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BRIEF COMMUNICATIONS

A thalidomide–hydroxyurea hybrid increases HbF production in sickle cell mice and reduces the release of proinflammatory cytokines in cultured monocytes

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Fetal hemoglobin (HbF) induction by hydroxyurea (HU) therapy is associated with decreased morbidity and mortality in sickle cell anemia (SCA) patients, but not all patients respond to or tolerate HU. This provides a rationale for developing novel HbF inducers to treat SCA. Thalidomide analogs have the ability to induce HbF production while inhibiting the release of tumor necrosis factor-alpha. Molecular hybridization of HU and thalidomide was used to synthesize 3- (1,3-dioxoisoindolin-2-yl) benzyl nitrate (compound 4C). In this study, we show that compound 4C increases HbF production in a transgenic SCA mouse model and reduces the production of pro-inflammatory cytokines by SCA mouse monocytes cultured ex vivo. Therefore, compound 4C is a novel drug designed to treat SCA with a unique combination of HbF-inducing and anti-inflammatory properties.

Fetal hemoglobin (HbF, $\alpha_2\gamma_2$) is the major hemoglobin in fetal life. The switch from γ-globin to β-globin occurs shortly after birth, when HbF is replaced by HbA ($\alpha_2\beta_2$), the main hemoglobin in adulthood. Patients with sickle cell anemia (SCA) display higher levels of HbF than healthy individuals, whose HbF concentration is less than 1% of total hemoglobin. HbF does not interact with HbS and limits polymerization of HbS within red blood cells (RBCs) [1], which provides a sound rationale for the development of HbF inducers as treatment options for SCA [2].

Hydroxyurea (HU), a known HbF inducer and ribonucleotide reductase inhibitor, is a drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of SCA. Beneficial effects of HU appear to be associated with its nitric oxide (NO)-donating property, and more importantly, with the induction of HbF production through NO-dependent activation of soluble guanylyl cyclase in erythroid cells [3,4]. In SCA patients, an increase in HbF levels by HU therapy is associated with decreased mortality and a reduced incidence of clinical complications such as the frequency of acute painful episodes and acute chest syndrome [5]. Unfortunately, some patients do not respond to or are unable to tolerate HU [4]. In light of these findings, it has been proposed that thalidomide and its analogs, lenalidomide and pomalidomide, can emerge as a promising novel class of drugs for the treatment of SCA through their combined ability to reduce inflammation and to induce HbF production. The mechanism through which thalidomide increases HbF involves p38 mitogen-activated protein kinase activation and H4 histone acetylation [6]. In addition, these drugs also can inhibit the release of tumor necrosis factor-alpha (TNF-α) and others molecules involved in the inflammatory process [7–10].

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Using a molecular modification approach, our group has previously described 3-(1,3-dioxoisoindolin-2-yl) benzyl nitrate (compound 4C), a novel candidate drug. It was synthesized through molecular hybridization of HU and thalidomide to obtain both NO donor and analgesic properties [10]. A recent study showed that priapism, a known complication of SCA, was reversed by compound 4C in transgenic SCA mice and combined endothelial NO synthase and neuronal NO synthase double gene-deficient mice [11]. In this study, we show that compound 4C increases HbF production in a transgenic SCA mouse model and reduces the production of proinflammatory cytokines by SCA mouse monocytes ex vivo.

Methods

Animals and treatment

Knockout-transgenic SCA mice were bred at Augusta University according to institutional guidelines [12]. Animals were housed in temperature-controlled facilities on a 12-hour light/dark cycle with ad libitum food and water access. Six-week-old male SCA mice were treated with compound 4C (300 µmol/kg/d) or its vehicle (0.1% dimethylsulfoxide) daily (Monday through Friday) for 8 weeks via intraperitoneal injection. All animal procedures were approved by Augusta University's Animal Care and Use Committee (permit number: BR10-07-349).

Hematological parameters

Whole blood was collected by intracardiac puncture from ketamine/xylazine-anesthetized mice in vacutainer EDTA tubes (BD Biosciences, Franklin Lakes, NJ, USA). Blood count was performed with a CBC-Diff Veterinary Hematology System (Heska Corporation, Loveland, CO, USA). Reticulocyte counts were determined by supravital staining with methylene blue.

HbF measurement

HbF was measured by analytic high-performance liquid chromatography using a weak cation-exchange column SynChropak CM-300 (Eprogen) on the Waters Empower 32 high-performance liquid chromatography system (Millipore).

Interleukin (IL)-1β, IL-6, and KC measurement in monocyte cultures from SCA mice

Cells were purified of peripheral blood using Ficoll (GE Healthcare, Pittsburgh, PA, USA) gradient separation. Mononuclear cells were placed in plastic dishes with Dulbecco's modified Eagle's medium (Life Technologies, Carlsbad, CA, USA) with 10% calf bovine serum (ATCC, Manassas, VA, USA) and incubated for 2 hours in humidified air (5% CO_2 at 37°C). Purity of monocytes was >90% as determined by May–Giemsa staining (Fisher Scientific, Pittsburgh, PA, USA). Compound 4C was added to the monocyte cultures at different concentrations (100 or 300 µmol/L) for 30 minutes before stimulation with lipopolysaccharide (LPS; 1 µg/mL; Sigma-Aldrich, St. Louis, MO, USA). After 20 hours of incubation, supernatants were collected and stored at −80°C until analysis. IL-1β, IL-6, and KC (also known as cytokine CXCL1, a murine analog of human IL-8) were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA).

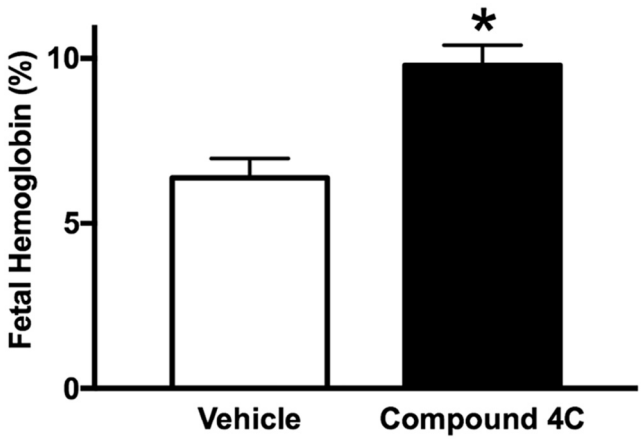

Figure 1. HbF protein levels from SCA mice treated with or without compound 4C (300 µmol/kg/d for 8 weeks). Data are shown as the mean ± SEM for seven to 10 mice in each group. *$p < 0.05$ compared with the vehicle-treated group.

Results

Compound 4C increases HbF protein levels in SCA mice. Eight weeks of treatment with compound 4C increased the protein levels of HbF by approximately 53% in SCA mice compared with mice treated with vehicle ($p < 0.05$; Fig. 1). This increase in HbF was accompanied by only a slight decrease in hemoglobin, RBC, and white blood cell (WBC) counts in the experimental group (Table 1).

Compound 4C reduces the production of IL-1ß, IL-6, and KC in supernatants of monocyte cultures from SCA mice. Pretreatment of SCA mouse monocyte cultures with compound 4C at concentrations of 100 and 300 µmol/L caused a significant reduction of IL-1β (57% and 93%, respectively; $p < 0.05$; Fig. 2A), IL-6 (70% and 97%, respectively; $p < 0.05$; Fig. 2B), and KC at the 300 µmol/L concentration (89%; $p < 0.05$; Fig. 2C) compared with vehicle-treated cultures. The magnitude of the anti-inflammatory properties of compound C at the higher concentration equaled or exceeded dexamethasone's suppressive effect on LPS-induced cytokine production (Fig. 2). Incubation with HU at similar

Table 1. Hematological parameters of SCA mice treated with compound 4C

Parameter	Vehicle	Compound 4C
RBCs (×10⁶/µL)	4.7 ± 0.4	3.9 ± 0.3
Hb (g/dL)	7.2 ± 0.4	6.3 ± 0.4
HCT (%)	21.4 ± 1.4	16.6 ± 1.3
Reticulocytes (%)	42.6 ± 4.1	39.6 ± 5.2
WBCs (×10⁶/µL)	11.4 ± 1.0	10.4 ± 0.8
MCV (fL)	45.9 ± 1.6	42.8 ± 2.0
MCH (pg)	15.6 ± 0.5	17 ± 1.6

Data are shown as the mean ± SEM of five to 10 mice in each group ($p > 0.05$ for all parameters).
HCT = Hematocrit; MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume.

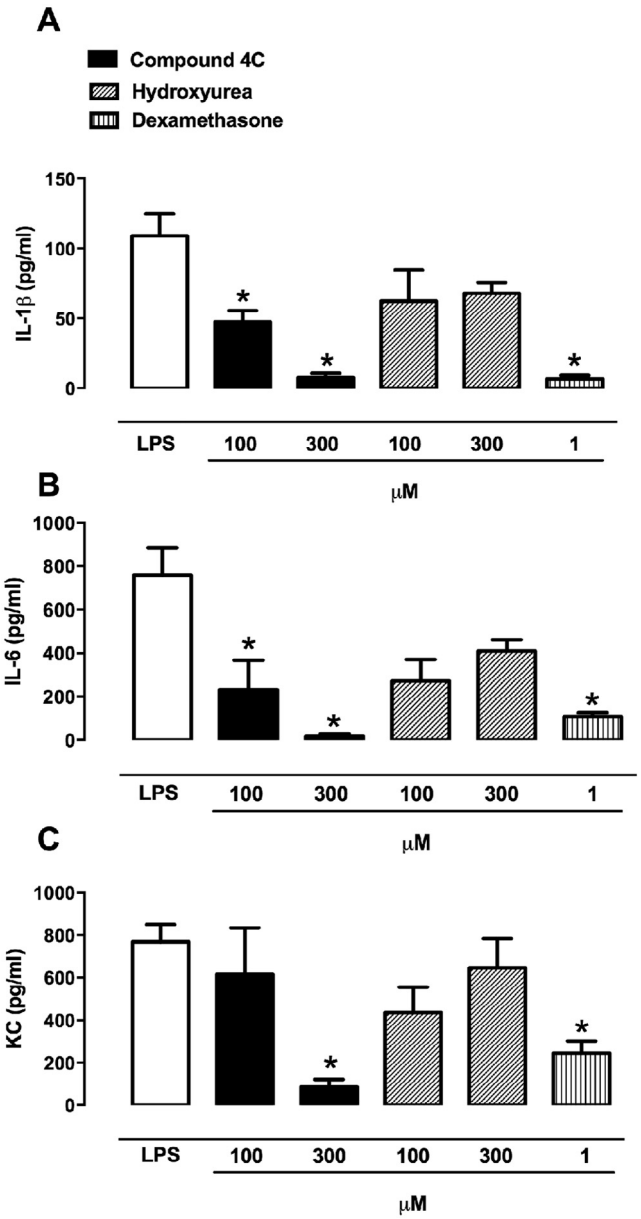

Figure 2. **(A)** IL-1β, **(B)** IL-6, and **(C)** KC levels in LPS-stimulated SCA monocyte cultures in the presence of compound 4C (100 or 300 μmol/L), HU (100 or 300 μmol/L), or dexamethasone (1 μmol/L) as a positive control of anti-inflammatory drug. Data are shown as the mean ± SEM of three to six mice in each group. *$p < 0.05$ compared with the vehicle-treated group.

concentrations (100 or 300 μmol/L) had no significant effect on the production of IL-1β, IL-6, or KC (Fig. 2).

Discussion

Advances in our understanding of the pathophysiology of SCA have highlighted the importance of HbF in modulating the severity of the disease and increased awareness of the significant role of inflammation in the sickle vaso-occlusive process [13]. Compound 4C emerges as a novel drug: designed to treat SCA, it has the capacity to address both pathophysiological aspects of the disease as an HbF inducer with anti-inflammatory properties that combine the molecular structures of HU and thalidomide [10].

The HbF-inducing effect of compound 4C is similar to what our group has observed in transgenic SCA mice treated with HU and the thalidomide analog pomalidomide in a previous study [8]. This further supports the contribution of the pharmacophoric group of thalidomide in compound 4C to the induction of HbF and suggests that it may be as effective as HU in augmenting HbF. In the future, we believe that compound 4C may become an alternative for SCA patients who are unresponsive to or unable to tolerate HU.

The exact mechanisms behind the HbF-inducing effects of compound 4C remain to be determined. Progress in understanding the mechanism of action of HU has identified that NO-donating compounds seem to increase γ-globin mRNA transcription and HbF expression in erythroid cells [3], which could justify this effect through the NO-donating group in compound 4C. In addition, the pharmacophoric group of the thalidomide molecule may behave in a manner similar to pomalidomide, which has been demonstrated to induce HbF production by reducing the levels of key transcriptional repressors of γ-globin gene expression in erythroblasts, such as BCL11A, SOX6, GATA1, KLF1, and LSD1 [9]. Our data showed a slight decrease in Hb levels and RBC and WBC counts after treatment with compound 4C, but a possible myelosuppressive effect was not expected, because this new hybrid does not have the pharmacophoric group from HU responsible for the inhibition of ribonucleotide reductase. The effect may nonetheless be caused by this new molecule, but more studies are needed to confirm.

In addition, compound 4C displays anti-inflammatory properties that may prove useful in the treatment of SCA. Previous studies have shown that thalidomide can decrease IL-6 levels in volunteers with experimental endotoxemia [14], as well as IL-1β production in whole blood cell cultures stimulated with LPS [15]. Regarding chemotaxis, a phthalimide analog coupled to a nitrate subunit reduced KC production induced by carrageenan in the paws of mice [16]. Chronic inflammation in SCA patients has been characterized by elevated plasma levels of TNF-α and IL-8 [17], increased IL-1β production by peripheral blood mononuclear cells from SCA patients [18], and increased circulating IL-6 levels in SCA vaso-occlusive crisis [19]. Furthermore, there is evidence that Cxcl1/KC is a key inflammatory mediator of vaso-occlusion in an SCA murine model of hemolytic transfusion reaction [20]. Our results expand our previously published observation that compound 4C is able to inhibit TNF-α production in LPS-stimulated monocytes from SCA mice [10], whereas HU lacks the ability to change IL-1β, IL-6, and KC production in murine monocyte cultures. This compound can reduce chemokine production, a possible advantage compared with lenalidomide and pomalidomide, which have been shown not to change the production of chemokine IL-8 [21]. Therefore, the anti-inflammatory properties of compound 4C can

be attributed to its pharmacophoric group from the thalidomide molecule and may be useful against the chronic inflammatory state in SCA patients. In fact, previous studies have reported that HU might induce the gene expression and synthesis of proinflammatory cytokines [22–24].

This study is limited by the use of ex vivo measurements of cytokines to document the anti-inflammatory properties instead of measuring circulating cytokines in the same murine model. There is still no method available to measure circulating levels of compound 4C to allow the study of drug pharmacokinetics. Therefore, we cannot determine the concentrations of compound 4C achieved, nor can we exclude the possibility that ideal HbF-inducing and anti-inflammatory properties may not be reached at the same concentrations of compound 4C. Nevertheless, our data will be helpful in the design of further in vivo investigations aiming at obtaining circulating concentrations of compound 4C with an anti-inflammatory effect that can be as potent as dexamethasone.

Conclusions

In summary, our study shows that 8 weeks of treatment with compound 4C increased HbF production in SCA mice. Moreover, compound 4C also decreased the IL-1β, IL-6, and KC production in LPS-stimulated monocytes from this model of SCA. Compound 4C is a novel drug designed to treat SCA with a unique combination of HbF-inducing and anti-inflammatory properties.

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