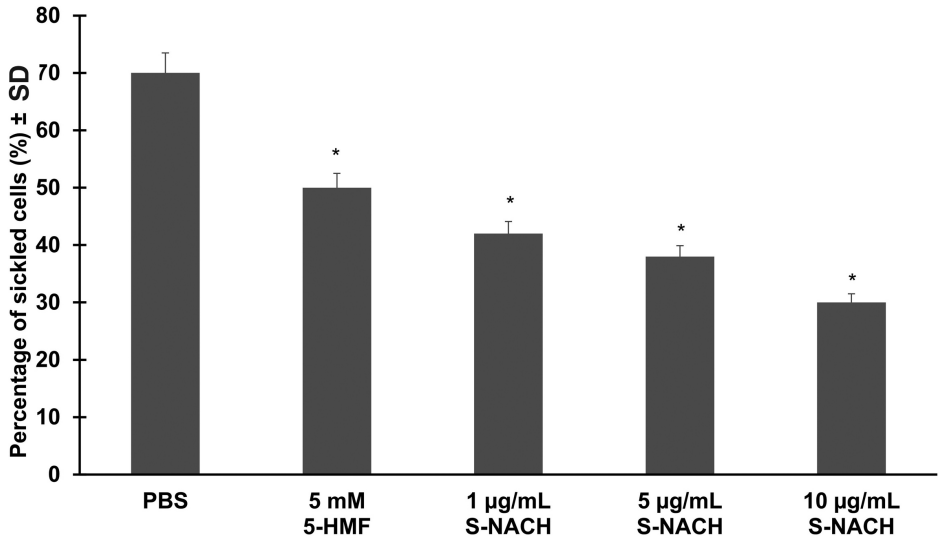

Figure 5. S-NACH treatment decreases sickling of red blood cells *ex vivo* under hypoxia. Total blood harvested from sickle cell disease mice (n=8) was mixed with a sulfated non-anticoagulant heparin derivative (S-NACH) at the dose of 1, 5, or 10 µg/mL and incubated in 2% O_2 at 37 °C for 1 h. Blood smears were made, stained, and the morphology of red blood cells (RBC) was analyzed. Hypoxia increased the percentage of sickled RBC. S-NACH treatment decreased the sickling of RBC in a dose-dependent manner. Phosphate-buffered saline (PBS) was used as a negative control and 5-hydroxymethyl-2-furfural (5-MF) was used as a positive control. **P*<0.05. SD: standard deviation

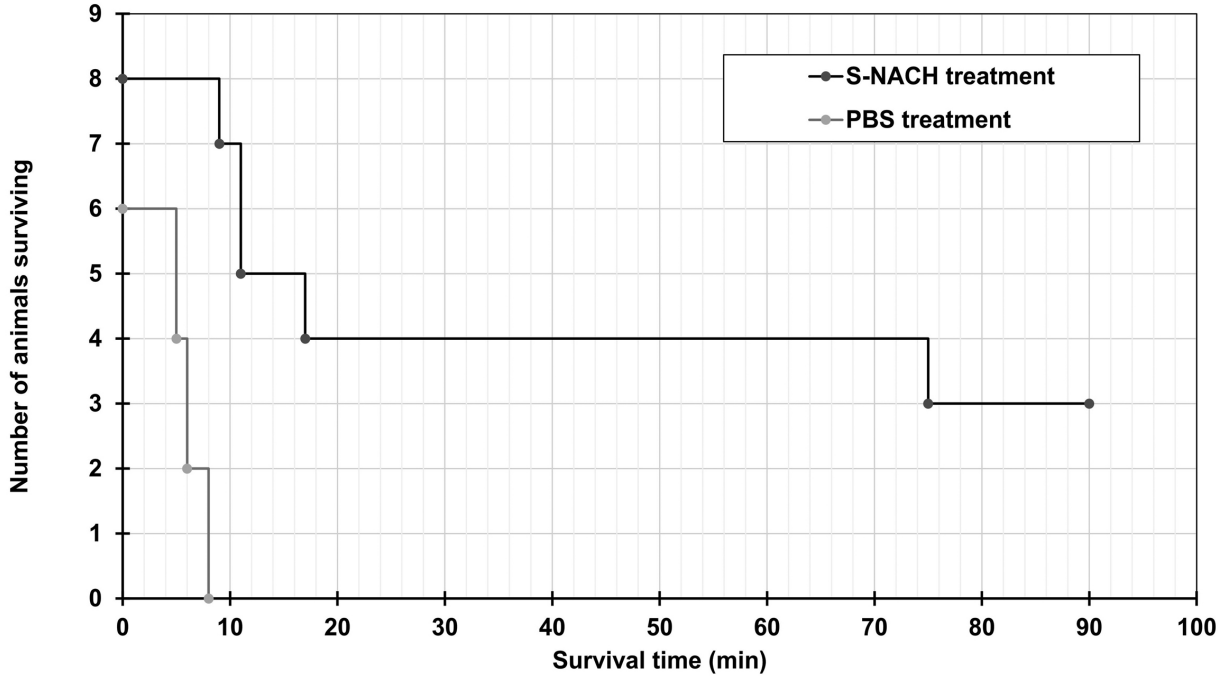

Figure 6. S-NACH treatment increases the survival of sickle cell disease mice under hypoxia. Sickle cell disease (SCD) mice were treated with phosphate-buffered saline (PBS, n=6) or 10 mg/kg sulfated non-anticoagulant heparin derivative (S-NACH, n=8). After 30 min, mice were incubated in a hypoxia chamber (5% O_2), 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.

S-NACH increased the levels of TFPI in plasma, decreased RBC sickling under normoxia and hypoxia, and reduced the levels of the 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 it as a promising bona fide multimodal candidate drug worthy of additional investigations for acute and chronic disease management in SCD patients. In summary, our data demonstrate direct and support pleiotropic effects of 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 underdeveloped areas of the world.[52]

Disclosures

SAM holds a US patent on S-NACH[28] and other related US patents. None of the authors has any conflicts of interest.

Contributions

SAM designed the study and is the Principal Investigator; OA, NHED, VM-C and MAT conducted the experiment; OA did the data analysis; OA and NHED contributed equally to the manuscript write up and data interpretation.All authors have approved the final version of the manuscript.

Acknowledgments

We appreciate Dr. Kelly A. Keating, Pharmaceutical Research Institute (PRI), for her excellent editing of this manuscript.

Funding

This project was funded by Vascular Vison Pharmaceuticals Co. to PRI and an NIH STTR Phase 1 grant (1R41HL147737-01-A1, NIH/NHLB "Multi-modal Mechanisms of Novel Sulfated Non-Anticoagulant Heparin (S-NACH) in Sickle Cell Disease Management") subaward to Children's Hospital of Philadelphia (CHOP), university of Pennsylvania.

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