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RESEARCH PAPER

Olinciguat, a stimulator of soluble guanylyl cyclase, attenuates inflammation, vaso-occlusion and nephropathy in mouse models of sickle cell disease

Boris Tchernychev[1] | Huihui Li[2] | Sung-Kyun Lee[2] | Xin Gao[2] | Raghunath Ramanarasimhaiah[1] | Guang Liu[1] | Katherine C. Hall[1] | Sylvie G. Bernier[1] | Juli E. Jones[1] | Susanne Feil[3] | Robert Feil[3] | Emmanuel S. Buys[1] | Regina M. Graul[1] | Paul S. Frenette[2] | Jaime L. Masferrer[1]

[1]Cyclerion Therapeutics Inc., Boston, Massachusetts, USA

[2]Departments of Medicine and Cell Biology, Albert Einstein College of Medicine, New York, New York, USA

[3]Interfaculty Institute of Biochemistry, University of Tübingen, Tübingen, Germany

Correspondence
Emmanuel S. Buys, Cyclerion Therapeutics Inc., 501 Boylston Street Floor 10, Boston, MA 02116, USA.
Email: ebuys@cyclerion.com

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Background and Purpose: Reduced bioavailability of NO, a hallmark of sickle cell disease (SCD), contributes to intravascular inflammation, vasoconstriction, vaso-occlusion and organ damage observed in SCD patients. Soluble guanylyl cyclase (sGC) catalyses synthesis of cGMP in response to NO. cGMP-amplifying agents, including NO donors and phosphodiesterase 9 inhibitors, alleviate TNFα-induced inflammation in wild-type C57BL/6 mice and in 'humanised' mouse models of SCD.

Experimental Approach: Effects of the sGC stimulator olinciguat on intravascular inflammation and renal injury were studied in acute (C57BL6 and Berkeley mice) and chronic (Townes mice) mouse models of TNFα-induced and systemic inflammation associated with SCD.

Key Results: Acute treatment with olinciguat attenuated increases in plasma biomarkers of endothelial cell activation and leukocyte-endothelial cell interactions in TNFα-challenged mice. Co-treatment with hydroxyurea, an FDA-approved SCD therapeutic agent, further augmented the anti-inflammatory effect of olinciguat. In the Berkeley mouse model of TNFα-induced vaso-occlusive crisis, a single dose of olinciguat attenuated leukocyte-endothelial cell interactions, improved blood flow and prolonged survival time compared to vehicle-treated mice. In Townes SCD mice, plasma biomarkers of inflammation and endothelial cell activation were lower in olinciguat- than in vehicle-treated mice. In addition, kidney mass, water consumption, 24-h urine excretion, plasma levels of cystatin C and urinary excretion of N-acetyl-β-d-glucosaminidase and neutrophil gelatinase-associated lipocalin were lower in Townes mice treated with olinciguat than in vehicle-treated mice.

Abbreviations: IVM, intravital microscopy; HbAA, Townes non-disease control mice carrying the normal human β-globin gene; HbSS, Townes SCD mice; NAG, N-acetyl-βbeta-d-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; NT, naïve non-treated; PAI-1, plasminogen activator inhibitor-1; PDE9, phosphodiesterase 9; RBCs, red blood cells; SAA, serum amyloid A; SAP, serum amyloid P component; SCD, sickle cell disease; sGC, soluble guanylyl cyclase; VOC, vaso-occlusive crisis; V_{RBC}, red cell velocity.

Boris Tchernychev and Huihui Li contributed equally.

Conclusion and Implications: Our results suggest that the sGC stimulator olinciguat attenuates inflammation, vaso-occlusion and kidney injury in mouse models of SCD and systemic inflammation.

KEYWORDS
inflammation, kidney injury, olinciguat, sickle cell disease, soluble guanylate cyclase, vaso-occlusive crisis

1 | INTRODUCTION

Sickle cell disease (SCD) is characterised by chronic inflammation driven by ongoing haemolysis and hypoxia (Conran & Belcher, 2018) Activation of vascular endothelium and upregulation of endothelial adhesion receptors on the surface of the vascular wall enables heterotypic interactions between sickled red blood cells (RBCs), activated leukocytes and endothelial cells, leading to vaso-occlusion of the venous microcirculation and eventual organ damage (Canalli et al., 2005; Frenette, 2004; Turhan et al., 2002).

Reduced bioavailability of **nitric oxide** (NO) in patients with SCD, caused in part by haemolysis, further promotes intravascular inflammation and vaso-occlusion (Kato et al., 2017; Reiter et al., 2002) Lower NO bioavailability results in decreased activation of **soluble guanylyl cyclase** (sGC), a heterodimeric haem-containing enzyme that catalyses synthesis of the second messenger **cGMP** (Derbyshire & Marletta, 2012; Horst & Marletta, 2018). cGMP modulates many physiological processes, including inflammation and vasorelaxation (Ahluwalia et al., 2004; Buys et al., 2018) Evaluation of NO donors and inhibitors of cGMP-specific **phosphodiesterase 9 (PDE9)** in **TNF**α-challenged C57BL/6 mice and genetic mouse models of SCD, showed that both these modulators of cGMP signalling can attenuate activation of vascular endothelium in preclinical models of SCD-associated inflammation (Almeida et al., 2012; Jasuja et al., 2014, 2016) Similarly, treatment of mice with the current standard of care, **hydroxyurea** attenuates activation of vascular endothelium, most likely by acting as a NO precursor (Almeida et al., 2012, 2015; Gladwin et al., 2002; King, 2004). Furthermore, the combination of hydroxyurea with PDE9 inhibitors demonstrates an additive effect and protects SCD mice against vaso-occlusive crisis (VOC) induced by TNFα-challenge (Almeida et al., 2012; Jasuja et al., 2014, 2016) or by transient hypoxia. (McArthur et al., 2020). Recently, the co-administration of hydroxyurea was shown to potentiate the ability of an sGC stimulator or activator to decrease TNFα-induced leukocyte recruitment in a mouse model of vaso-occlusion (Ferreira et al., 2020).

Olinciguat is a novel clinical-stage sGC stimulator that enhances the synthesis of cGMP in response to NO binding (Buys et al., 2018; Zimmer et al., 2020). sGC stimulators are allosteric modulators that bind to and stimulate haem-containing, NO-responsive sGC. In contrast to sGC activators that only bind to haem-free sGC and act independently of NO, stimulators act synergistically with NO and thus are powerful amplifiers of endogenous, physiological NO signalling and can maintain the local and transient nature of NO signalling. Here we evaluated the effect of olinciguat treatment on inflammation and endothelial cell activation in mouse models of SCD.

What is already known

- Reduced NO signalling is a hallmark of sickle cell disease.
- cGMP modulates inflammation and vasorelaxation, and increasing its bioavailability attenuates activation of vascular endothelium.

What does this study add

- Olinciguat (soluble guanylyl cyclase stimulator) alleviated intravascular inflammation and vaso-occlusion in sickle cell disease models.
- Olinciguat also attenuated renal injury in the Townes mouse model of sickle cell disease.

Clinical significance

- Olinciguat could alleviate acute and chronic complications and symptoms of sickle cell disease.
- Further study of olinciguat on inflammation and organ protection in sickle cell disease is warranted.

2 | METHODS

2.1 | Mice

All animal care and experimental procedures were approved by Institutional Animal Care and Use Committees (Cyclerion (15-010), Albert Einstein College of Medicine (20180409), University of Tübingen (IB 2/15)). Wild-type C57BL/6 mice (8–10 weeks old) were purchased from Envigo Labs (Indianapolis, IN). Unless otherwise

stated, mice were group housed in polycarbonate cages with filter tops and acclimatised for at least 3 days before the study started. All animals were housed under controlled conditions of temperature (22 ± 4°C) and a relative humidity of 30% to 70% and placed in a 12:12-h light–dark cycle room (lights on at 6:30 a.m.) at an AAALAC-accredited animal research facility. All animals were allowed ad libitum access to water and standard rodent chow (Harlan Teklad, Indianapolis, IN; Irradiated Teklad Global 16%). Endpoints relevant to SCD pathophysiology were assessed in genetically modified mice. Animal experiments were performed in accordance with all relevant ethical regulations and in adherence to ARRIVE guidelines (Percie du Sert et al., 2020; Lilley et al., 2020).

Genetically identical cohorts of male SCD mice were generated by transplanting bone marrow from Berkeley SCD mice (Tg [Hu-miniLCRα1$^{G}\gamma^{A}\gamma\delta\beta^{S}$] Hbα-deficient Hbβ-deficient) (Pászty et al., 1997) into lethally irradiated C57BL/6 mice as previously described. (Chang et al., 2010) C57BL/6 mice engrafted with the bone marrow from heterozygous mice (Tg [Hu-miniLCRα1$^{G}\gamma^{A}\gamma\delta\beta^{S}$] Hbα-deficient Hbβ-heterozygous) were studied as non-disease controls. Only mice carrying more than 97% of donor chimerism at 3 to 5 months after bone marrow transplantation were studied for intravital microscopy. Female Townes SCD mice homozygous for human sickle ^{s}β-globin gene (HbSS) and age-matched Townes non-disease control (HbAA) mice (Ryan et al., 1997) were obtained from The Jackson Laboratory (Bar Harbor, ME). The time courses of the three experimental setups described are provided in Figure S1.

2.2 | Acute TNFα-induced inflammation in C57BL/6 mice

Inflammation in C57BL/6 mice was induced by i.p. injection of mouse TNFα (50 ng per mouse). Olinciguat (10 mg kg^{-1}) and vehicle (60% PEG in water; 0.2 ml per mouse) were administered p.o., 1 h prior to TNFα challenge. The olinciguat dose was selected to reach exposures in mice similar to those observed in clinical use. In normotensive rats, a dose of olinciguat eliciting a similar exposure to the exposure achieved in this study decreased MAP by ∼11 mmHg. (Zimmer et al., 2020) We did not observe any acute effects of olinciguat or hydroxyurea treatment on the behaviour of SCD mice that would suggest a severe hypotensive response. Hydroxyurea (250 mg kg^{-1} p.o.) was given just before TNFα injection. Four hours after induction of inflammation, animals were killed (CO_2 asphyxiation) and blood was collected into K_2EDTA tubes from the inferior vena cava for analysis of plasma biomarkers.

2.3 | Intravital microscopy

The microvasculature of male C57BL/6 mice and chimeric C57BL/6 mice engrafted with bone marrow from Berkeley SCD mice were studied using intravital microscopy (IVM). (Thunemann et al., 2014) Inflammation was induced by i.p. injection of mouse TNFα (0.5 μg per mouse) 90 min before cremaster muscle surgery. Mice were treated with olinciguat (10 mg kg^{-1}), hydroxyurea (100 mg kg^{-1}) or the combination of olinciguat (administered p.o. 30–60 min prior to TNFα treatment) plus hydroxyurea (administered p.o. at the time of TNFα treatment). Mice were anaesthetized by i.p. injection of fentanyl (0.1 mg kg^{-1}), midazolam (2 mg kg^{-1}) and medetomidine (20 mg kg^{-1}) or with 2% chloralose and 10% urethane and placed on a prewarmed (36°C) IVM stage. The cremaster muscle was exteriorized, cleaned of connective tissue and spread flat on the pedestal of a custom-built imaging platform. The cremaster was continuously superfused with either saline buffer (composition, in mmol L^{-1}; 118.4 NaCl, 20 $NaHCO_3$, 3.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$) or with bicarbonate-buffered saline (composition, in mmol L^{-1}; 135 NaCl, 5 KCl, 27 $NaHCO_3$, 0.64 $MgCl_2$, pH 7.4) warmed to 35°C to 36°C and aerated with a mixture of 95% N_2 and 5% CO_2. Leukocyte-endothelial cell interactions in the postcapillary venules of mouse cremaster muscle were visualised using an upright Nikon (MM-40) microscope equipped with a 40 × water immersion objective. From each animal, 8–30 venules were recorded for 1 to 2 min (10 frames per second; SPOT™ Pursuit camera, Diagnostic Instruments, Inc.) using a charge-coupled video camera (Hamamatsu) and video recorder (Sony SVHS, SVO-9500) or using an upright Examiner.Z1 microscope (ZEISS) equipped with a water immersion objective (W Plan Achromat 40×/1.0). Offline analysis with ImageJ1.51j was performed to determine vessel diameter, leukocyte rolling velocity (μm s^{-1}), flux of rolling leukocytes (cells min^{-1}) and adherent leukocytes (cells min^{-1}). To calculate adhesion efficiency, adhesion was divided by rolling flux for each individual vessel. Microsoft® Excel® and OriginPro were used for further data evaluation.

Venular diameters were measured using a video calliper. Centerline red cell velocity (V_{RBC}) for each venule recorded was measured using an optical Doppler velocimeter (Texas A&M). IVM recordings started 120 min after TNFα challenge and were performed for 40 to 60 min followed by offline analysis of leukocyte-endothelial cell interactions. All Berkeley SCD mice were monitored until death. Survival time was determined from the time of TNFα treatment to the time mice stopped breathing.

2.4 | SCD inflammation

The therapeutic effect of olinciguat on established SCD inflammation was studied in 18-week-old Townes mice following chronic administration of the compound. HbSS mice were treated with either vehicle or olinciguat (10 mg kg^{-1} by midday gavage and compound formulated in the diet) for 8 weeks. The olinciguat dose was selected to reach and maintain exposures in mice similar to those observed in the clinic. Age-matched Townes mice carrying the normal human β-globin gene (HbAA) served as non-disease control. Twenty-four-hour water consumption and urine output were measured using metabolic cages 7 weeks after treatment initiation. After 8 weeks of treatment, all mice were killed (CO2 asphyxiation), and blood and kidneys were collected for biomarker analysis.

2.5 | Plasma and urine analysis

Plasma sE-, sL- and sP-selectins, sICAM-1, serum amyloid A (SAA), serum amyloid P component (SAP), IL-6, IL-1β, plasminogen activator inhibitor-1 (PAI-1), cystatin C and urinary N-acetyl-β-d-glucosaminidase (NAG) and neutrophil gelatinase-associated lipocalin (NGAL) levels were measured using enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems. Urinary albumin and creatinine levels were analysed using ELISA and an enzymic method, respectively (Crystal Chem, Elk Grove Village, IL). Urinary protein concentrations were determined using a Coomassie Plus (Bradford) assay (Thermo Scientific, Waltham, MA).

2.6 | Data and statistical analysis

Experimental design, analysis and reporting of data are consistent with *BJP* guidance for publication. Values are presented as mean ± SEM. Data were analysed using one-way ANOVA with Fisher-LSD post-test where appropriate. For the parameter of adhesion efficiency, the data set was not normally distributed, and thus a Kruskal–Wallis test was used followed by an uncorrected Dunn's test. An outlier analysis was performed across all data sets. A ROUT (robust regression and outlier removal) with a Q = 1% was set as the criteria for exclusion of a data point. Statistical significance for the Kaplan–Meier survival curve was assessed using a log-rank (Mantel–Cox) test. Naïve non-TNFα challenged Berkeley SCD mice were studied as a VOC-free control (no mortality observed). Differences between groups were considered significant at $P < .05$. In all experiments, animals were randomised to groups and order of treatment was randomised. Data recording and analysis were performed in a blinded fashion. Data was not normalised or transformed. Where possible, studies were designed to generate groups of equal size, using randomisation and blinded analysis. Results are presented in scatter plots where appropriate. Due to the large number of datapoints in Figures 2 and 3 and because the scatter plots did not reveal unusual or interesting aspects of the data not obvious from the bar chart, results are displayed as bar charts.

The TNFα model (Figures 1 and 2), is an acute (5-h) model of TNFα-induced inflammation in C57BL/6 mice. Based on our

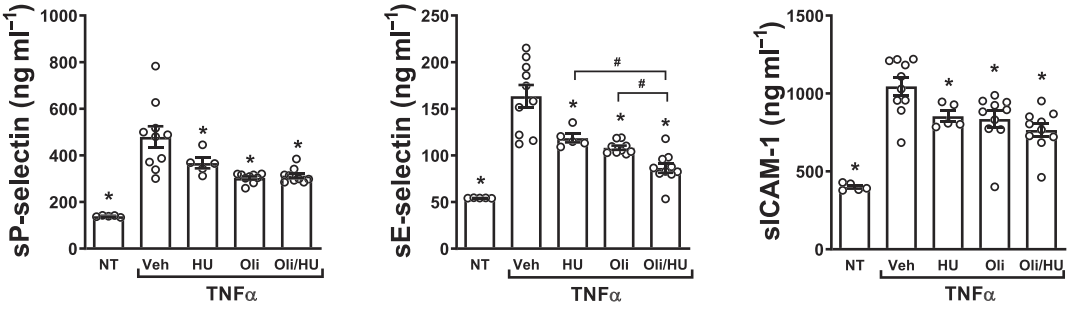

FIGURE 1 Concentrations of sP-selectin (left), sE-selectin (middle) and sICAM-1 (right) in the plasma from non-treated control mice (NT) and mice treated with either vehicle (Veh), hydroxyurea (HU), olinciguat (Oli) or the olinciguat plus hydroxyurea combination (Oli/HU) before induction of inflammation with mouse TNFα. Data are presented as mean ± SEM, n = 10 per group. *$P < .05$, significantly different from vehicle; #$P < .05$, significantly different as indicated

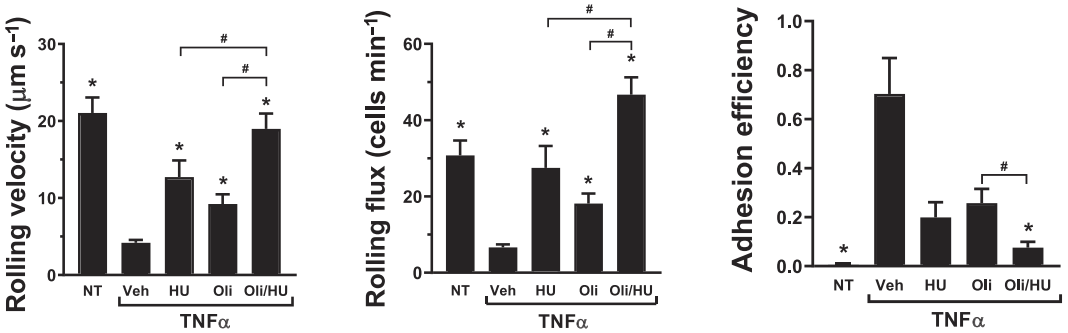

FIGURE 2 Leukocyte-endothelial cell interactions in C57BL/6 mice with acute inflammation. Effect of vehicle (Veh), olinciguat (Oli), hydroxyurea (HU) or olinciguat plus hydroxyurea (Oli/HU) combination on leukocyte rolling velocity (left), leukocyte rolling flux (middle) and leukocyte adhesion efficiency (right, calculated from adhesion and rolling flux data) was studied in C57BL/6 mice with TNFα-induced inflammation. Naïve C57BL/6 mice were studied as a non-treated (NT) control. Data are presented as mean ± SEM. Five to 21 venules from each animal (three to four animals per condition) were recorded for 1 min. The group size was n = 21 for NT, n = 64 for Veh, n = 19 for HU, n = 34 for Oli and n = 33 for Oli/HU. *$P < .05$, significantly different from vehicle; #$P < .05$, significantly different as indicated

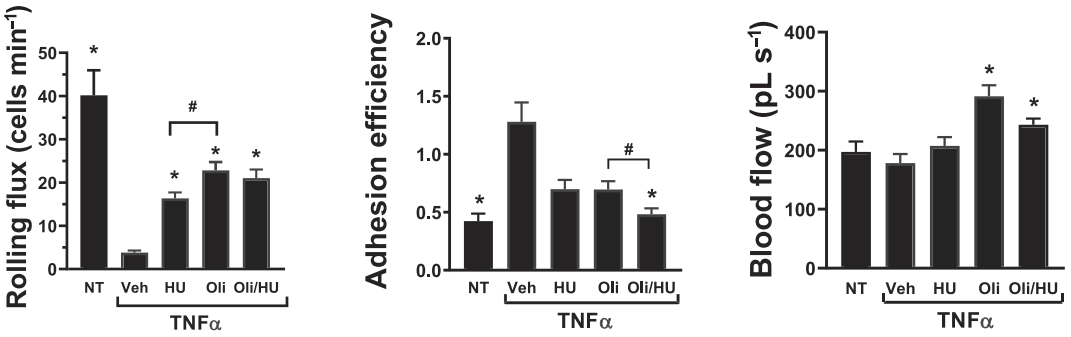

FIGURE 3 Leukocyte-endothelial cell interactions and blood flow in chimeric Berkeley SCD mice with VOC after a challenge with TNFα. Effect of vehicle (Veh), olinciguat (Oli), hydroxyurea (HU) or olinciguat plus hydroxyurea (Oli/HU) combination on leukocyte rolling flux (left), leukocyte adhesion efficiency (middle) and venous blood flow (right) was studied in Berkeley SCD mice with TNFα-induced VOC. Naïve non-treated (NT) Berkeley SCD mice were studied as a VOC-free control. Data are presented as mean ± SEM, n = 5, 6, 6, 5 and 6 in NT, Veh, Oli, HU and OLI/HU, respectively. *P < .05, significantly different from vehicle; #P < .05, significantly different as indicated

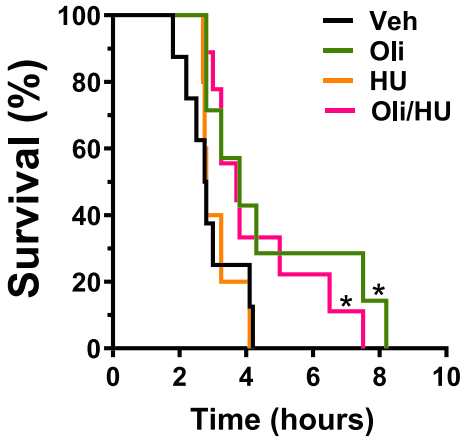

FIGURE 4 Kaplan–Meier survival curve after induction of VOC in Berkeley SCD mice with mouse TNFα. Berkeley SCD mice were pretreated with either vehicle (Veh), olinciguat (Oli), hydroxyurea (HU) or olinciguat plus hydroxyurea (Oli/HU) combination before induction of VOC. Data are presented as mean ± SEM, n = 8, 7, 5 and 9 in Veh, Oli, HU and OLI/HU, respectively *P < .05, significantly different from vehicle

previous experience with this model and a priori power analysis, we determined that a sample size of 10 would be sufficient to evaluate the effect of hydroxyurea, olinciguat or the olinciguat-hydroxyurea combination, on plasma levels of sP-selectin, sE-selectin and sICAM-1. To analyse leukocyte rolling velocity, leukocyte rolling flux and leukocyte adhesion efficiency, 5 to 21 random venules from 3 to 4 animals in each group were recorded for 1 min. In every experiment, contemporaneous vehicle controls were included. The group size was n = 21 for naïve non-treated (NT), n = 64 for Veh, n = 19 for hydroxyurea , n = 34 for olinciguat and n = 33 for the olinciguat / hydroxyurea combination. These experiments were initially designed as a screening study and, therefore, a priori power analysis was not performed.

In the Berkeley study (Figures 3 and 4), previous work had indicated that data are less variable and the dynamic window is greater, in untreated control mice than in mice challenged with TNFα. Sample sizes were based on previously reported data (Chen et al., 2016) and a priori power analysis. It is important to note that survival in naïve non-TNFα-challenged (NT) Berkeley SCD mice is 100% and that increasing sample size in that group would not affect the analysis; furthermore, NT mice were not included in the statistical analysis for the data described in Figure 4.

The Townes study (Figures 5–10) was a chronic 2-month study in three groups: non-disease HbAA control group (n = 10), sickle cell

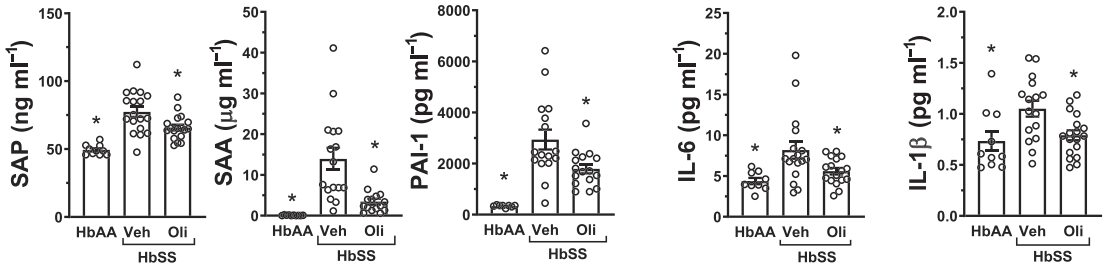

FIGURE 5 Effect of olinciguat on biomarkers of SCD inflammation. Townes SCD mice (HbSS) were treated with either vehicle (Veh) or olinciguat (Oli) for 8 weeks. Untreated Townes control mice (HbAA) were studied as non-disease control. Plasma concentrations of SAP, SAA, PAI-1, IL-6 and IL-1β were measured after 8 weeks of treatment. Data are presented as mean ± SEM, n = 10, 18 and 18 in HbAA, Veh and Oli, respectively. *P < .05, significantly different from vehicle

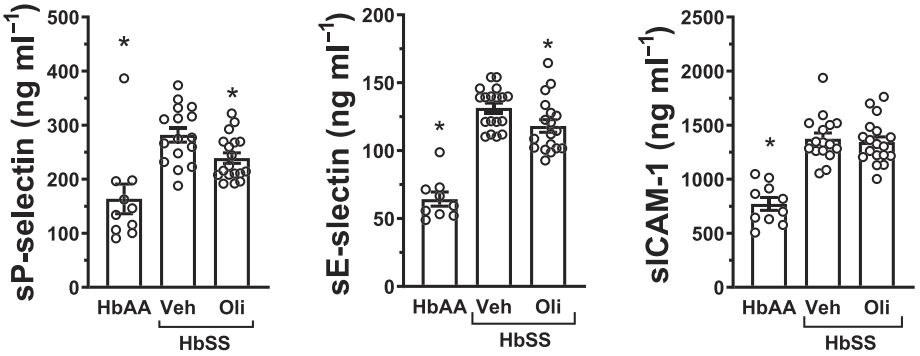

FIGURE 6 Effect of olinciguat on biomarkers of endothelial cell activation in sickle cell mice. Townes SCD mice (HbSS) were treated with either vehicle (Veh) or olinciguat (Oli) for 8 weeks. Untreated Townes control mice (HbAA) were studied as non-disease control. Plasma concentrations of sP-selectin (left), sE-selectin (middle) and sICAM-1 (right) were measured after 8 weeks of treatment. Data are presented as mean ± SEM, n = 10, 18 and 18 in HbAA, Veh and Oli, respectively. *P < .05, significantly different from vehicle

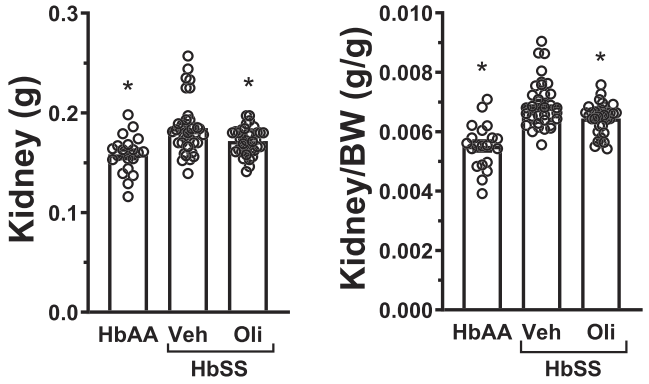

FIGURE 7 Effect of olinciguat on kidney weight. SCD mice (HbSS) were treated with either vehicle (Veh) or olinciguat (Oli) for 8 weeks. Untreated Townes control (HbAA) were studied as non-disease control. Kidneys were collected and weighed at the end of the study after 8 weeks of treatment. Data represent combined weights of left and right kidneys before (left) and after (right) normalisation to body weights. Data are presented as mean ± SEM, n = 10, 18 and 18 in HbAA, Veh and Oli, respectively. *P < 0.05, significantly different from vehicle

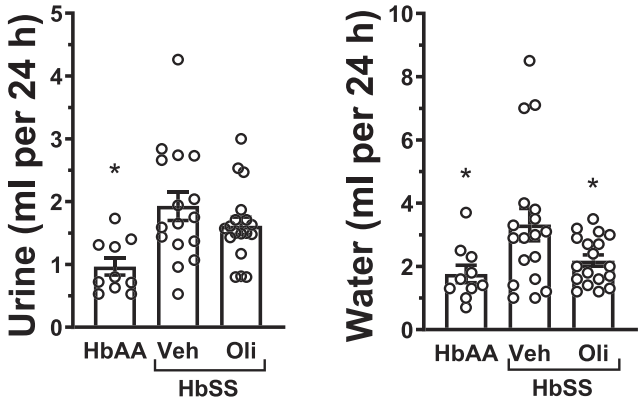

FIGURE 8 Effect of olinciguat on urine excretion (left) and water intake (right). Twenty-four-hour urine output and water consumption in Townes control (HbAA) and Townes SCD (HbSS) mice treated with either vehicle (Veh) or olinciguat (Oli) were determined using metabolic cages in the eighth week of treatment. Data are presented as mean ± SEM, n = 10, 18 and 18 in HbAA, Veh and Oli, respectively. *P < 0.05, significantly different from vehicle

HbSS group treated with vehicle (n = 18) and SCD HbSS mice treated with olinciguat (n = 18) in which multiple biomarkers were analysed. These biomarkers differ from each other in respect to their variability as well as their dynamic window. In addition, in preliminary experiments aimed at characterising the model, the variability for these biomarkers appeared smaller in healthy HbAA mice than in sickle cell HbSS mice. Therefore, we studied fewer HBAA mice than HBSS mice. A priori power analyses indicated 10–20 HbSS mice, depending on the biomarkers assessed, provided sufficient statistical power to query differences between groups for those biomarkers that have either a narrow dynamic window, a high variability or both.

2.7 | Materials

Recombinant mouse TNFα was obtained from R&D Systems, Inc. (Minneapolis, MN). Olinciguat was supplied by Cyclerion Therapeutics (Cambridge, MA). Chloralose, hydroxyurea and urethane were obtained from Sigma-Aldrich (St Louis, MO) and other anaesthetics were supplied as follows: fentanyl (Fentadon) from Dechra Veterinary Products Deutschland GmbH (Aulendorf, Germany); midazolam (Midazolam-hameln) from Hameln pharma plus GmbH (Hameln, Germany); medetomidine (Dormitor) from Vetoquinol GmbH (Ismaning, Germany).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to Pharmacology (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to Pharmacology 2019/20 (Alexander Fabbro et al., 2019; Alexander, Kelly et al., 2019).

3 | RESULTS

3.1 | Effect of olinciguat and hydroxyurea on TNFα-induced endothelial cell activation in C57BL/6 mice

The endothelial adhesion receptors E-selectin, P-selectin and ICAM-1 play a central role in the vascular complications associated with SCD. Upregulation of these receptors on the surface of activated endothelial cells enables interaction of sickled RBCs, leukocytes and platelets with the vascular wall that in turn precipitates painful VOCs, organ damage and acute chest syndrome episodes in SCD patients.

We evaluated the effect of olinciguat and of hydroxyurea, a standard-of-care treatment for SCD patients, on the expression of sP-selectin, sE-selectin and sICAM-1 induced by treating C57BL/6 mice with mouse TNFα. Plasma levels of these receptors reflect their expression by the endothelium and were thus used as biomarkers of endothelial cell activation (Frijns et al., 1997) Plasma levels of sP-selectin, sE-selectin and sICAM-1 increased from baseline upon a challenge of C57BL/6 mice with TNFα, suggesting activation of endothelial cells (Figure 1). Compared with vehicle-treated mice, pretreatment of mice with either olinciguat, hydroxyurea or their combination attenuated the TNFα-induced increase in plasma levels of sP-selectin, sE-selectin and sICAM-1 (Figure 1). Plasma levels of sE-selectin were lower upon co-treatment with olinciguat plus hydroxyurea than when treated with olinciguat or hydroxyurea alone (Figure 1).

To evaluate the effect of olinciguat on leukocyte-endothelial cell interactions, mediated in part by endothelial selectins and ICAM-1, IVM of the cremaster microcirculation was performed. Compared to naive controls, leukocyte rolling velocity and flux were decreased and leukocyte adhesion efficiency increased in C57BL/6 mice challenged with TNFα, suggesting that TNFα increased the expression of endothelial adhesion receptors (Figure 2). Leukocyte rolling velocities were higher in mice subjected to a TNFα-challenge and pretreated with either olinciguat or hydroxyurea than in those treated with vehicle. Furthermore, leukocyte rolling velocities were even higher in mice treated with the olinciguat plus hydroxyurea combination than in mice treated with olinciguat or hydroxyurea alone (Figure 2). Leukocyte rolling flux was greater in mice treated with olinciguat or hydroxyurea than in vehicle-treated mice and even greater in mice treated with the olinciguat plus hydroxyurea combination than in mice that received olinciguat or hydroxyurea alone (Figure 2). Leukocyte adhesion efficiencies in postcapillary venules of mouse cremaster muscles were lower in mice pretreated with the olinciguat plus hydroxyurea combination than in vehicle-treated mice. The adhesion

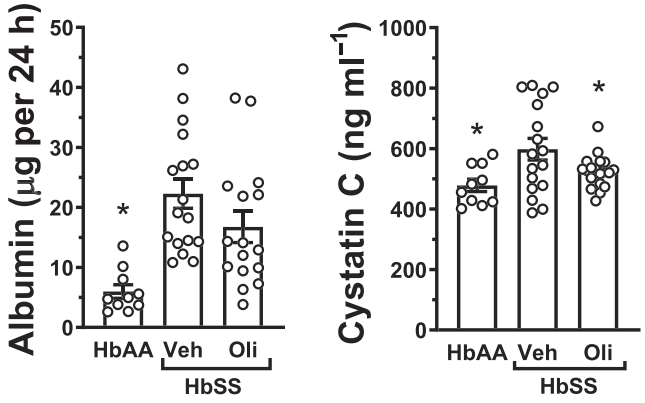

FIGURE 9 Effect of olinciguat on biomarkers of glomerular injury. Townes SCD (HbSS) mice were treated with either vehicle (Veh) or olinciguat (Oli) for 8 weeks. Untreated Townes control (HbAA) mice were studied as non-disease control. Twenty-four-hour urinary albumin excretion (left) and plasma levels of cystatin C (right) were analysed in the eighth week of treatment. Data are presented as mean ± SEM, n = 10, 18 and 18 in HbAA, Veh and Oli, respectively. *P < .05, significantly different from vehicle

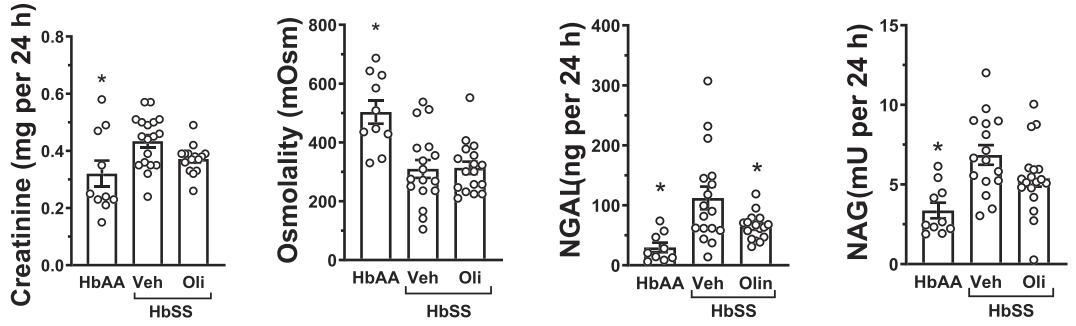

FIGURE 10 Effect of olinciguat on creatinine excretion, osmolality and biomarkers of tubular injury in Townes SCD (HbSS) mice treated with either vehicle (Veh) or olinciguat (Oli). Untreated Townes control (HbAA) mice were studied as non-disease control. Twenty-four-hour urinary creatinine excretion, osmolality, NGAL and NAG were analysed in the eighth week of treatment. Data are presented as mean ± SEM, n = 10, 18 and 18 in HbAA, Veh and Oli, respectively. *P < .05, significantly different from vehicle

efficiencies were also lower in mice treated with olinciguat plus hydroxyurea than in mice treated with olinciguat alone (Figure 2). Taken together, these results indicate that olinciguat can attenuate the TNFα-induced endothelial cell activation and can be additive to hydroxyurea to decrease adhesion of leukocytes to the vascular wall.

3.2 | Effect of olinciguat and hydroxyurea on TNFα-induced VOCs in Berkeley SCD mice

To evaluate the effect of olinciguat on experimental VOCs, chimeric Berkeley SCD mice were challenged with mouse TNFα. The leukocyte rolling flux was lower in Berkeley SCD mice challenged with TNFα and treated with vehicle than in non-challenged control Berkeley SCD mice (Figure 3). In TNFα-challenged Berkeley SCD mice pretreated with either olinciguat, hydroxyurea or the combination of olinciguat plus hydroxyurea, leukocyte rolling flux was higher than was measured in vehicle-treated TNFα-challenged Berkeley SCD mice, suggesting that both olinciguat and hydroxyurea can attenuate the TNFα-induced expression of endothelial selectins (Figure 3). Challenging Berkeley SCD mice with TNFα resulted in significantly greater leukocyte adhesion efficiency than in non-TNFα-challenged Berkeley SCD mice. This effect was attenuated by pretreatment of TNFα-challenged Berkeley SCD mice with the olinciguat plus hydroxyurea combination (Figure 3). Leukocyte adhesion efficiency to vascular wall of postcapillary venules was also lower in TNFα-challenged Berkeley SCD mice treated with the olinciguat plus hydroxyurea combination than in mice treated with olinciguat alone (Figure 3). Blood flow was greater in TNFα-challenged Berkeley SCD mice treated with either olinciguat or the olinciguat plus hydroxyurea combination than in TNFα-challenged Berkeley SCD mice treated with vehicle (Figure 3).

Previously, it was shown that an acute TNFα-induced VOC results in mortality of Berkeley SCD mice (Chang et al., 2010; Jasuja et al., 2014). A similar effect was observed in our study; however, survival was prolonged with olinciguat treatment or the combination of olinciguat plus hydroxyurea (Figure 4). Together, these data demonstrate that treatment with olinciguat can attenuate the vaso-occlusion associated with SCD and that it protects against crises that are mediated by sickled RBCs, leukocytes and activated endothelial cells in a mouse model of SCD.

3.3 | Olinciguat attenuates chronic SCD inflammation

SCD is characterised by anaemia which leads to chronic intravascular inflammation due to haemolysis, hypoxia and recurrent reperfusion injury (Conran & Belcher, 2018; Kato et al., 2017) Therefore, the therapeutic effect of olinciguat on biomarkers of inflammation in 18-week-old Townes mice with established SCD was explored. Plasma concentrations of inflammatory biomarkers including SAP, SAA, PAI-1, IL-6 and IL-1β were significantly greater in vehicle-treated Townes SCD mice (HbSS) than in Townes non-disease control (HbAA) mice (Figure 5). Plasma levels of these biomarkers were lower in HbSS mice treated for 8 weeks with olinciguat than in vehicle-treated HbSS mice (Figure 5). Similar to biomarkers of inflammation, levels of endothelial cell activation biomarkers including sP-selectin, sE-selectin and sICAM-1 were higher in vehicle-treated HbSS mice than in HbAA control mice (Figure 6). At 8 weeks, levels of plasma sE-selectin and sP-selectin, but not sICAM-1, were lower in HbSS mice treated with olinciguat than in vehicle-treated HbSS mice (Figure 6). Thus, treatment of Townes SCD mice with olinciguat attenuated biomarkers of chronic SCD inflammation and reduced expression of endothelial adhesion receptors that promote SCD-associated vaso-occlusion and organ damage.

3.4 | Effect of olinciguat on SCD nephropathy

Nephropathy associated with SCD is characterised by renal hypertrophy, hyperfiltration and glomerulosclerosis (Nath & Hebbel, 2015) Therefore, the effect of olinciguat treatment on renal mass, water consumption and 24-h urine output in Townes SCD mice was evaluated. In 18-week-old SCD mice, kidney weights were greater in vehicle-treated HbSS than in HbAA control mice (Figure 7), indicating renal hypertrophy in the vehicle-treated mice. The observed increase in renal mass was less pronounced in HbSS mice treated for 8 weeks with olinciguat (Figure 7). Compared to non-disease control HbAA mice, vehicle-treated HbSS mice developed polyurea concomitant with increased water consumption (Figure 8). By Week 8 of treatment, water consumption levels were lower in olinciguat-treated HbSS mice than in those treated with vehicle (Figure 8).

To evaluate the effect of olinciguat on glomerular injury, urinary protein albumin excretion and plasma levels of cystatin C were measured at the end of the 8-week treatment period. Urinary excretion of albumin was higher in HbSS mice than in HbAA mice. Albuminuria trended lower in olinciguat than in vehicle-treated SCD mice, but differences were not statistically significant (Figure 9). Post-treatment plasma levels of cystatin C were lower in olinciguat-treated HbSS mice than in vehicle-treated HbSS mice, suggesting that olinciguat can attenuate impairment of glomerular filtration in a mouse model of SCD (Figure 9).

In addition to glomerulopathy, SCD patients with renal disease develop extensive tubular injury (Ataga & Orringer, 2000) characterised early in life by an inability to concentrate urine (hyposthenuria) and elevated total creatinine excretion (Ataga & Orringer, 2000; Sharpe & Thein, 2011). In addition, biomarker levels of distal (NGAL) and proximal (NAG) tubule injury are increased in the urine of patients with SCD nephropathy compared with healthy subjects. (Sundaram et al., 2011) Because tubular abnormalities associated with SCD are reproduced in SCD mouse models (Kasztan et al., 2017; Ryan et al., 1997), biomarkers of tubular dysfunction and injury in Townes SCD mice were analysed. In control SCD mice, urinary creatinine excretion was greater, and the urine osmolality was lower in vehicle-treated HbSS mice than in HbAA control mice (Figure 10). Olinciguat treatment had no effect on SCD hyposthenuria

compared with controls. However, urinary creatinine excretion tended to be lower in HbSS mice receiving olinciguat than in HbSS mice treated with vehicle (Figure 10). Next, the effect of olinciguat treatment on urinary biomarkers of tubular injury was evaluated. Consistent with published data (Kasztan et al., 2017), urinary excretion of NGAL and NAG were greater in vehicle-treated HbSS mice than in HbAA control mice (Figure 10). Treatment of HbSS mice with olinciguat attenuated the increase in urinary excretion of NGAL and tended to attenuate the increase in urinary excretion of NAG (Figure 10). Together, these data suggest that treatment with olinciguat had reno-protective effects and attenuated SCD-associated kidney injury in Townes SCD mice.

4 | DISCUSSION

In patients with SCD, inflammation associated with haemolysis promotes vaso-occlusion and organ damage. Impaired signalling through the NO-sGC-cGMP pathway is an important mechanism contributing to the pathophysiology of SCD. sGC is expressed in a broad range of cells and tissues, and the product of sGC activity, cGMP, is a key intracellular signalling molecule that plays a role in regulating important physiological processes including smooth muscle relaxation and inflammation. Previous work in mouse models of SCD identified beneficial effects of cGMP-amplifying agents, including NO donors and PDE9 inhibitors (Almeida et al., 2012; Jasuja et al., 2014, 2016). The action of NO donors is independent of endogenous NO production, which is local and transient in nature. PDE inhibitors are not specific to NO-derived cGMP, and they inhibit degradation of cGMP arising from both sGC and particulate guanylate cyclases. In contrast, sGC stimulators are low MW compounds that target the NO-sGC-cGMP pathway specifically and synergistically enhance NO-driven cGMP signalling with endogenous NO. sGC stimulators, as a class, have shown benefit both in the clinic (Armstrong et al., 2020; Ghofrani et al., 2013) and across a broad range of preclinical disease models (Buys et al., 2018; Stasch & Hobbs, 2009). Olinciguat is a novel sGC stimulator currently in Phase II clinical development in patients with SCD (Zimmer et al., 2020).

Here we report that olinciguat attenuated transient and firm adhesion of leukocytes to activated endothelium in C57BL/6 mice with TNFα-induced inflammation. Furthermore, co-treatment with hydroxyurea, the current standard of care in children and adults with SCD, augmented the effect of olinciguat on leukocyte endothelial cell interactions. The observed attenuation of endothelial cell activation by olinciguat supports functional data and implies that olinciguat reduces expression of endothelial adhesion receptors that mediate leukocyte rolling and firm adhesion to the vascular wall. The effect of olinciguat on plasma levels of sE-selectin was augmented by co-administration of hydroxyurea. Similarly, co-administration of hydroxyurea was recently reported to potentiate the beneficial effects of other sGC agonists in a TNFα-induced mouse model of vaso-occlusion (Ferreira et al., 2020).

Expression of adhesion receptors by activated endothelium is a critical step in the initiation of a VOC, which is a major morbidity among SCD patients (Manwani & Frenette, 2013). Therefore, olinciguat and olinciguat plus hydroxyurea in combination were tested in the model of inflammation-induced VOC. To address our hypothesis, we studied previously characterised chimeric SCD mice generated by engrafting C57BL/6 mice with the bone marrow from Berkeley SCD mice (Turhan et al., 2002). Challenging these mice with TNFα, when combined with a surgical insult for IVM preparation, triggers VOC (Chang et al., 2010; Hidalgo et al., 2009; Turhan et al., 2002). Similar to C57BL/6 mice, treatment of SCD mice with olinciguat alone or in combination with hydroxyurea before TNFα challenge attenuated leukocyte endothelial cell adhesion. The additive effect with hydroxyurea was observed only on adhesion efficiency. Olinciguat—but not hydroxyurea —also increased blood flow and doubled survival time of chimeric SCD mice challenged with TNFα. The TNFα challenge in Berkeley SCD mice resulted in 100% mortality at 4 h, reflecting the severity of this model. Similar to hydroxyurea, the PDE9 inhibitor BAY73-6691 failed to increase survival time in this model (Almeida et al., 2012). It remains to be determined why the beneficial effect of these NO-cGMP modulators on leukocyte-endothelial cell interactions and blood flow did not translate to an increase in survival time as it did for the sGC stimulator olinciguat. Due to the acute nature of the TNFα experiments, we did not assess the effects of hydroxyurea and/or sGC stimulators on foetal haemoglobin.

The effect of cGMP-amplifying agents on leukocyte endothelial cell interactions in TNFα-challenged C57BL/6 and chimeric SCD mice has been studied previously. In one study, inhibition of cGMP-specific PDE9 with PF-04447943 alone had no significant effect on leukocyte adhesion and rolling (Jasuja et al., 2014). In contrast, combined treatment with PF-04447943 plus hydroxyurea prior to TNFα-challenge decreased leukocyte adhesion and increased neutrophil rolling flux and velocity in C57BL/6 mice. Furthermore, plasma levels of soluble adhesion receptors including sP- and sE-selectin and sICAM-1 were also reduced by co-administration of PF-04447943 and HU. In another study, treatment of chimeric SCD mice with the PDE9 inhibitor BAY73-6691 decreased leukocyte adhesion with no effect on leukocyte rolling flux and mortality. Co-administration of hydroxyurea with BAY73-6691 in SCD mice significantly decreased expression of endothelial selectins and ICAM-1, increased leukocyte rolling flux and velocity, reduced leukocyte adhesion and improved survival (Almeida et al., 2012). The same study also demonstrated that hydroxyurea exerts its beneficial synergistic effect via NO production and activation of cGMP synthesis by sGC.

Although expression of PDE9 is mostly restricted to brain and haematopoietic cells, sGC is expressed in many organs including lungs, heart, kidneys and liver. Preclinical studies demonstrate that sGC stimulation of cGMP synthesis has a beneficial effect and protects these organs against damage (Buys et al., 2018; Evgenov et al., 2006). Aside from acute painful crisis, SCD is characterised by chronic organ injury caused by recurrent vaso-occlusive episodes that promote inflammation, reperfusion injury and, ultimately, organ

damage. The kidney is one of the most affected organs in SCD patients, and renal dysfunction is a risk factor for mortality in SCD patients (Saborio & Scheinman, 1999; Yeruva et al., 2016). Both decreased GFR and increased albuminuria have been described in SCD patients (Guasch et al., 1996). Studies in different models of kidney injury have shown that stimulation of sGC offers reno-protective effects (Krishnan et al., 2018; Shea et al., 2020; Stasch et al., 2015; Zimmer et al., 2020). Here we studied Townes SCD mice to evaluate the effect of chronic administration of olinciguat on SCD inflammation and nephropathy. Post-treatment plasma levels of biomarkers of inflammation and endothelial cell activation were higher in Townes SCD mice than in non-disease control mice but were lower in olinciguat-treated mice than in vehicle-treated SCD mice. Plasma levels of sP-selectin and sE-selectin were also lower in Townes SCD mice treated with olinciguat. These data suggest that chronic treatment with olinciguat attenuates inflammation and endothelial cell activation associated with SCD. Consistent with previously published data (Ryan et al., 1997), Townes SCD mice developed nephropathy characterised by renal hypertrophy, increased urine output, and glomerular and tubular injury. Olinciguat attenuated the increase in renal mass and decreased water consumption. Biomarkers of glomerular injury, including albuminuria, trended lower in SCD mice treated with olinciguat than in vehicle-treated Townes SCD mice. Plasma levels of cystatin C, a biomarker of renal function, were also lower in olinciguat than in vehicle-treated Townes SCD mice. Although we did not measure these biomarkers over time and the exact mechanisms underlying the protective effects observed remain to be elucidated, together these data suggest that, consistent with reno-protective effects demonstrated by the sGC stimulator **praliciguat** in diabetic kidney disease (Hanrahan et al., 2020), olinciguat may be able to attenuate renal injury associated with SCD.

In addition to glomerular injury, patients with SCD-associated nephropathy often develop tubular dysfunction. This pathology is often characterised by hyposthenuria, a low urine osmolality caused by impaired water reabsorption in the renal tubules. Tubular creatinine secretion is also increased in patients with SCD nephropathy (Ataga & Orringer, 2000). In Townes SCD mice, baseline urine osmolality was lower and urinary creatinine excretion and urinary biomarkers of tubular injury were higher than in non-disease control mice. Although treatment of Townes SCD mice with olinciguat had no effect on hyposthenuria, urinary creatinine excretion tended to be lower in Townes SCD mice treated with olinciguat than in those treated with vehicle. Urinary biomarkers of tubular injury, including NGAL were also lower in olinciguat-treated mice. The lack of the effect of olinciguat on hyposthenuria is perhaps not surprising. Clinical data indicate that reversible SCD hyposthenuria develops early in childhood and progresses with age, becoming irreversible in adulthood (Sharpe & Thein, 2011). In this regard, it is possible that in the 18-week-old SCD mice we studied, the defect in tubular water reabsorption was already irreversible. Interestingly, as observed in our study, pharmacological inhibition of the endothelin receptor in Townes SCD mice also reduced urinary biomarkers of tubular injury without improving hyposthenuria (Kasztan et al., 2017). Finally, interventions that lower systemic blood pressure have been reported to attenuate nephropathy in SCD patients (Quinn et al., 2017; Thrower et al., 2019). Importantly, sGC stimulators such as olinciguat can lower blood pressure, which may represent another pharmacological mechanism, in addition to effects on inflammation and endothelial cell activation, by which olinciguat could provide reno-protective benefits. Although we did not directly measure systemic blood pressure, animals appeared healthy and there was no evidence of lethargy, decreasing body weights or other signs of severe hypotension. We cannot, however, exclude the possibility that the haemodynamic effects of stimulating sGC may have affected autoregulation of renal blood flow, renal biomarkers of inflammation or renal function in the mouse models of SCD we studied.

In conclusion, our findings in two mouse models of SCD provide preclinical evidence that stimulation of sGC with olinciguat attenuated sickle cell inflammation, endothelial cell activation and VOCs. In addition, our data indicate that olinciguat had a reno-protective effect and attenuates SCD-associated nephropathy in mouse SCD models. Thus, olinciguat may have the potential to alleviate acute and chronic complications in patients with SCD. More specifically, olinciguat may have the potential to protect these patients from sickle cell-related nephropathy, a common and burdensome complication. More broadly, sGC stimulators have shown promising reno-protective effects, both preclinically and in patients with diabetic kidney disease (Hanrahan et al., 2020). Further preclinical and clinical studies are needed to better understand the effect of olinciguat on inflammation and organ protection in SCD and beyond.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: BT, JEJ, SF, RF, PSF, JLM; Performed the experiments and contributed to acquisition of data: BT, HL, S-KL, XG, RR, GL, KCH, SGB, SF; Supervised procedures: JEJ, RF, PSF, JLM; Contributed to data analysis: BT, JEJ, SF, RF, ESB, RMG, PSF, JLM; Drafted the manuscript: BT, JEJ, SF, RF. ESB, RMG, PSF, JLM; Participated in critically revising the draft of the manuscript: BT, HL, S-KL, XG, RR, GL, KCH, SGB, JEJ, SF, RF. ESB, RMG, PSF, JLM.

CONFLICT OF INTEREST

BT, RR, GL, KH, SGB, JEJ, ESB, RMG and JLM are current or former employees of and may own stock/stock options in Cyclerion Therapeutics.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Robert Feil https://orcid.org/0000-0002-7335-4841
Emmanuel S. Buys https://orcid.org/0000-0002-1763-9359

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