Hydroxyurea with AKT2 inhibition decreases vaso-occlusive events in sickle cell disease mice.

Running title: Combination therapy of HU and an AKT2 inhibitor

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ABSTRACT

Heterotypic cell-cell adhesion and aggregation mediate vaso-occlusive events in patients with sickle cell disease (SCD). Although hydroxyurea (HU), an inducer of fetal hemoglobin, is the main therapy for treatment of SCD, it is unclear whether it has immediate benefits in acute vaso-occlusive events in SCD patients. Using real-time fluorescence intravital microscopy, we demonstrated that short-term co-administration of HU and Akti XII, an AKT2 inhibitor, efficiently reduced neutrophil adhesion and platelet-neutrophil aggregation in venules of TNF-α- or hypoxia/reoxygenation-challenged Berkeley (SCD) mice. Importantly, compared with HU or Akti XII treatment alone, short-term treatment with both agents significantly improved survival in those mice. We found that the level of plasma nitric oxide species was elevated by HU but not Akti XII, AKT2 phosphorylation levels in activated neutrophils and platelets were reduced by Akti XII but not HU, and the expression of endothelial E-selectin and intercellular adhesion molecule 1 was decreased by either agent. Our results suggest that short-term co-administration of HU and Akti XII has immediate benefits for acute vaso-occlusive events and survival in SCD mice exceeding those seen for single therapy.

Key point: Co-administration of hydroxyurea and an AKT2 inhibitor has beneficial effects on acute vaso-occlusive events and survival in SCD mice.

INTRODUCTION

Sickle cell disease (SCD), an inherited blood disorder, results from a homozygous mutation at the 6th position (Glu6Val) of the β-globin chain (hemoglobin S, HbS) or from compound heterozygous forms like HbSC and HbS-β-thalassemia.[1] HbS tends to polymerize in the deoxygenated state, and this leads to the sickling and hemolysis of red blood cells.[2,3] Importantly, decreased nitric oxide (NO) bioavailability and increased oxidative stress contribute to the pathophysiology of SCD, including activation of endothelial cells (ECs), inflammation, and organ damage.[4] Recurrent vaso-occlusive events, the hallmark of SCD, are mediated by adhesion and aggregation of red blood cells, leukocytes, and platelets on activated ECs[5,6] and cause pain crises in SCD patients.[7] Hydroxyurea (HU), the only FDA-approved drug for treatment of SCD, stimulates HbF production,[8] serves as an NO donor,[9,10] and inhibits tissue factor expression in leukocytes.[11] Although the mechanism by which HU acts is still not fully understood, previous studies showed that treatment of SCD patients with HU is associated with the production of NO and increases HbF levels in a NO-dependent manner.[9,10] Consistently, studies using a humanized SCD (Berkeley) mouse model revealed that short-term treatment with HU can have immediate benefits on vaso-occlusive events, independently of HbF production.[12] The authors further demonstrated that combination therapy of HU and a phosphodiesterase 9 inhibitor efficiently inhibit acute vaso-occlusion in SCD mice.[12]

Intravital microscopic studies showed that neutrophil-platelet interactions on activated ECs are the major determinant of vascular occlusion during thromboinflammatory disease in which inflammation is coupled to thrombosis.[5,13,14] Although the mechanisms mediating neutrophil-platelet interactions remain poorly understood, previous studies showed that platelet and neutrophil AKT2 play critical roles in regulating platelet P-selectin exposure and activation of β2 integrins,[5,15] thereby mediating neutrophil-EC and neutrophil-platelet interactions under inflammatory conditions. Importantly, we found that basal levels of AKT phosphorylation are significantly increased in neutrophils and platelets isolated from SCD patients compared with healthy donors and that short-term treatment with Akti XII, an AKT2 specific inhibitor, reduces neutrophil adhesion and platelet-neutrophil aggregation in venules of Berkeley mice, resulting in improved blood flow rates.[5] Despite high homology (80%) in protein sequences of the three isoforms, our recent studies clearly indicated that AKT2 could be a dominant isoform regulating heterotypic cell-cell interactions and microvascular occlusion under inflammatory conditions.

In the present study, we investigated whether short-term co-administration of HU and Akti XII has beneficial effects on acute vaso-occlusive events and survival in Berkeley mice challenged with TNF-α or hypoxia/reoxygenation.

MATERIALS AND METHODS

For a full description of all methods, see Supplemental Methods.

Mice. WT (C57BL/6, 6 weeks old), hemizygous (Tg(Hu-miniLCRα1 $^G\gamma^A\gamma\delta\beta^S$) $Hba^{-/-}$ $Hbb^{+/-}$), and Berkeley sickle (Tg(Hu-miniLCRα1 $^G\gamma^A\gamma\delta\beta^S$) $Hba^{-/-}$ $Hbb^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Berkeley mice were generated by transplantation of bone marrow cells isolated from Berkeley mice into lethally irradiated WT mice as described previously.[16] The University of Illinois Institutional Animal Care and Use Committee approved all animal care and experimental procedures.

Intravital microscopy and survival times. Berkeley mice were injected via a tail vein with saline or HU, 100 µg/g body weight (BW) and subsequently treated with intraperitoneal injection of TNF-α (500 ng), 3 hours prior to imaging. In other experiments, Berkeley mice were placed into an 8% O_2 chamber for 3 hours to induce hypoxia, followed by 3 hours of reoxygenation in room air. The mice were treated with vehicle or HU (100 µg/g BW) at the beginning of reoxygenation via tail vein injection. Akti XII was administered through a jugular vein right before infusion of Dylight 488-conjugated anti-CD42c (platelet glycoprotein Ibβ) and Alexa Fluor 647-conjugated anti-Gr-1 (Ly-6G/Ly-6C) antibodies. Images were recorded and analyzed as described in Supplemental Methods. During or after intravital microscopic studies, survival times for each mouse were measured. Each time point began at TNF-α injection (Figure 1A) or reoxygenation (Figure S1A) and ended when the mouse died or at 6 hours after TNF-α injection or reoxygenation.

Statistics. Data were analyzed using the GraphPad Prism 5 software by ANOVA with Dunnett's test, Student's *t*-test, and Mantle-Cox log-rank test. A P value less than 0.05 was considered significant.

RESULTS

Co-administration of HU and Akti XII efficiently reduces neutrophil adhesion and platelet-neutrophil interactions in venules and prolongs survival times in TNF-α-challenged Berkeley mice

It was reported that treatment with HU at 100 µg/g BW partially inhibits neutrophil adhesion to the venules of TNF-α-challenged Berkeley mice.[12] We recently demonstrated that platelets and neutrophils isolated from mice treated with 10 µg/g BW of Akti XII showed reduced phosphorylation of AKT2, but not AKT1 and AKT3, *ex vivo* and that 10 µg/g BW of Akti XII markedly decreased neutrophil adhesion and platelet-neutrophil aggregation in TNF-α-challenged Berkeley mice.[5] Using a lower dose of Akti XII (3 µg/g BW), we performed intravital microscopy to determine the combined effect of co-administration of HU and Akti XII on vaso-occlusive events in Berkeley mice. The mice were treated with iv injection of a single dose of HU (100 µg/g BW) prior to ip injection of TNF-α (Figure 1A). Surgical procedures were carried out at 2.5 hours after TNFα challenge, followed by infusion of Akti XII. We observed that treatment with Akti XII or HU alone significantly reduced the number of adherent neutrophils with minimal effect on the rolling influx (Figure 1B-D, Videos 1-4). Compared to HU or Akti XII alone,

6

combination treatment significantly increased the number of rolling neutrophils and further decreased neutrophil adhesion to ECs. As determined by the fluorescence intensities of anti-CD42c antibodies, HU- or Akti XII-treated Berkeley mice exhibited no effect on platelet-neutrophil interactions (Figure 1E). In contrast, the mice treated with both agents showed a near complete inhibition of platelet-neutrophil interactions. These results suggest that co-administration of HU and Akti XII efficiently inhibits vaso-occlusive events in TNF-α-challenged Berkeley mice.

Intraperitoneal injection of TNF-α into Berkeley mice and subsequent surgical trauma lead to death within several hours of TNF-α injection as a result of acute vaso-occlusive events.[12,16] We found that compared to the vehicle control, treatment with HU alone offered no increased survival, whereas treatment with Akti XII (3 µg/g BW) improved survival in the Berkeley mice (Figure 1F). Fifty percent of mice died at 3.6, 3.9, 4.8, and 5.2 hours after TNF-α injection in vehicle-, HU-, Akti XII-, and both HU- and Akti XII-treated mice, respectively (P = 0.0012 between HU and HU + Akti XII and P = 0.031 between Akti XII (3 µg/g BW) and HU + Akti XII). Most mice treated with 10 µg/g BW of Akti XII survived 6 hours after TNF-α injection, supporting our recent findings that AKT2 could be a novel target for treatment of thromboinflammatory disease.

Combination therapy efficiently inhibits neutrophil transmigration into the lung alveoli in TNF-α-challenged Berkeley mice

It was reported that increased vascular permeability may cause acute inflammation in the lung of Berkeley mice.[17] Thus, we performed histochemistry to measure neutrophil transmigration into the alveoli. The number of transmigrated neutrophils was decreased

in HU- or Akti XII-treated Berkeley mice, compared with the vehicle control (Figure 1G-H). Co-administration of HU and Akti XII further diminished neutrophil transmigration, suggesting that combination therapy has beneficial effects on pulmonary inflammation in TNF-α-challenged Berkeley mice, which may explain the improved survival after short-term treatment.

Co-administration of HU and Akti XII has beneficial effects on heterotypic cell-cell interactions and markedly improves survival in hypoxia/reoxygenation-challenged Berkeley mice

Previous studies showed that platelet-neutrophil aggregation is induced in blood isolated from Berkeley mice challenged with hypoxia (8% oxygen for 3 hours) and subsequent reoxygenation (room air for another 3 hours).[18] Using this hypoxia/reoxygenation model, the same intravital microscopy was performed. Compared with TNF-α-challenge, hypoxia/reoxygenation challenge showed increased rolling and decreased adhesion of neutrophils in venules of Berkeley mice (Figure S1B-D). Platelets adhered to neutrophils as a single cell and did not form thrombi (Figure S1E). The vehicle-treated mice died within 4.2 hours after reoxygenation (1.2 hours after surgery, Figure S1F). However, very few neutrophils transmigrated into the lung alveoli of hypoxia/reoxygenation-challenged Berkeley mice (data not shown). Compared with HU or Akti XII alone, co-administration of HU and Akti XII showed a significant increase in the rolling influx (Figure S1C). Strikingly, all mice treated with both agents survived until the end of the experiment (6 hours after reoxygenation, Figure S1F). Although the mechanisms mediating hypoxia/reoxygenation-induced death of Berkeley

8

mice may be different from those in TNF-α-challenged mice, our results suggest that co-administration of HU and Akti XII has beneficial effects on vaso-occlusive events and survival in hypoxia/reoxygenation-challenged Berkeley mice.

The expression of E-selectin and ICAM-1 is affected by treatment with HU or Akti XII

EC E-selectin and ICAM-1 are critical for neutrophil rolling and adhesion, respectively, during vascular inflammation.[19] To determine the effect of HU and/or Akti XII treatment on the expression of those proteins, we performed immunohistochemistry using cremaster muscles taken from the mice after intravital microscopy. The expression of E-selectin and ICAM-1 was significantly decreased by HU or Akti XII treatment, compared to the vehicle control (Figure 2A-D). E-selectin expression was further reduced in the mice treated with both HU and Akti XII, whereas no further reduction of ICAM-1 expression was observed. Since co-administration of HU and Akti XII is more beneficial than single treatment in inhibiting cell-cell interactions and survival in TNF-α-challenged Berkeley mice, these results suggest that the function of blood cells, such as neutrophils and platelets, is also impaired by both agents.

The level of plasma NO metabolites is affected by short-term treatment with HU

Previous studies showed that the effect of oral administration of HU on the level of plasma NO metabolites could be different dependent on SCD patients.[10] Thus, we further measured the level of plasma nitrites and nitrates (NOx) in Berkeley mice after intravital microscopy. Blood was drawn immediately after the mice died or at the end of

the experiment. We found that plasma NOx levels were increased by 2.4- or 2.1-fold in mice treated with HU alone or both HU and Akti XII, respectively, compared to the vehicle control (Figure 2E). There was no difference between HU and double-treated groups. Treatment with Akti XII did not influence plasma NOx levels. Similar results were also obtained in hypoxia/reoxygenation-challenged Berkeley mice (Figure S1G) in which the levels of circulating NOx were relatively higher than those in TNF-α-challenged Berkeley mice. In controls, treatment of WT (C57BL/6) and $Hbb^{+/-}$ (hemizygous) mice with HU alone significantly increased plasma NOx levels (Figure S2), suggesting that iv injection of HU (100 µg/g BW) serves as a NO donor in mice.

The level of cellular NO inhibits ICAM-1 expression in activated ECs

Because most actions of NO are intracellular and NO donors are known to inhibit ICAM-1 expression in activated ECs,[20] we determined whether sodium nitroprusside (SNP), a well-known NO donor,[21] affects ICAM-1 expression in TNF-α-activated ECs. Due to technical difficulties in isolating mouse vascular ECs, human umbilical vein ECs (HUVECs) were used in this study. Immunoblotting analysis showed that SNP treatment during TNF-α stimulation dose-dependently decreased ICAM-1 expression in HUVECs (Figure S3A-B). Complete inhibition was observed when HUVECs were stimulated with TNF-α in the presence of 10 µM SNP. These results support our speculation that NO produced by short-term treatment with HU may inhibit ICAM-1 expression in TNF-α-activated cremaster muscle ECs.

Treatment with Akti XII or both HU and Akti XII reduces the phosphorylation levels of neutrophil and platelet AKT2 *ex vivo*

We reported that AKT2 phosphorylation is significantly reduced in platelets and neutrophils isolated from WT mice treated with 10 µg/g BW of Akti XII.[5] To further examine whether HU and a low concentration of Akti XII (3 µg/g BW) affect AKT2 phosphorylation in neutrophils and platelets *ex vivo*, Berkeley mice were treated with HU and Akti XII at 3 and 0.5 hours, respectively, prior to cell isolation. We found that the phosphorylation level of AKT2, but not AKT1, was significantly decreased in fMLF-stimulated neutrophils (Figure 2F-G) and thrombin-activated platelets (Figure 2H-I) isolated from the mice that received Akti XII alone or both HU and Akti XII, compared to the cells isolated from vehicle- or HU-treated mice. There was no difference between Akti XII and double-treated groups.

Using platelets and neutrophils isolated from Berkeley mice, we further examined whether SNP affects AKT2 phosphorylation during cell activation. We observed that SNP treatment at a concentration of 10-100 µM showed different effects on AKT2 phosphorylation in platelets and neutrophils; inhibition of AKT2 phosphorylation in thrombin-activated platelets, but enhancement of AKT2 phosphorylation in fMLF-stimulated neutrophils (Figure S3C-F). These results imply that dependent on the cellular level, NO may differentially regulate AKT2 phosphorylation in activated platelets and neutrophils. We found that treatment with 100 µM SNP significantly increased the cellular NOx levels in isolated platelets or neutrophils (Figure S3G-H). Pretreatment of platelets with 10-100 µM SNP, compared to vehicle or 1 µM SNP, elevated cellular NOx levels in a concentration-dependent manner following thrombin stimulation, whereas

only the highest concentration (100 μM) of SNP increased the NOx levels in fMLF-stimulated neutrophils. Interestingly, platelet and neutrophil activation affected cellular NOx levels differently after pretreatment with 100 μM SNP; platelet activation significantly increased NOx levels, but neutrophil activation markedly decreased it. This may result from a large amount of reactive oxygen species produced from activated neutrophils which can disrupt NO homeostasis.[22]

DISCUSSION

Previous studies suggested that increased oxidative stress consumes NO, decreasing NO levels in Berkeley mice and that decreased NO bioavailability aggravates oxidative stress in SCD patients.[23,24] It was reported that HU-induced HbF production is mediated by activation of soluble guanylyl cyclase in a NO-dependent manner.[9] Consistently, transgenic Berkeley mice expressing increased levels of HbF exhibited a significant increase in the level of NO metabolites.[23] Recent studies showed that the beneficial effects of iv injection of HU (100 μg/g BW) in TNF-α-challenged Berkeley mice was abolished when co-administered with a NO scavenger,[12] suggesting that short-term treatment with HU serves as a NO donor. However, it is unclear whether such a high dose of HU can be used for iv injection in SCD patients because of the potential toxicity and whether short-term treatment of SCD patients with HU increases circulating NO levels. In the present study, we found that compared to the vehicle control, HU treatment significantly increased plasma NOx levels in Berkeley mice, which is likely to reduce oxidative stress. Further, our *ex vivo* immunofluorescence microscopy

showed that NO produced by HU treatment might inhibit ICAM-1 expression in TNF-α-activated cremaster ECs, which was supported by our *in vitro* studies using SNP-treated HUVECs. Although HU treatment did not affect AKT2 phosphorylation in activated platelets and neutrophils *ex vivo*, our *in vitro* studies using SNP implied that NO might differentially regulate AKT2 phosphorylation in platelets and neutrophils. Future studies are required in SCD mice and patients to determine how much NO is produced by varying doses and administration times of HU and whether clinically approved NO donors have beneficial effects on acute vaso-occlusive events. Further, it should be examined how long-term treatment with HU affects plasma/cellular NO levels which may differentially regulate the function of intravascular cells dependent on the cellular amount.

Studies with AKT2-null mice revealed that neutrophil AKT2 induces generation of reactive oxygen species by activating NADPH oxidase 2 and mediates heterotypic cell-cell interactions under thromboinflammatory conditions and that platelet AKT2 is important for granular secretion and platelet aggregation.[5,13,15,25] Therefore, inhibition of AKT2 in intravascular cells would be expected to attenuate oxidative stress and impair neutrophil adhesion and platelet-neutrophil interactions in vessels, thereby enhancing the beneficial effect of HU in Berkeley mice. Mechanistically, our results suggest that administration of HU and Akti XII each inhibits inflammatory conditions: short-term treatment with HU (100 µg/g BW) significantly increases plasma NO levels, short-term treatment with Akti XII (3 µg/g BW) decreases AKT2 phosphorylation in neutrophils and platelets without affecting plasma NO levels, and administration of either agent reduces surface expression of ICAM-1 and E-selectin on activated ECs. Thus, it is thought that

the combined effects of both agents could result in immediate benefits – further inhibition of cell-cell interactions and significant improvement of survival in TNF-α- or hypoxia/reoxygenation-challenged Berkeley mice.

In addition to HU, numerous inhibitors have been tested in SCD mice and patients to reduce vaso-occlusive events. Some novel agents that are currently in clinical trials induce HbF production (Decitabine and HQK-1001),[26,27] inhibit the activation and adhesive function of neutrophils (GMI-1070)[28] and platelets (Prasugrel)[29], impair blood coagulation (Tinzaparin),[30] and decrease oxidative stress (Omega-3 fatty acids and N-acetyl cysteine).[31,32] In particular, the randomized phase II study with GMI-1070 (Rivipansel), a pan selectin inhibitor, has shown that when given to SCD patients early in the hospitalization for treatment of vaso-occlusive events, it reduces requirement for parenteral opioid analgesia and displays a trend to decrease time to resolution as indicated by readiness for discharge from hospital.[28] Previous studies demonstrated that inhibition or deletion of AKT2 significantly decrease neutrophil and platelet activation, thereby reducing neutrophil-EC and platelet-neutrophil interactions and improving blood flow rates during vascular inflammation.[5,15] Furthermore, the present study reveals that co-administration of HU and Akti XII has beneficial effects on acute vaso-occlusive events and survival in TNF-α- or hypoxia/reoxygenation-challenged Berkeley mice. Because of the enrichment in insulin-responsive tissues, AKT2 is required to maintain normal glucose homeostasis.[33] We found that one infusion of Akti XII at 10-30 µg/g BW into mice inhibited phosphorylation of AKT2, but not AKT1 and AKT3, in activated platelets and neutrophils[5] and does not affect glucose tolerance (Figure S4). Although future studies are necessary to assess the potential toxicity of targeting AKT2, our

14

results provide important evidence that an AKT2 specific inhibitor may be a short-term supplemental therapy for immediate benefits on acute vaso-occlusive events and survival in SCD patients.

AUTHORSHIP

Contribution: A.B. designed and performed research, collected and analyzed data, and wrote the manuscript; J.L., K.K., and N.S. performed research; and J.C. initiated and designed research, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1. Co-administration of HU and Akti XII efficiently inhibits neutrophil adhesion and platelet-neutrophil aggregation in venules, improves survival, and impairs neutrophil transmigration into alveoli in TNF-α-challenged Berkeley mice. TNF-α was intraperitoneally injected into Berkeley mice to induce severe inflammatory conditions. Intravital microscopy was performed as described in Methods. Neutrophils and platelets were labeled by infusion of Alexa Fluor 647-conjugated anti-Gr-1 and Dylight 488-conjugated anti-CD42c antibodies. (A) Timeline for the treatment and surgery (jugular cannulation and cremaster muscle exposure) in Berkeley mice. (B) Representative images of intravital captures at various time points. The time "0" was set as the image capture was initiated at each vessel. (C-D) Number of rolling and adherent neutrophils. (E) The integrated median fluorescence intensities of anti-CD42c antibodies (F platelets) were normalized to the number of adherent neutrophils and plotted as a function of time. Data were obtained from 45-57 venules in 6-8 mice per group. (F) Survival curves of Berkeley mice during or after intravital microscopy. (G-H) Representative images from histochemistry of lung sections (n = 35-43 vessels from 4-5 mice per group). The number of transmigrated neutrophils (arrow heads) was quantified

16

in the field of view (110 mm^2). Bar = 20 µm. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus vehicle control, ANOVA and Dunnett's test. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ between two groups, Student's *t*-test. The survival rate was assessed with Mantel-Cox log-rank test.

Figure 2. The effects of HU and Akti XII on the expression of E-selectin and ICAM-1, plasma NOx levels, and AKT2 phosphorylation in TNF-α-challenged Berkeley mice. (A-D) Following intravital microscopy, the cremaster muscle was excised, fixed, and embedded in paraffin for immunohistochemistry. Sections of the muscle were labeled with rat anti-mouse E-selectin or ICAM-1 and then Dylight 488-labeled anti-rat IgG antibodies, followed by incubation with PE-labeled anti-PECAM-1 antibodies and a mounting reagent containing DAPI. (A and C) Representative images of E-selectin or ICAM-1 and PECAM-1 staining. (B and D) The geometric mean fluorescence intensities (MFI) of E-selectin or ICAM-1 expression in venules of Berkeley mice. Data represent the mean ± SD (n = 45-57 venules in 6-8 mice per group). (E) Following intravital microscopy, plasma NOx levels were measured as described in Methods. Data represent the mean ± SD (n = 6-8 mice per group). (F-I) Berkeley mice were treated with saline, HU, Akti XII, or both agents as described in Methods. Neutrophils and platelets were isolated and stimulated with fMLF (N-formyl-methionyl-leucyl-phenylalanine) and thrombin, respectively. Immunoblotting was performed using equal amounts of protein (50 µg), followed by densitometry using Image J. Representative blots (F and H) and quantitation of AKT2 phosphorylation after normalization to total AKT expression in neutrophils (G) and platelets (I). Data represent the mean ± SEM (n

= 6 mice per group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus vehicle control, ANOVA and Dunnett's test. #$P < 0.05$ and ##$P < 0.01$ between two groups, Student's *t*-test.

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