Beneficial Effects of Soluble Guanylyl Cyclase Stimulation and Activation in Sickle Cell Disease are Amplified by Hydroxyurea: *In Vitro* and *In Vivo* Studies


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Soluble guanylyl cyclase stimulation in sickle cell disease.

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Number of text pages: 31
Number of tables: 0
Number of figures: 3
Number of Supplementary Tables: 2
Number of Supplementary Figures: 5
Number of references: 52
Number of words in Abstract: 238
Number of words in Introduction: 750
Number of words in Discussion: 1327

NON-STANDARD ABBREVIATIONS: cGMP, cyclic guanosine monophosphate; DAMP, damage-associated molecular pattern; HbF, fetal hemoglobin; HbS, sickle hemoglobin; FN, fibronectin; HU, hydroxyurea; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; RBC, red blood cell; SCA, sickle cell anemia; SCD, sickle cell disease; sGC, soluble guanylyl cyclase; TNF, tumor necrosis factor-α; WBC, white blood cells.

Recommended Section: Drug Discovery and Translational Medicine
ABSTRACT

The complex pathophysiology of sickle cell anemia (SCA) involves intravascular hemolytic processes and recurrent vaso-occlusion, driven by chronic vascular inflammation, which result in the disease’s severe clinical complications, including recurrent painful vaso-occlusive episodes. Hydroxyurea, the only drug frequently used for SCA therapy, is a cytostatic agent, although it appears to exert nitric oxide/soluble guanylyl cyclase (sGC) modulating activity. As new drugs that can complement, or replace, the use of hydroxyurea are sought to further reduce vaso-occlusive episode frequency in SCA, we investigated the effects of the sGC agonists, BAY 60-2770 (sGC activator) and BAY 41-2272 (sGC stimulator), in the presence or absence of hydroxyurea, on SCA vaso-occlusive mechanisms and cell recruitment, ex vivo and in vivo. These agents significantly reduced stimulated human SCA neutrophil adhesive properties, ex vivo, in association with the inhibition of surface β2-integrin activation. A single administration of BAY 60-2770, or BAY 41-2272, decreased TNF-cytokine induced leukocyte recruitment in a mouse model of SCA vaso-occlusion. Importantly, the in vivo actions of both agonists were significantly potentiated by the co-administration of hydroxyurea. Erythroid cell fetal hemoglobin (HbF) elevation is also a major goal for SCA therapy. BAY 41-2272, but not BAY 60-2770, at the concentrations employed, significantly induced gamma-globin gene transcription, in association with HbF production in cultured erythroleukemic cells. In conclusion, sGC agonist drugs could represent a promising approach as therapy for SCA, either for use as stand-alone treatments, or in combination with hydroxyurea.

Key words: cGMP, hydroxyurea, inflammation, nitric oxide, sickle cell disease
SIGNIFICANCE STATEMENT

This preclinical study demonstrates that stimulators and activators of sGC are potent inhibitors of the adhesion and recruitment of leukocytes from humans and in mice with sickle cell anemia (SCA), and may represent a promising approach for diminishing vaso-occlusive episode frequency in SCA. Hydroxyurea, a drug already frequently used for treating SCA was found to potentiate the beneficial effects of sGC agonists in in vivo studies, implying that these classes of compounds could be used alone or in combination therapy.
Introduction

SCA, an inherited disease, is caused by the generation of abnormal sickle hemoglobin (HbS), which polymerizes at low oxygen levels, making the red blood cell more rigid and sickle shaped (Eaton and Hofrichter, 1987). SCA incurs numerous clinical complications, including frequent painful vaso-occlusive episodes that often require hospitalization, acute chest syndrome, stroke, renal damage, pulmonary hypertension, and a shortened lifespan (Steinberg, 2009). Sickle hemoglobin polymerization promotes a vast range of pathophysiological alterations, including changes in red blood cell function, extra and intra-vascular hemolysis, and chronic inflammation and, consequently, vaso-occlusion (Conran and Belcher, 2018). Vaso-occlusive processes (responsible for much of the morbidity of SCA) occur principally in the microcirculation (Steinberg, 2016) and are triggered by the interactions of red blood cells, endothelial cells, activated leukocytes, platelets, and plasma proteins via a mechanism in which inflammation, hypoxic events, oxidative stress, and reduced nitric oxide (NO) availability play driving roles (Zhang et al., 2016). In particular, in vivo studies employing mice with sickle cell disease (SCD mice) suggest that the recruitment of activated leukocytes to blood vessel walls is crucial for initiating these multi-cellular interactions (Turhan et al., 2002; Hidalgo et al., 2009).

Hydroxyurea (HU; or hydroxycarbamide) is one of the few therapeutic options currently employed for reducing the frequency of vaso-occlusive episodes in SCA patients. Augmentation of fetal hemoglobin (HbF) production in SCA patients, even at low levels, can decrease sickle hemoglobin polymerization and significantly improve the disease’s clinical course. Hydroxyurea, a cytostatic agent, induces HbF production in erythroid lineage cells, thus diminishing sickling events (Charache et al., 1995). Mounting data also indicate that hydroxyurea acts as an NO-donor compound, boosting
cGMP levels *in vivo*, and can react with heme proteins to generate NO (Pacelli et al., 1996; Glover et al., 1999). Additionally, numerous agents are currently in various stages of clinical development for SCA therapy, many of which have been identified using a pathophysiology-based approach that targets at least one of the mechanisms that contributes to the pathology of the disease (Conran and Belcher, 2018).

Reduced bioavailability of NO, due principally to the consumption of vascular NO by cell-free hemoglobin during hemolysis, may contribute to SCA vaso-occlusive processes (Reiter et al., 2002). As the consumption of NO following acute hemolytic processes leads to rapid inflammatory responses that result in substantial leukocyte recruitment to the blood vessel wall (Almeida et al., 2015), stimulation of sGC (the enzyme target of NO) and intracellular cGMP elevation, could represent an approach to reducing inflammation, leukocyte recruitment, and consequent vaso-occlusive processes in SCA. Indeed, amplification of NO-dependent signaling using inhibitors of phosphodiesterase-9 (PDE9) significantly diminishes vaso-occlusive processes in SCD mice (Almeida et al., 2012; McArthur et al., 2019), and the amelioration of NO bioavailability improves microvascular functions, increases survival, and prevents lung injury during hypoxia in SCD mice (de Franceschi et al., 2003; Kaul et al., 2008).

The sGC enzyme functions, normally, in its reduced (ferrous) oxidation state; however, oxidative conditions can make the sGC heme unresponsive to NO or even result in its loss from the enzyme (Stasch et al., 2006). Depending on its oxidative state, the enzyme can be pharmacologically stimulated by either sGC stimulators or sGC activators. BAY 41-2272 is a prototype compound of a class of NO-independent sGC stimulators; this molecule binds to a regulatory site on the α-subunit of sGC and stimulates the native non-oxidized enzyme without NO, but also synergistically with NO. BAY 41-2272 can inhibit platelet aggregation *in vitro* and leukocyte adhesion *in vivo* (Hobbs and Moncada,
2003; Ahluwalia et al., 2004). On the other hand, for stimulation of the oxidized sGC, where there is the loss of function of the heme group, heme-independent sGC activators are required (Stasch et al., 2006). BAY 60-2770 is an NO- and heme-independent sGC activator (Pankey et al., 2011) that, given the oxidative stress associated with SCD (van Beers and van Wijk, 2018), could be of therapeutic benefit in this disease.

It is conceivable that a multi-drug approach to treating SCA will evolve in the coming years, where drugs can be used alone, or in combination with others, such as hydroxyurea, to amplify NO-dependent signalling and diminish inflammation (Conran and Torres, 2019), amongst other mechanisms. The aim of this study was to investigate the effects of BAY 60-2770 (sGC activator) and of BAY 41-2272 (sGC stimulator), administered in the absence or presence of hydroxyurea, on the inflammatory mechanisms that contribute to SCA vaso-occlusive processes, in vitro and in vivo. In addition, modulation of HbF production in erythroid cells by these agents was evaluated.
Materials and Methods

Reagents

BAY 60-2770 and BAY 41-2272 were provided by Bayer AG (Wuppertal, Germany). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was purchased from Cayman Chemical (Ann Arbor, MI, USA) and hemin was obtained from Frontier Scientific (Newark, NJ, USA). Recombinant human and murine TNF-α (TNF), and human fibronectin were from R&D Systems (Minneapolis, MN, USA). Dulbecco’s Modified Eagle Medium, fetal bovine serum, penicillin and streptomycin were from Gibco-Invitrogen (New York, NY, USA). Trizol was from Invitrogen (Carlsbad, CA, USA). All other reagents were from Sigma Aldrich (San Luis, MO, USA), unless otherwise stated.

Separation of human neutrophils

Blood samples were collected in citrate from healthy individuals and from SCA (HbSS) individuals, following informed consent and approval of this study by the Ethics Committee of the University of Campinas (CAAE: 36984214.1.0000.5404). Neutrophils (> 95% purity, > 98% viability) were separated from peripheral blood samples using a Ficoll-Paque gradient (English and Andersen, 1974) and resuspended in RPMI medium (Vitrocell Embriolife, Campinas, Brazil) for immediate use in assays. Demographic and hematological data for patients that participated in the study are presented in Supplementary Table 1.

Adhesion assays

Static neutrophil adhesion assays: Briefly, after specified incubations, neutrophils (2 X 10^6 cells/mL in RPMI medium) were seeded onto 96-well plates coated with 20 μg/mL
fibronectin. Cells were allowed to adhere for 30 min at 37°C, 5% CO₂ (Canalli et al., 2008), after which non-adhered cells were discarded and wells washed thrice with PBS. Fifty µL of RPMI were added to each well containing cells, and varying concentrations of the original cell suspension were added to empty wells to form a standard curve. Cell adhesion was calculated as a percentage by measuring the myeloperoxidase content of each well and comparing with the appropriate standard curve.

Microfluidic Assays: The Venaflux™ platform (Cellix Ltd, Dublin, Ireland) was used to measure neutrophil adhesion under flow conditions. Biochip (Vena8 biochips, Cellix Ltd) microchannels (400-µm wide) were pre-coated (overnight, 2-8°C) with recombinant fibronectin (20 µg/ml) and nonspecific binding sites were subsequently blocked with 1% bovine serum albumin / PBS. Neutrophils (2.5 x 10⁶ cells/ml) were perfused over microchannels (initial flow rate of 3.3 nL/sec; initial shear stress of 0.5 d/cm²) for 3 min. One hundred and eighty images were acquired (1/sec) for each channel using a Zeiss microscope (x20 lens; Gottingen, Germany) and DeltaPix Camera (Nibe, Denmark). Neutrophil adhesion to microchannels at 3 min was calculated using the DucoCell analysis program (Cellix Ltd), recording the mean number of neutrophils adhered to an area of 0.09 mm².

K562 Cell culture and CD34⁺ isolation

Erythroleukemic K562 cells, acquired from the American Type Culture Collection (Manassas, VA, USA), were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. Cultures were incubated at 37°C in an atmosphere of 5% CO₂ in air with extra humidity. K562 cells were incubated with agents on the first day of sub-culture and the medium was not changed during the 96 h induction period. Hydroxyurea, BAY 41-2272 and BAY 60-
2770 were dissolved in minimal quantities of dimethyl sulfoxide (DMSO). All cells used for culture were in the log phase of exponential growth and alterations in cell viability or growth phase were monitored during treatment using trypan blue exclusion assays. Control cultures were grown in the presence of the equivalent quantities of drug vehicle (DMSO or sterile water) to those utilized in the treated culture. CD34+ hematopoietic stem cells were isolated from three healthy donor volunteers, according to (Almeida et al., 2008).

**Flow cytometry**

**Neutrophils:** Isolated neutrophils (1.0 x 10^6 cells/mL in RPMI) were incubated with APC-conjugated mouse anti-human CD11b (clone M1/70, eBioscience) and FITC-conjugated mouse anti-human CD11a (clone HI111, eBioscience) to evaluate expressions of the CD11b (Mac-1 subunit) and CD11a (LFA-1 subunit) molecules on the neutrophil surface. For detection of activation-specific epitopes on the CD11a and CD11b molecules, cells were incubated with mouse anti-human CD11a (MEM-83, eBioscience) and FITC-conjugated rat anti-mouse IgG1, and PE-conjugated mouse anti-human CD11b Ab (CBRM1/5, eBioscience), respectively (30 min, 4° C, in the dark). Ten thousand events were acquired on a FACScalibur (BD Biosciences) using the 488 nm laser, and employing a FSC/SSC gate (CellQuest Software, BD Biosciences). Data are expressed as MFI and were compared to a negative isotype control using the FlowJo analysis software (Tree Star, Ashland, OR, USA).

**K562 cells:** HbF protein expression was monitored in K562 (1 x 10^5) cells following permeabilization (Fix & Perm Cell permeabilization kit, Life Technologies Corp., MD, USA) and incubation in phosphate-buffered saline with FITC-conjugated anti-fetal hemoglobin antibody (Life Technologies Corp.), according to the manufacturer’s
instructions. Events were acquired and analyzed as described above for neutrophil flow cytometry.

**Generation of control and SCD chimeric mice**

Male C57BL/6 mice were obtained from the animal breeding facility at the University of Campinas, Brazil, and were housed 4-5 mice per cage with free access to food and water. Animals were fed on a 22% protein diet (NUVILAB – CR1 irradiated) without additional arginine supplementation. Chimeric sickle cell disease (SCD) mice and chimeric C57BL/6 mice were generated from the transplantation of bone marrow from Berkeley transgenic sickle cell disease mice (Tg[Hu-miniLCR1GAS] Hba/Hbb/) or C57BL/6 mice into lethally-irradiated male C57BL/6 mice (6 weeks of age), as previously described (Turhan et al., 2002; Almeida et al., 2012). Only chimeric SCD mice expressing 97% human globin (phenotyped by polyacrylamide gel electrophoresis (Turhan et al., 2002)) at three months post-transplantation were used at 3 to 5 months after transplantation; these mice are hereafter referred to as SCD mice and control mice, respectively. All experimental procedures were approved by the Animal Care and Use Committee of the University of Campinas (CEUA/UNICAMP; Protocols: 3121-1, 4439-1, 5358-1) and performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. All efforts were made to minimize animal suffering and to use the minimum number of animals to produce replicable results.

**Intravital microscopy protocols**

Inflammation was induced in mice by the injection of murine TNF (0.5 μg intraperitoneally/ 200 μL) and mice were concomitantly treated with hydroxyurea (100
mg/kg intravenously), BAY 41-2272 (10 µg/mouse), BAY 60-2770 (10 µg/mouse intravenously), or a combination of two compounds, or vehicle (2.5% v/v DMSO). Compounds were administered (either individually or in combination) in a single injection of 150 µL. At 2 hours after treatment administrations, mice were anesthetized and tracheostomized. The cremaster muscle was surgically exteriorized, before continuously superfusing with bicarbonate-buffered saline (37°C, pH 7.4), equilibrated with a 95% N\textsubscript{2} and 5% CO\textsubscript{2} mixture. Microvessels (6-15 for each mouse, 15-30 µm in diameter) were visualized at 3 hours after surgery using an Axio Imager D2 microscope (Carl Zeiss Microscopy, Jena, Germany; 63X magnification) custom-designed for intravital microscopy and images were recorded for 30-90 seconds (40 frames/sec) using an Axiocam 503 monochromatic camera (Carl Zeiss Microscopy). Rolling, adhesion and extravasation of leukocytes (WBC) were monitored and analyzed for 30-45 minutes after surgery. Definitions of leukocyte rolling, adhesion, and extravasation are described in (Turhan et al., 2004). Concentrations of BAY 41-2272 and BAY60-2770 administered in vivo were determined using drug response dosing experiments in C57BL/6 mice (Supplementary Figure 1) and based on previous studies (Wang et al., 2013; Ghosh et al., 2016). The hydroxyurea dose employed was based on previous in vivo studies from our groups (Almeida et al., 2012; Almeida et al., 2015).

**Quantification of plasma cGMP**

Blood was collected from mice within 5 h of administration, or not, of the drugs studied. Plasma was separated from the blood (2500 g for 10 minutes) and samples were then stored at -80°C. Plasma cGMP was quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits (GE Healthcare, Chicago, IL, USA).
Quantitative real-time polymerase chain reaction and gene expression analysis

Total RNA was extracted from cells of interest using Trizol reagent, or an RNA extraction kit (RNeasy mini kit, Qiagen, Hilden, Germany), according to the manufacturers’ protocols. cDNA was synthesized from total RNA extracts with RevertAid H minus First Strand cDNA synthesis kit (ThermoFisher Scientific, MA, USA). Synthetic oligonucleotide primers were designed to amplify cDNA for genes encoding γ-globin (HBG), soluble guanylate cyclase alpha subunit (GUCY1A1), soluble guanylate cyclase beta subunit (GUCY1B1) and β-actin (ACTB), and glyceraldehyde phosphate dehydrogenase (GAPDH) (Primer Express; Applied Biosystems, Foster City, CA, USA). Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA); for HBG, ACTB and GAPDH primer sequences and concentrations, see (Dos Santos et al., 2012). Primer sequences for amplifying GUCY1A1 were; forward, 5’-ATGCACTGTACACTCGCTTCGA-3’ and reverse, 5’-CAACGACGCCAGCAAAAAAC-3’. Primer sequences for GUCY1B1 were; forward, 5’-GCCAGGTTCAGTAGATGGTG-3’ and reverse, 5’- GGCATCCGCTGTCTATGC-3’. All samples were assayed in a 12 μL volume containing 10 ng of cDNA (3.0 μL), 6.0 μL of SYBR Green Master Mix PCR (Applied Biosystems), and 3.0 μL of specific primers in a MicroAmp Optical 96-well reaction plate (Applied Biosystems) using the StepOne Plus (Applied Biosystems), as previously described. Gene expression was quantified using the Gnorm program. Two replicas were run on the plate for each sample. For HBG gene expression, results are expressed as mRNA levels, normalized according to the expressions of ACTB and GAPDH. For the GUCY1A1/ GUCY1B1 genes, relative expression was calculated as the fold change in mRNA quantity, according to the $2(-\Delta\Delta Ct)$ method, and normalized to the expression of ACTB.
Statistical analysis

Values are expressed as means ± standard error of mean (SEM). Data were confirmed as parametric, or not, and comparisons were performed using ANOVA with multiple comparisons post-test, as appropriate and as specified. Differences among groups were considered significant at p≤0.05.

Results

sGC stimulation and sGC activation inhibit *ex vivo* SCA human neutrophil adhesive mechanisms

As previously reported (Canalli et al., 2008; Miguel et al., 2011), neutrophils isolated from SCA individuals often demonstrate an increased capacity to adhere to fibronectin ligand, *in vitro*, when compared to neutrophils from healthy individuals without SCA (CON). Given that stimulation of cGMP-dependent signaling has demonstrated beneficial effects on vaso-occlusive mechanisms (Almeida et al., 2012), we first semi-quantified the expressions of the genes encoding the sGC alpha and beta subunits in neutrophils from healthy individuals (Supplementary Figure 2). As gene expression of both sGC subunits was confirmed in these cells, albeit at lower levels than those observed in CD34+ haematopoietic stem cells, we then looked at how sGC agonism may modulate neutrophil adhesive properties. Neutrophils from individuals with SCA were then incubated with either BAY 60-2770 or BAY 41-2272 (90 min) before co-stimulating with recombinant TNF cytokine (200 ng/ml, 30 min) or hemin (50 µM, 30 min) and determining cellular adhesion to fibronectin using static adhesion assays (30 min, 37°C, 5% CO2). TNF and hemin each augmented the adhesion of SCA neutrophils (Figure 1A-B). Both BAY 60-2770 (1-10 µM) and BAY 41-2272 (10-100 µM) significantly decreased the TNF-stimulated adhesion of SCA neutrophils to fibronectin (Fig. 1A), while only BAY 41-
2272, at concentrations of 10 and 100 µM, but not 1 µM, significantly inhibited hemin-induced SCA adhesion to fibronectin (Figure 1B). With regard to neutrophils from healthy control individuals, neither BAY 41-2272 nor BAY 60-2770, at the same concentrations, significantly inhibited either TNF- or hemin-induced healthy control neutrophil static adhesion to fibronectin (Supplementary Figure 3A-B).

The inhibiting effects of the sGC activator/stimulator on TNF-stimulated SCA neutrophil adhesion to fibronectin were confirmed using microfluidic assays, which afforded cell flow through biochip channels of similar widths to those of small blood vessels (400 µm), utilizing a shear stress of 0.5 dynes/cm² for 3 min. Under conditions of flow, the adhesion of neutrophils from SCA individuals was significantly inhibited by BAY 60-2770 and by BAY41-2272 (10 µM; Figure 1E). In contrast, neither BAY 41-2272 nor BAY 60-2770, at the concentrations employed, significantly inhibited TNF-stimulated adhesion of healthy control neutrophils in the microfluidic assay (Supplementary Figure 3E).

Cell viability assays (MTT) demonstrated that, at the concentrations depicted, neither BAY 60-2770 (1-10 µM) nor BAY 41-2272 (1-100 µM) affected neutrophil viability (data not shown).

**Hydroxyurea potentiates the effects of sGC activation on ex vivo human neutrophil adhesion to fibronectin**

Hydroxyurea is suggested to have NO donor properties. To investigate the influence of hydroxyurea on the effects of sGC stimulation and activation, SCA neutrophils were co-incubated with BAY 60-2770 or BAY 41-2272 (10 µM; 90 min) and hydroxyurea (500 µM, 15 min), before observing their adhesion to FN under static conditions (30 min, 37°C, 5% CO2). Under the static conditions used, hydroxyurea alone did not significantly
inhibit either TNF or hemin-induced SCA neutrophil adhesion to fibronectin (Figure 1C-D). Co-treatment of TNF-stimulated SCA neutrophils with BAY 60-2770 (10\(\mu\)M, 90 min) together with hydroxyurea further decreased their adhesion to fibronectin, but did not significantly alter the effect of BAY 41-2272 (10 \(\mu\)M; 90 min) on TNF-stimulated SCA neutrophil adhesion (Figure 1C). In contrast, co-incubation SCA neutrophils with hydroxyurea did not modulate the effects of either of the compounds on hemin-induced neutrophil adhesion to fibronectin (Figure 1D; P>0.05).

When TNF-stimulated neutrophils from healthy control individuals were co-incubated with BAY 60-2770 (10 \(\mu\)M) together with hydroxyurea (500 \(\mu\)M, 15 min), a significant inhibition of cell adhesion to fibronectin occurred (Supplementary Figure 3C); however, no potentiation of the effects of BAY 41-2272 (100 \(\mu\)M) on either TNF- or hemin-induced healthy control neutrophil adhesion was observed (Supplementary Figure 3C-D).

**The inhibiting effects of BAY 41-2272, but not BAY 60-2770, on TNF-induced SCA neutrophil adhesion are reversed by sGC oxidation**

The effect of the oxidation of the heme moiety of sGC on the ability of hydroxyurea, BAY41-2272 and BAY 60-2770 to inhibit TNF-induced SCA neutrophil adhesion to fibronectin was evaluated using microfluidic assays. Neutrophils were pretreated with either hydroxyurea (500 \(\mu\)M, 45 min), BAY 60-2770 or BAY 41-2272 (10 \(\mu\)M, 90 min), in the presence or absence of ODQ (100 \(\mu\)M; sGC oxidant), and co-stimulated with TNF (30 min), before perfusing over fibronectin-coated channels (Figure 1E). Hydroxyurea significantly decreased TNF-induced adhesion to fibronectin under microfluidic conditions; however, the slight reversal of this effect by ODQ was not statistically significant (p>0.05). Expectedly, ODQ was able to reverse the effect of BAY 41-2272 on
SCA neutrophil adhesion (Figure 1E), consistent with the mechanism of action of sGC stimulators, while sGC oxidation did not reverse the inhibiting effect of BAY 60-2770 on SCA neutrophil adhesion in response to TNF stimulation.

Since significant inhibition of TNF-stimulated healthy control neutrophil adhesion to fibronectin, following BAY 60-2770 or BAY 41-2272 (10 µM, 90 min) pre-incubation, was not observed under flow conditions, co-incubation with ODQ had no significant effects on these mechanisms (Supplementary Figure 3E).

**sGC activation and sGC stimulation inhibits TNF-induced Mac-1 and LFA-1 integrin activation.**

Neutrophil adhesive interactions with the extracellular matrix and endothelium are largely mediated by the actions of the LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) integrins on the cell surface. The expressions of the CD11a and CD11b subunits on the surface of healthy control neutrophils, following incubation of the cells with TNF cytokine (200 ng/ml, 30 min) and after pre-incubation (30 min), or not, with hydroxyurea (100 µM), BAY 41-2272 (10 µM) or BAY 60-2770 (10 µM) were determined by flow cytometry. The activation states of these integrin subunits were also determined by using activation epitope-specific antibodies.

While TNF did not significantly modulate CD11a and CD11b surface expression (Supplementary Table 2) on healthy control neutrophils, under the conditions used, this cytokine significantly augmented the binding activities of these integrin subunits on neutrophils (Figure 1F-G). In turn, similarly to hydroxyurea, both BAY 41-2272 and BAY 60-2770 significantly abrogated this TNF-induced increase in LFA-1 and MAC-1 integrin activity (Figure 1F-G).
sGC activation and sGC stimulation reduce leukocyte recruitment in the microvasculature of TNF-stimulated chimeric SCD mice.

TNF-induced leukocyte recruitment to the microvascular wall triggers vaso-occlusive mechanisms in mice with SCD. To evaluate the effects of intracellular cGMP modulation on leukocyte recruitment, we administered hydroxyurea (100 mg/kg i.v.), BAY 60-2770 (10 µg/mouse; i.v.), and/or BAY 41-2272 (10 µg/mouse; i.v.) to chimeric SCD mice, immediately before inducing leukocyte recruitment with TNF (0.5 µg, intraperitoneally). Leukocyte recruitment to the microvasculature of the cremaster muscle was then observed at 180 min post-TNF administration by intravital microscopy.

As previously demonstrated, and under the conditions employed, administration of a single dose of hydroxyurea modulated leukocyte recruitment in the microvasculature, significantly reducing the leukocyte adhesion and extravasation induced by TNF, without significant alterations in rolling activity (Figure 2A-C; Supplementary Figure 4). The effects of BAY 60-2770 or BAY 41-2272, at the concentrations used, were very similar to those of hydroxyurea, although BAY 41-2272 inhibited leukocyte recruitment, apparently, less efficiently (P>0.05) than BAY 60-2770 (Figure 2A-C; Supplementary Figure 4). Importantly, co-administration of hydroxyurea together with either of the sGC modulators significantly potentiated their effects on leukocyte recruitment, reducing leukocyte rolling, and further decreasing cell adhesion and extravasation in relation to either hydroxyurea alone or the agonists alone (Figure 2A-C).

In control mice, the administration of hydroxyurea (100 mg/kg i.v.) and BAY 41-2272 (10 µg/mouse; i.v.) also significantly decreased TNF-induced leukocyte adhesion and extravasation (Supplementary Figure 5B-C) in the microvasculature, in association with an elevation in leukocyte rolling (Supplementary Figure 5A). In contrast, BAY 60-
2770 (10 µg/mouse; i.v.) significantly abrogated TNF-induced rolling, adhesion and extravasation (Supplementary Figure 5A-D).

**Effects of BAY 41-2272 and BAY 60-2770 on plasma cGMP in chimeric SCD mice**

To look at the ability of the sGC stimulator/activators to modulate cGMP levels in SCD mice, when administered immediately before TNF stimulation (0.5 µg i.p.), we collected plasma from mice within 5 h of administration. Figure 2D demonstrates that a single dose of hydroxyurea (100 mg/kg, i.v.) significantly increased plasma cGMP. Augmentations in plasma cGMP following BAY 60-2770 and BAY 41-2272 (10 µg/mouse, i.v.) were not found to be statistically significant. Hydroxyurea (100 mg/kg, i.v.), BAY 60-2770 and BAY 41-2272 (10 µg/mouse, i.v.) also elevated plasma cGMP in control mice (Supplementary Figure 5E), but this increase was only significant for BAY 60-2770 treated mice.

**BAY 41-2272 induces gene expression of γ-globin and fetal hemoglobin protein production in erythroid lineage cells.**

Given the fact that drugs with HbF-elevating properties are highly beneficial in SCA, and that evidence suggests a role for cGMP-dependent signalling in γ-globin regulation, we looked at the ability of BAY 41-2272 and BAY 60-2770 to increase the expression of the gene encoding γ-globin, *HBG*, in the erythroleukemic cell line, K562. Having confirmed the expressions of the genes encoding the sGC subunits in K562 cells (Supplementary Figure 2), these cells were cultured in the presence or absence of hydroxyurea (100 µM), BAY 60-2770 (10 µM), BAY 41-2272 (10 µM), or DMSO vehicle (0.1% vol/vol) for 96 h. *HBG* expression in cells was determined by qRT-PCR and HbF protein expression was measured by flow cytometry (Figure 3).
As expected, hydroxyurea increased \textit{HBG} expression in the K562 cells by approximately 2-fold at 48-96 h of culture (Figure 3A), in association with a significant increase in HbF protein expression at 72-96 h (Figure 3B). Surprisingly, co-culture of the cells with BAY 41-2272 triggered \textit{HBG} expression and HbF production much more efficiently than hydroxyurea at all time points, augmenting HbF expression by more than 3-fold by 96 h of culture. In contrast, BAY 60-2770, at the concentrations employed (data for 1 and 100 µM BAY 60-2770 not shown), did not show such an upregulating effect on \textit{HBG} expression and, consequently HbF production, in the erythroleukemic cells.

Interestingly, the effects of hydroxyurea and BAY 41-2272 on HbF production were both accompanied by inhibition of the proliferation (but not induction of cell death) of the K562 cells (Figure 3C), consistent with the known cytostatic property of hydroxyurea.

**Discussion**

Sickle cell anemia is now recognized as a global health problem (Kato et al., 2018) and while curative therapy for SCA exists in the form of hematopoietic stem cell transplantation, its availability is very restricted (Yawn et al., 2014). Despite concerted efforts by researchers and the pharmaceutical industry to develop novel drugs for the treatment of SCA (Telen, 2016; Conran and Torres, 2019), only three substances have been FDA approved for SCA therapy since hydroxyurea’s approval over 20 years ago, and it is probable that multi-drug approaches, possibly still involving the use of hydroxyurea, will evolve for the management of the disease (Carden and Little, 2019). Recruitment of leukocytes to the vascular wall constitutes a major trigger for vaso-occlusion in SCA, and occur in response to inflammatory stimuli such as TNF cytokine,
cell-free heme (or hemin, a DAMP) and ischemia/reperfusion processes (Turhan et al., 2002; Kalambur et al., 2004; Belcher et al., 2014). As such, inhibiting the adhesion of leukocytes to the vascular endothelium, under inflammatory conditions, may represent an important approach for preventing SCA vaso-occlusion (Chang et al., 2010), and its consequent complications.

Neutrophils from SCA individuals generally demonstrate augmented adhesive properties, compared to neutrophils from healthy individuals, and this adhesion is further augmented by cellular activation by the inflammatory molecules, TNF cytokine and hemin, both of which are elevated in the circulation of SCA individuals (Lanaro et al., 2009; Schaer et al., 2013). Neutrophils express the genes encoding sGC, albeit at significantly lower levels than those encountered in CD34+ hematopoietic cells. As stimulation of cGMP-dependent signalling may have beneficial consequences on leukocyte function (Almeida et al., 2012; Conran and Torres, 2019) in SCA, we investigated the effects of the sGC activator and sGC stimulator, BAY 60-2770 and BAY 41-2272, respectively, on the ex vivo adhesive properties of neutrophils from patients with SCA. BAY 60-2770 at relatively low in vitro concentrations (1-10 µM) effectively inhibited the adhesion of SCA neutrophils to fibronectin, following TNF-induced activation, but not hemin-induced SCA neutrophil adhesion. Notably, BAY 41-2272 (from 10 µM) significantly inhibited the adhesive properties of SCA neutrophils, when activated by hemin, in addition to diminishing TNF-induced SCA neutrophil adhesion. BAY 41-2272 and BAY 60-2770 are preclinical tool compounds and have not been tested in patients to allow direct comparison of exposure and pharmacokinetics with the in vitro doses employed (Zenzmaier et al., 2015), but concentrations of these agonists of 1-30 µM are consistently employed in in vitro studies (Zenzmaier et al., 2015). Consistent with the sGC activating and stimulating abilities of BAY 60-2770 and BAY 41-2272 (Follmann
et al., 2013), respectively, oxidation of sGC by ODQ slightly potentiated the inhibiting effect of BAY 60-2770 on neutrophil adhesion, while reversing the effect of BAY 41-2272.

Flow cytometry assays suggest that the observed effects of both sGC agonists on TNF-induced neutrophil adhesion appear to be mediated by the suppression of the activation of the Mac-1 and LFA-1 integrins on the neutrophil cell surface, in keeping with previous reports of a role for the NO/cGMP pathway in regulating leukocyte integrin function (Conran et al., 2001; Conran et al., 2003). In vivo, BAY 60-2770 and BAY 41-2272, when administered alone, significantly diminished leukocyte recruitment to the TNF-stimulated microvasculature of SCD mice (as demonstrated by decreased leukocyte adhesion and extravasation at the microvessel walls). Of note, while the in vitro effects of BAY 41-2272 on neutrophil adhesive properties appeared to be superior, in vivo, the effects of BAY 60-2770 were apparently slightly more efficient under the conditions employed.

Hydroxyurea has previously been shown to inhibit leukocyte recruitment in SCD mice, following a single administration, and via a mechanism mediated by cGMP-dependent signalling (Almeida et al., 2012). In vitro, co-incubation of SCA neutrophils with hydroxyurea potentiated the effects of BAY 60-2770 on their adhesion. Given that Bay 60-2770 activates oxidized sGC in an NO-independent manner (Pankey et al., 2011), the amplification of its effect by hydroxyurea was somewhat surprising. Furthermore, in vivo, the concomitant administration of hydroxyurea to SCD mice amplified the effects of both BAY 60-2770 and BAY 41-2272 on leukocyte recruitment in the microvasculature, further inhibiting leukocyte adhesion to blood vessel walls, when used in combination with a sGC activator/stimulator. The beneficial effects of BAY 60-2770 and BAY 41-2272 in the microvasculature of mice with SCD were associated with
improvements in circulating cGMP levels. As such, the potentiation of the effects of BAY 60-2770 by hydroxyurea suggests that, in this model, hydroxyurea may in fact modulate sGC activity, in conjunction with the sGC activator, rather than provide NO supplementation. Another explanation could be that constant shifts can occur in the redox equilibrium, modulating the balance of oxidized and native sGC and, therefore, the sensitivity of this enzyme to NO (Breitenstein et al., 2017). However, to confirm these notions, further investigations are necessary.

As mentioned, a major effect of hydroxyurea is its ability to induce HbF (fetal hemoglobin, α2γ2) production in erythroid cells, in turn reducing sickle hemoglobin polymerization and red cell sickling (Platt et al., 1984). Drugs in development for the treatment of SCA should ideally be able to elevate HbF levels if their use is proposed to replace that of hydroxyurea in a chronic regimen. In fact, activation of the sGC-PKG pathway can upregulate the expression of the γ-globin gene, in turn increasing HbF generation (Ikuta et al., 2001); furthermore, there is evidence that the HbF-inducing ability of hydroxyurea in erythroid cells could well be mediated by inducing sGC activity (Cokic et al., 2003; Cokic et al., 2008). As such, we compared the abilities of BAY 60-2770 and BAY 41-2272, with that of hydroxyurea, to induce γ-globin (HBG) gene transcription and the expression of HbF protein in a erythroleukemic cell line. Hydroxyurea (100 µM) significantly induced HBG gene expression within 48 h of culture, in association with increased HbF generation after 72 h. sGC stimulation with BAY 41-2272 (10 µM) significantly increased both HBG gene and HbF protein expression within a similar time frame, and apparently even more efficiently than hydroxyurea. Like hydroxyurea, BAY 41-2272 decreased erythroid cell proliferation without inducing cell death, indicating that BAY 41-2272 may mediate this effect via a cytostatic action. In contrast, BAY 60-2770, at the concentrations employed (1-100 µM),
induced neither HBG gene nor HbF protein expression. Given the similar, or even slightly superior effects of the sGC activator *in vivo*, the effects of these agonists on HBG gene and HbF protein expression should be further investigated.

One limitation of this study is that the intravenous effects of both sGC agonists on murine blood pressure were not assessed. However, it is well established that sGC stimulators and sGC activators have dose-dependent blood pressure lowering effects. Moreover, intravenous doses of 100 µg/kg of BAY 41-2272 significantly decrease blood pressure in anaesthetized rats (Straub et al., 2002), while, in conscious rats, both BAY412272 and BAY60-2770, when given intravenously (300µg/kg), are reported to have no significant effect on blood pressure (Fullhase et al., 2015). Olinciguat, another sGC stimulator, which is currently in phase 2 clinical development for use in sickle cell anemia patients (NCT03285178)(Zimmer et al., 2020), reduces blood pressure in humans and in hypertensive and normotensive rats, but also successfully reduces inflammatory mechanisms in TNF-stimulated mice (Buys et al., 2018; Zimmer et al., 2020). As such, further preclinical and clinical studies should be careful evaluate the extent of any effect of sGC agonists on blood pressure, when considering the use of these agents for the treatment of SCA.

In conclusion, this study suggests that both sGC activation and stimulation could represent approaches to reduce leukocyte recruitment to the endothelium and, therefore, reduce vaso-occlusive episodes in SCD. While sGC activation could potentially reduce leukocyte recruitment, without concomitant hydroxyurea administration, this class of drugs could be of use for reducing vaso-occlusive episodes in patients not on hydroxyurea therapy. Alternatively, if both HbF induction and abrogation of vaso-occlusive events are a goal for therapy, combination therapy with both sGC stimulators and hydroxyurea could offer a potent approach for SCD management.
Acknowledgements

The authors would like to thank the Instituto de Pesquisas Energéticas Nucleares (IPEN), São Paulo, for support with animal procedures.

Authorship Contributions

Participated in research design: NC, PS, JPS, DB, CBA, FFC and PSF
Conducted experiments: WAF, HC, CL, FCL, LIM, FG, LT, EMFG and PLB
Contributed new reagents or analytic tools: PS, JPS, DB, CFFP, FFC and STOS
Performed data analysis: NC, WAF, HC, STOS
Wrote the manuscript: NC and PS
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Footnotes

Funding: This research study was funded by Bayer AG Pharmaceuticals, Germany. WAF received a Post-Doctoral Fellowship grant from Bayer Pharma AG. HC, FG, LT, EMFG, PLB, and LIM are recipients of fellowships from Fundação de Amparo à Pesquisa do Estado de S. Paulo (FAPESP) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. PSF is supported by R01 grants from the National Institutes of Health (HL069438, DK056638, DK116312 and DK112976).

Potential Conflicts of Interest: WAF received a Post-Doctoral fellowship from Bayer AG Pharmaceuticals. NC received grant funding from Bayer AG Pharmaceuticals through a restricted research grant. PSF serves as consultant for Pfizer, has received research funding from Ironwood Pharmaceuticals and is shareholder of Cygnal Therapeutics. JPS is senior advisor of Bayer AG. DB and PS are full time employees of Bayer AG Pharmaceuticals.

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Legends for Figures

**Figure 1. Effects of BAY 60-2770 and BAY 41-2272 on the adhesion of neutrophils from SCA individuals to fibronectin ligand.** Static adhesion assays: Neutrophils isolated from SCA individuals (N=5-12) were incubated for 90 min with BAY 60-2770 (BAY60) or BAY 41-2272 (BAY41) before stimulating with (or without) (A) TNF cytokine (200 ng/ml, 30 min co-incubation) or (B) hemin (50 µM, 30 min co-incubation) and observing adhesion of neutrophils to fibronectin (FN) ligand (30 min, 37°C, 5% CO₂). Subsequently, the effects of the co-incubation of cells with hydroxyurea (HU; 500 µM, 15 min) on (C) TNF cytokine (200 ng/ml, 30 min) and (D) hemin (50 µM, 30 min) -induced adhesion of SCA neutrophils (N=5-12) to fibronectin ligand (30 min, 37°C, 5% CO₂) were determined. (E) Microfluidic adhesion assay: Adhesion of neutrophils from SCA individuals (N=6-7) to fibronectin ligand in 400-µm-wide channel biochips, using a shear rate of 0.5 dynes/cm² (3 min, 37°C), was determined after co-incubating cells with HU (500 µM, 45 min), BAY 41-2272 (90 min) or BAY 60-2770 (90 min) in the presence or absence of the sGC inhibitor, ODQ (100 µM), before co-stimulating, or not, with TNF cytokine (200 ng/ml, 30 min, 37°C); Neutrophils adhered to fibronectin represents the number of neutrophils adhered in a field of 0.09mm². *, P<0.05; ***, P<0.001, compared to basal adhesion. #, P<0.05; ##, P<0.01, ###, P<0.001, compared to TNF-stimulated or hemin-stimulated adhesion. ●, P<0.05; ●●, P<0.01, compared to BAY60 or BAY41 alone (as appropriate; ANOVA and Sidak’s multiple comparison test). Effects of BAY 60-2770 and BAY 41-2272 on the activation conformation of the CD11a (F) and CD11b (G) integrin subunits on neutrophils isolated from healthy subjects. Cells were incubated with hydroxyurea (HU; 100 µM), BAY 41-2272 (10 µM) or BAY 60-2770 (10 µM) for 30 min (37°C, 5 % CO₂) before the addition, or not, of TNF (200 ng/mL; 30 min). Flow
cytometry was employed to compare antibody binding to the activated epitopes of CD11a and CD11b (antibody clones MEM-83 and CBRM1/5, respectively). Integrin subunit activation is shown as the mean ± SEM, N=6-8 for each group. *, P<0.05, compared to basal; #, P<0.05, compared to TNF alone (ANOVA and Sidak’s multiple comparison test).

Figure 2. BAY 60-2770 and BAY 41-2272 inhibit leukocyte recruitment to the microvasculature in a murine model of SCD inflammatory vaso-occlusion. Vasooclusive-like processes were induced in SCD mice by administration of TNF (0.5 µg, i.p.) Mice concomitantly received, or not, administrations of saline vehicle, hydroxyurea (HU, 100 mg/Kg), BAY 60-2770 (10µg/mouse), and/or BAY 41-2272 (10µg/mouse). (A) Leukocyte rolling; (B) leukocyte adhesion and (C) leukocyte extravasation were quantified in venules of SCD mice (4-6 mice per group; 6-10 venules per mouse) at 180 min after the administration of TNF. **, P<0.01; ***, P<0.001, compared to TNF alone. #, P<0.05, ##, P<0.01, ###, P<0.001, compared to HU alone. Ø, P<0.05, compared to BAY 60-2770 alone. ⚫⚫⚫, P<0.001, compared to BAY 41-2272 alone (D) Measurement of plasma cGMP in SCD mice following administration of TNF (0.5 µg i.p., 180 min) concomitantly with saline vehicle or hydroxyurea (HU, 100 mg/Kg), BAY 41-2272 (BAY41, 10 µg/mouse) or BAY 60-2770 (BAY60, 10 µg/mouse). Plasma cGMP was measured by ELISA. N= 3-7 mice per group; *, P < 0.05, compared to TNF alone (ANOVA and Dunn’s multiple comparison test).

Figure 3. Effects of soluble guanylyl cyclase activity modulation on γ-globin and fetal hemoglobin (HbF) expression in K562 erythroleukemic cells in culture. Cells were co-incubated with hydroxyurea (HU; 100 µM), BAY 60-2770 (1-10 µM), BAY
41-2272 (1-10 µM) or DMSO vehicle (0.1% v/v) for 96 hours (37°C, 5% CO₂): (A) 
*HBG* gene expression was determined by qRT-PCR, normalized to *ACTB* and *GAPDH* expression; (B) HbF was determined by flow cytometry and (C) cell proliferation was determined (BAY 60-2770 and BAY 41-2272, 10 µM). (A) *, P<0.05, **, P<0.01***, P<0.001, compared to untreated cells (CTRL); #, P<0.05; ##, P<0.01, ###, P<0.001, compared to DMSO alone; ●●●, P<0.001, compared to BAY 60-2770 (1 µM); φ φ φ, compared to BAY 60-2770 (10 µM); (B) **, P<0.01, ***, P<0.001, compared to CTRL and DMSO; Φ, P<0.05, compared to HU; ●●, P<0.01; ●●●, P<0.001, compared to BAY 60-2770 (1 µM); φ φ φ, compared to BAY 60-2770 (10 µM). (C) **, P<0.01, ***, P<0.001, compared to CTRL; ###, P<0.001, compared to DMSO alone and BAY 60-2770 (10 µM); Φ, P<0.01, compared to HU. Two-way ANOVA, Tukey’s multiple comparisons test.
Figure 1
Figure 2
Figure 3