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COMBINED HYDROXYUREA AND ET_A RECEPTOR BLOCKADE REDUCES RENAL INJURY IN THE HUMANIZED SICKLE CELL MOUSE

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Abstract

Aim: The objective of this study is to determine if ambrisentan (ET_A selective antagonist) and hydroxyurea (HU) treatment has a synergistic effect on renal injury in SCN when compared to HU treatment alone. The premise of the study is based on recent findings show that endothelin-1 (ET-1) contributes to the pathophysiology of nephropathy in sickle cell disease (SCD) and that ET_A receptor blockade improves renal function and protects against renal injury. Hydroxyurea (HU) is commonly prescribed for the treatment of SCD and has been shown to reduce renal injury in patients with SCD.

Methods: Male 12-week-old humanized sickle mice (HbSS) and their genetic controls (HbAA) were treated with vehicle, HU, ambrisentan, or HU with ambrisentan for 2 weeks and renal structure and function were assessed.

Results: Vehicle treated HbSS mice exhibited significant proteinuria compared to vehicle treated HbAA mice. HbSS mice also displayed significantly elevated plasma ET-1 concentrations and decreased urine osmolality compared to HbAA controls. Proteinuria was significantly lower in both HU and ambrisentan treated animals compared to vehicle treated HbSS mice; however, there was no additional improvement in HbSS mice treated with combined ambrisentan and HU. The combination of HU and ambrisentan resulted in significantly lower KIM-1 excretion, glomerular injury, and interstitial inflammation than HU alone.

Conclusion: These findings indicate that HU and ET_A receptor blockade produce similar reductions in renal injury in the humanized sickle mouse suggesting that both treatments may converge on the same mechanistic pathway.

Keywords

sickle cell disease; sickle nephropathy; endothelin; chronic kidney disease

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CONFLICT OF INTEREST

The authors have nothing to disclose.

INTRODUCTION

Sickle cell disease (SCD) is a prevalent monogenic hemoglobin disorder that affects millions of people across the globe.¹ The autosomal recessive disorder is characterized by a point mutation in the β -globin gene wherein a valine is substituted for glutamic acid, resulting in polymerization of mutant hemoglobin S (HbS) under hypoxic conditions.² Polymerization causes circulating erythrocytes to assume an abnormal “sickle” shape, which initiates a series of pathophysiological events leading to hemolytic anemia, vaso-occlusive crises, and inflammation.^{3,4} As advancements in therapeutic strategies have improved longevity in patients with SCD, sickle cell nephropathy (SCN) has been recognized as a significant contributor of mortality and morbidity in these patients.^{5,6} Of the approximate 70% of SCD patients that experience renal dysfunction indicated by overt proteinuria, an estimated 20% will develop end stage renal disease and require renal transplantation or long-term dialysis.^{7,8}

Chronic erythrocyte sickling and hemolysis that results in local ischemia, progressive glomerulopathy, and hematuria characterize the underlying renal pathology in SCD.^{9,10} Clinical manifestations of renal dysfunction in SCD can be observed in patients as early as infancy and includes defects in urine-concentrating ability, gross proteinuria, and hyperfiltration.³ As the disease progresses, these pathologies are concomitant with a gradual decline in glomerular filtration rate, augmented albuminuria, and distal nephron dysfunction.¹¹ Currently, therapies that offer substantial renal protection for SCD patients are limited and some, such as hematopoietic stem cell transplantation, have limited applicability and substantial risk that can be potentially life threatening.

Known primarily for its potent, vasoconstrictor properties, endothelin-1 (ET-1) is an endothelial-derived signaling peptide whose paracrine and autocrine functions are mediated by two G-protein coupled receptors: endothelin receptor type A (ET_A) and endothelin receptor type B (ET_B).¹² Renal endothelin system activation is augmented in SCD patients and recent studies suggest that ET-1 contributes to the development and progression of SCN via enhanced ET_A activation.^{13–15} One potential mechanism of glomerular protection appears to be related to reduced production of reactive oxygen species.¹³ While ET_B receptor inhibition exacerbates ET-1-induced inflammation and apoptosis, selective blockade of the ET_A receptor reduces reactive oxygen species generation, reduces renal injury, and improves overall renal function in SCN.^{13,14,16} Furthermore, blocking ET_A receptor activity long-term protects against the development of ET-1-induced renal glomerular injury and reduces inflammation in SCN; however, all aspects of tubular injury are not restored.¹⁴ In addition, despite its efficacy in SCN, selective ET_A receptor blockade has also been demonstrated to promote fluid retention and vascular permeability via enhanced ET_B activation.¹⁷

One of only two currently FDA approved therapies, hydroxyurea is a myelosuppressive agent commonly prescribed for the treatment of SCD.¹⁸ Hydroxyurea stimulates fetal hemoglobin production, thereby reducing erythrocyte sickling and subsequent vaso-occlusive events.^{18,19} In addition to fetal hemoglobin induction, hydroxyurea promotes red blood cell hydration and reduces leukocyte and reticulocyte production via inhibition of

ribonucleotide reductase, an enzyme that promotes DNA synthesis.^{18,20} The clinical efficacy of hydroxyurea in SCD patients has been demonstrated through primary and secondary stroke prevention, reduced pulmonary hypertension, and decreased mortality and morbidity.^{21–23} In addition, hydroxyurea treatment improves renal function and reduces albuminuria, serum creatinine, and reticulocyte production in SCN.^{24,25} Notwithstanding its clinical efficacy in SCN, hydroxyurea treatment can exhibit adverse side effects, such as marrow suppression and neutropenia, which makes it unsuitable for many patients.^{26,27}

The purpose of the current study was to compare selective ET_A receptor blockade and hydroxyurea in a model of SCN and determine if combined therapy would provide any advantage over either drug alone. To investigate this theory, we used the humanized sickle mouse that spontaneously develops progressive renal injury and proteinuria over time. As hydroxyurea is a known chemotherapy agent and has been shown to induce chromosome fragility through destabilization of replication forks, we treated experimental animals for two weeks to minimize potential long-term genomic effects.²⁸

RESULTS

Nearly all experiments and data analysis were performed in both HbAA and HbSS mice for all treatment groups. Since there were no significant effects of treatments in HbAA mice, we have moved these data to the supplemental file and data are presented here from only HbSS mice to highlight the effects of each individual treatment. Physical characteristics of the experimental mice were assessed after two weeks of treatment with vehicle, hydroxyurea, the selective ET_A antagonist ambrisentan, or combined hydroxyurea with ambrisentan. Drug treatment had no significant effects on kidney-to-body- mass ratio, water consumption, or systolic blood pressure in HbSS mice or their genetic controls (Table 1, Supplemental Table1). In addition, all HbSS mice had similar splenomegaly that was significantly greater than similarly treated HbAA mice (Table 1). To assess the effect of drug treatment on systemic endothelin system activation, we measured plasma ET-1 in all groups. Plasma ET-1 was significantly elevated in vehicle treated HbSS mice compared to vehicle treated HbAA controls (Figure 1A). This elevation was attenuated by hydroxyurea and ambrisentan treatment alone but remained significantly elevated in HbSS mice treated with both drugs.

To determine the effects of short-term hydroxyurea treatment and ET_A receptor blockade on glomerular dysfunction in SCD, we measured common indices of glomerular injury after two weeks of drug treatment in HbSS and HbAA mice. Vehicle treated HbSS mice exhibited significant glomerular injury, as indicated by elevated proteinuria, albuminuria, and glomerular sclerosis (Figure 1, Figure 2). Proteinuria, albuminuria, and glomerular sclerosis were all significantly reduced by drug treatment in HbSS mice compared to vehicle treated sickle mice (Figure 1B,1C, Figure 2).

Ischemic insults are particularly deleterious on the proximal tubule because of its substantial metabolic demand compared to other nephron segments. Normally minimally expressed in adult uninjured kidneys, kidney injury molecule 1 (KIM-1) is a transmembrane protein located on proximal tubule epithelia that aid in the phagocytosis of cellular debris and dead cells in the post-ischemic kidney.²⁹ Thus, to evaluate the effect of hydroxyurea and selective

ET_A antagonist treatment on proximal tubule injury in the HbSS model, we assessed KIM-1 excretion in vehicle and drug-treated HbSS and HbAA mice. KIM-1 excretion was significantly elevated in vehicle treated HbSS mice compared to vehicle treated HbAA mice. Hydroxyurea and ambrisentan treatment both attenuated KIM-1 excretion compared to vehicle treated HbSS mice, but these effects were not statistically significant. However, the reduction of KIM-1 excretion with combined hydroxyurea and ambrisentan treatment was statistically significant compared to vehicle treated HbSS mice (Figure 3A). We also assessed urine concentrating ability by measuring urine osmolality in HbSS and HbAA mice. While vehicle treated HbSS mice exhibited significant defects in urine concentrating ability compared to vehicle treated HbAA mice, this effect was not restored by any short-term drug treatment (Figure 3B). Hydroxyurea treatment, however, reduced urine volume compared to vehicle treated sickle mice (Figure 3C). These data suggest that combined hydroxyurea with a selective ET_A antagonist may be more beneficial for reducing tubular injury compared to either treatment alone. As renal iron accumulation is common and may contribute to tubular injury in SCD patients,^{30–32} we examined tubule iron deposition in treated mice. While there was significant iron deposition in vehicle treated HbSS mice compared to HbAA mice, none of the treatments attenuated this phenotype (Figure 4).

Augmented renal oxidative stress and inflammation has been shown to be principal mediators in the pathology of sickle cell nephropathy.^{33,34} Thus, to better elucidate the potential therapeutic mechanisms of action for hydroxyurea, ambrisentan, and combined treatment, we assessed urinary thiobarbituric acid reactive substances (TBARS) excretion, a marker of oxidative stress, and immune cell infiltration. Renal oxidative stress, indicated by urinary TBARS excretion, was significantly elevated in vehicle treated HbSS mice compared to HbAA mice (77.5± 16.5 nmol/day vs. 11.7±3.53 nmol/day, p=0.02). This was associated with a decline in renal function indicated by elevated blood urea nitrogen (Figure 5B). However, only selective ET_A antagonist treatment reduced oxidative stress in HbSS mice (Figure 5A). This was also accompanied by reduced renal cortical inflammation, indicated by reduced T-cell and macrophage infiltration (Figure 6 and Figure 7). Neither hydroxyurea nor combined treatment significantly reduced renal oxidative stress (Figure 5A). However, cortical inflammation was reduced by both hydroxyurea and combined treatments in HbSS mice, though only combined treatment provided a significantly greater reduction compared to hydroxyurea treatment alone (Figure 6 and Figure 7).

DISCUSSION

The selective ET_A antagonist, ambrisentan, and the myelosuppressive agent, hydroxyurea, have both been demonstrated to improve renal function and attenuate renal injury in SCD.^{14,24,35} However, neither treatment alone provides complete restoration of the renal injury phenotype seen in SCN and furthermore, both treatments are sometimes accompanied by adverse side effects.^{17–19,26} Many patients, however, cannot tolerate therapeutic doses of hydroxyurea or do not respond to treatment at all, leaving them limited treatment options for disease management.¹⁸ As many patients either do not take or do not respond to hydroxyurea treatment, selective ET_A antagonist ambrisentan might provide an effective alternative or combined therapy. In addition, until recently hydroxyurea was the only FDA approved therapy for the management of SCD and a majority of patients received treatment

at some point. Endari, an L-glutamine oral powder, was approved in 2017 in the United States FDA for patients with SCD and is believed to work, at least in part, by reducing oxidative stress.³⁶

The current study sought to determine if combined hydroxyurea therapy with selective ET_A antagonist treatment provided more robust renal protection than hydroxyurea alone. These data provide evidence that combined hydroxyurea with ambrisentan treatment does indeed reduce renal injury in SCD. Previous studies have shown elevated plasma ET-1 concentrations and urinary ET-1 excretion in patients with SCD.^{37,38} Similar increases in systemic and renal endothelin system activation have been demonstrated to contribute to the development and progression of SCN in the humanized sickle mouse model.³⁵ The physiological and pathophysiological effects of ET-1 are mediated through its two G-protein coupled receptors: ET_A and ET_B.³⁹ However, blocking only the ET_A receptor reduces renal injury in SCN.³⁵ Selective inhibition of ET_A receptor activity reduces glomerular reactive oxygen species generation, glomerular sclerosis, proteinuria, albuminuria and tubular injury in SCN.^{13,14} When given individually, hydroxyurea and ambrisentan reduced circulating plasma ET-1 levels, but the mechanism for this effect is unclear. Importantly, all treatments reduced glomerular injury, indicated by attenuated proteinuria, albuminuria, and glomerular sclerosis. The absence of plasma ET-1 reduction with combined treatment suggests a potential drug interaction between hydroxyurea and ambrisentan. Previous studies have shown that ET_B receptors, particularly those on endothelial cells, serve as a major contributor to the clearance of plasma ET-1.^{40,41} As combined treatment may affect ET_B receptor expression on the vascular endothelium, this could potentially be the mechanism for the blunted plasma ET-1 response seen in these mice. More studies are needed, however, to more thoroughly elucidate the mechanism behind this response. In addition, only combined hydroxyurea and selective ET_A antagonist treatment reduced proximal tubular injury indicated by a reduction in KIM-1 excretion in HbSS mice, although no significant improvements in urine-concentrating deficiency or tubular iron deposition were made. While previous studies from our lab have shown KIM-1 excretion significantly reduced by ambrisentan treatment in HbSS mice, discrepancies in our results could be attributed to variation in renal injury phenotype in the sickle colony and the differences in the number of experimental animals.¹⁴ These data suggest that combined hydroxyurea and ambrisentan treatment maybe a preferred therapy for a reduction in renal tubular injury in SCD patients.

Hydroxyurea is an effective commonly prescribed therapy for the treatment of SCD. Prolonged treatment with hydroxyurea decreases albuminuria and improves renal function in SCD patients.^{24,42} Its clinical effects have often been ascribed to its ability to stimulate fetal hemoglobin production and, interestingly, fetal hemoglobin induction has been demonstrated as necessary prerequisite to a reduction in end-organ damage in murine models of SCD.⁴³ However, clinically sufficient fetal hemoglobin induction by hydroxyurea typically takes approximately at minimum 3–6 months to occur, which suggests that our results may be fetal hemoglobin independent.^{44,45} In addition, studies have demonstrated a lower incidence of vaso-occlusive crises with hydroxyurea treatment in as short as 3 months of treatment at low to moderate doses.⁴⁶ These findings, too, were fetal hemoglobin independent. Regardless, there still remains a possibility that our hydroxyurea treated mice experienced some augmented fetal hemoglobin expression after only two weeks of treatment. There

also remains the possibility that selective ET_A antagonist treatment can potentially induce fetal hemoglobin expression. Fetal hemoglobin measurements were not performed in this cohort; so further experiments are required to completely determine any potential role of fetal hemoglobin in our results.

In our study, two weeks of hydroxyurea treatment reduced glomerular injury indicated by a reduction in proteinuria and albuminuria in HbSS mice. In addition, combined hydroxyurea and ambrisentan treatment reduced KIM-1 excretion, though urine osmolality was not significantly increased. Combined treatment also reduced immune cell infiltration in HbSS mice, although only ambrisentan treatment alone, however, reduced oxidative stress. To date, this is the only study demonstrating a reduction of renal injury in the Townes mouse model after two weeks of treatment with hydroxyurea. Certainly, studies have linked the fetal hemoglobin-induction capability of hydroxyurea with clinical efficacy; however, hydroxyurea has many pleiotropic effects that are fetal hemoglobin-independent and can be potential mechanisms-of-action for our results. Previous studies have demonstrated clinical improvements in hydroxyurea treated patients well before detectable levels of fetal hemoglobin had occurred.^{44,45} Short-term hydroxyurea treatment can also alter erythrocyte-endothelial cell interactions and reduce adhesion in SCD patients.⁴⁷ Hydroxyurea has also been shown to increase mean corpuscular volume independent of fetal hemoglobin induction in patients after only 8 weeks of treatment.⁴⁸ Changes in rheologic properties from hydroxyurea treatment can improve erythrocyte mobility, function, and longevity.⁴⁵ Lastly, oxidation of hydroxyurea by circulating heme groups can release the potent vasodilator, nitric oxide (NO).⁴⁹ NO production can stimulate local vasodilation, reduce endothelial cell adhesion, and reduce leukocyte adhesion. While these are all plausible mechanisms, more studies are needed to elucidate the mechanism of short-term hydroxyurea treatment on renal injury in SCN, as well as determine any potential adverse side effects.

In conclusion, our data provide evidence that combined hydroxyurea and ambrisentan treatment is beneficial for the treatment of SCN. While no overt synergistic improvement was seen with combined hydroxyurea and ambrisentan treatment on glomerular injury, tubular injury proved more promising as only combined treatment showed significant improvement. These results provide a basis for combined hydroxyurea and ambrisentan therapy as a more effective therapy for SCN than hydroxyurea alone.

METHODS

Animals

All experimental mice were maintained and studied in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental protocols and animal care were reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committees. For our studies, we used the humanized sickle cell mouse with knock-in human hemoglobin genes in place of the endogenous murine hemoglobin genes with notation: B6; 129-*Hba*^{tm1(HBA)Tow} *Hbb*^{tm2(HBG1,HBB*)Tow} / *Hbb*^{tm3(HBG1,HBB)Tow} / J, developed by Townes *et. al.*⁵¹ Mice homozygous at the β -globin locus for sickle hemoglobin or wild type hemoglobin were defined as humanized sickle mice (HbSS) and genetic control mice (HbAA), respectively.

Control animals were from the same colony. All experiments used 14wk old male mice for both HbSS and control groups. Experimental groups varied in number based on animal availability. Experimental animals were exposed to a 12hr light/dark cycle and were housed under conditions of constant temperature and humidity. All mice were provided unrestricted access to mouse chow (Teklad diets, Envigo, Huntingdon, UK) and water. Standard metabolic cages were used for all experimental groups. The 24-hour urine sample collections were performed for 2 days after animals were allowed to adapt to standard metabolic cages for 24 hours. Daily urinary excretion rate was calculated as an average of two 24-hour collections.

Drug Treatment Protocols

Hydroxyurea (50mg/kg/day; Sigma-Aldrich Lot #MKBR1926V; St. Louis, MO), selective ET_A receptor antagonist ambrisentan (10mg/kg/day; AbbVie, Inc. Lot #9309-BH-40R, Abbott Park, IL), combined hydroxyurea and ambrisentan, or vehicle was administered in the drinking water and the concentrations were adjusted daily based on water intake, as previously described.⁵² Drug treatments were administered for 14 consecutive days beginning from 12 weeks of age after which time the efficacy of treatment on nephropathy was assessed.

Blood Pressure Measurements

Tail-cuff blood pressure measurements were taken using a pneumatic transducer (BP-2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC). After 5 consecutive days of prior training, blood pressure measurements were taken for 5 consecutive days from 13 week old HbSS and their genetic HbAA controls, as previously described.⁵³ Data are presented as the average of 2–5 days.

Plasma and Urine Analyses

Plasma ET-1 concentrations were measured using ELISA according to manufacturer's instructions (QuantiGlo ET-1 Kit, R&D Systems, Minneapolis, MN). 24-hour urine collection samples were used for all urine analyses. Total urinary protein excretion was measured using the Bradford assay (BioRad Laboratories, Hercules, CA). Urinary levels of tubular injury marker KIM-1 were measured using a mouse KIM-1 ELISA kit (ab119596-KIM-1 [TIM-1] ELISA kit; Abcam, Cambridge, UK). Urinary albumin concentration was measured using an immunoperoxidase assay according to the manufacturer's instructions (Genway Biotech Inc., San Diego, CA). Urinary TBARS excretion was measured using a TBARS assay kit (TBARS Assay kit, Cayman Chemical, Ann Arbor, MI). Plasma urea concentration was measured using a blood urea nitrogen detection kit (Urea Nitrogen (BUN) Colorimetric Detection kit, Invitrogen, Waltham, MA).

Histological Analyses

Kidneys isolated from 14-week old experimental animals were immersed in 10% formalin for 24–48 hrs and embedded in paraffin. Embedded kidneys were sectioned (4- μ m thick) and stained with Gomori trichrome using standard protocols. Tissue sections were evaluated blindly according to the criteria used for quantification of the changes in renal structures, as

previously described.^{52–54} The extent of glomerular sclerosis was assessed on Gomori trichrome–stained sections using a grading score from 0 to 4; where 0 represents no sclerosis in the glomerulus, 1 represents up to 25% sclerosis of the glomerulus, 2 represents up to 50% sclerosis of the glomerulus, 3 represents up to 75% sclerosis of the glomerulus, and 4 represents 100% sclerosis of the glomerulus. Data are presented as an average score of 20–25 glomeruli assessed per stained section.

Immunohistological Analyses

Paraffin-embedded kidney sections were deparaffinized and hydrated to distilled water. Tissue sections were incubated in 1x antigen retrieval buffer (10x Antigen Retrieval Solution, DAKO #S1699). Slides were then placed in a blocking solution (Rodent Block M, Biocare Medical #RBM961H). For T cell detection, the primary antibody used was anti-CD3 (Abcam #ab16669). For macrophage detection, the primary antibody anti-F4/80 was used (MCA497GA, BioRad). Slides were developed in DAB-peroxidase substrate buffer. Slides counterstained with hematoxylin and incubated in Scott's bluing. Samples were then dehydrated and coverslipped. Positive cells were assessed as previously described.¹⁴

Statistical Analyses

All analyses were performed using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA). Two-way ANOVA with Tukey's *post hoc* correction for multiple comparisons was used for analyses. All data are expressed as means $\pm$ SEM with $p < 0.05$ being considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Combined hydroxyurea and selective ET_A antagonist treatment reduces glomerular injury in humanized sickle mice.

(A) Plasma ET-1 concentration ($P_{\text{interaction}}=0.008$) (B) Urinary protein excretion ($P_{\text{interaction}}=\text{NS}$) and (C) Urinary albumin excretion ($P_{\text{interaction}}=0.001$) were assessed in HbSS mice after two weeks of treatment with vehicle, hydroxyurea, hydroxyurea plus ambrisentan, or ambrisentan. Data are presented as mean $\pm$ SEM. * $p<0.05$ vs. vehicle.

Figure 2. Combined hydroxyurea and selective ET_A antagonist treatment attenuates glomerular sclerosis in humanized sickle mice.

(**A**) Quantification of glomerular sclerosis scores of Gomori's trichrome stained sections ($P_{\text{interaction}} = <0.0001$) (**B**). Representative images of glomerular sclerosis in treatment groups. Magnification: 40X. Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. vehicle.

Figure 3. Tubular injury is attenuated with combined hydroxyurea and selective ET_A antagonist treatment in humanized sickle mice.

(A) Urinary KIM-1 excretion ($P_{\text{interaction}}=0.004$) (B) Urine osmolality ($P_{\text{interaction}}=NS$) and (C) Urine excretion ($P_{\text{interaction}}=0.02$) were assessed in HbSS mice after two weeks of treatment. Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. vehicle.

Figure 4. Combined hydroxyurea and selective ET_A antagonist treatment does not reduce renal iron accumulation in humanized sickle mice.

(A) Quantification of tubular iron deposition detected by Prussian Blue staining ($P_{\text{interaction}}=0.02$) (B). Representative images of iron deposition in HbSS treated mice. Magnification: 40X. Data are presented as mean $\pm$ SEM. * $p<0.05$ vs. vehicle.

Figure 5. Renal oxidative stress is not attenuated with combined hydroxyurea and selective ET_A antagonist treatment.

(A) Urinary TBARS excretion and (B). Plasma blood urea nitrogen (BUN) ($P_{\text{interaction}} = \text{NS}$) were assessed in HbSS mice after two weeks of treatment. Magnification: 40X. Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. vehicle, † $p < 0.05$ vs. HU

Figure 6. Renal T cell infiltration is reduced by combined hydroxyurea and selective ET_A antagonist treatment.

(A) Quantification of cortical CD3+ cells in treated HbSS mice ($P_{\text{interaction}} < 0.0001$) (B) Representative images of CD3+ cell infiltration in the cortex (C) Quantification of medullary CD3+ cells in HbSS treated mice ($P_{\text{interaction}} = 0.03$) (D) Representative images of CD3+ cell infiltration in the medulla. Magnification: 40X. Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs. vehicle, † $p < 0.05$ vs. HU.

Figure 7. Renal macrophage infiltration is reduced by combined hydroxyurea and selective ET_A antagonist treatment.

(A) Quantification of cortical F4/80+ cell infiltration ($P_{\text{interaction}}=0.006$) (B) Representative images of F4/80+ cell infiltration in the cortex (C) Quantification of medullary F4/80+ cell infiltration in HbSS treated mice ($P_{\text{interaction}}=0.03$) (D) Representative images of F4/80+ cell infiltration in the medulla. Magnification: 40X. Data are presented as mean $\pm$ SEM. * $p<0.05$ vs. vehicle, † $p<0.05$ vs. HU, ‡ $p<0.05$ vs. HU+Amb.

TABLE 1.

Physical characteristics after 2 weeks of treatment in humanized sickle mice (HbSS)

	Vehicle	Hydroxyurea	Ambrisentan	Hydroxyurea+Ambrisentan
N	5–15	5–11	6	6–13
Body Weight, g	26.5±0.7	27.2±0.7	24.7±0.5	27.2±0.6
Right kidney/ body weight, mg/g	6.0±0.2	6.1±0.3	6.2±0.1	5.8±0.2
Left kidney/ body weight, mg/g	6.1±0.2	6.5±0.3	6.2±0.4	5.8±0.2
Systolic BP, mmHg	119±7	121±3	117±3	111±3
Water Intake, ml/day	5.5±0.5	5.7±0.5	5.0±0.4	5.0±0.4
Spleen-to-body weight ratio (%)	*6.29±0.40	*5.16±0.29	*5.351±0.29	*5.61±0.47

HU, hydroxyurea; Amb, ambrisentan; BP, blood pressure. $P_{\text{interaction}} = \text{NS}$ for all parameters.

*
p<0.05 vs HbAA vehicle;

p<0.05 vs HbSS vehicle.

Data are presented as mean ± SEM. Data for HbAA mice are shown in Supplemental Table 1.