Cyp7b1-inhibiting azoles enhance hematopoietic stem and progenitor cell mobilization in normal and sickle cell disease mice

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Abstract

Mobilized hematopoietic stem and progenitor cells (HSPC) are essential for transplantation-based therapies, including curative gene therapies for sickle cell disease (SCD). While granulocyte colony-stimulating factor (G-CSF, filgrastim) remains the standard mobilization agent, many patients respond inadequately, and it can trigger life-threatening vaso-occlusive crises in SCD. The CXCR4 antagonist AMD3100 (plerixafor) is routinely combined with G-CSF for non-SCD settings but is ineffective as a single agent in SCD, underscoring the urgent need for alternative strategies. We previously identified 27-hydroxycholesterol (27HC) as a physiological inducer of HSPC mobilization during pregnancy. Here, we show that exogenous 27HC enhances AMD3100-induced HSPC mobilization in mice, either alone or with G-CSF. Because 27HC is metabolized by the enzyme Cyp7b1, we tested whether pharmacological Cyp7b1 inhibition could mimic this effect. Treatment with clotrimazole, an antifungal and Cyp7b1 inhibitor, significantly enhanced AMD3100-induced HSPC mobilization in wild-type, SCD, and humanized mice. Importantly, intravenous administration of voriconazole, a clinically approved systemic antifungal with Cyp7b1-binding activity, similarly augmented AMD3100-induced HSPC mobilization in wild-type and SCD mice without altering steady-state hematopoiesis. These findings establish Cyp7b1-inhibiting azoles as novel and clinically relevant enhancers of HSPC mobilization, particularly for SCD patients who cannot safely receive G-CSF but require robust HSPC yields for gene therapy.

Introduction

Adult hematopoietic stem and progenitor cells (HSPC) reside in the bone marrow (BM) and sustain blood production throughout life,[1] and HSPC collection and transplantation are central to therapies for hematologic malignancies and blood disorders.[2] The most common HSPC collection strategy uses granulocyte colony-stimulating factor (G-CSF, filgrastim), which mobilizes HSPC from the BM into the peripheral blood (PB) for collection via apheresis. In donors and patients with poor responses to G-CSF alone, the CXCR4 antagonist AMD3100 (plerixafor) is added to enhance mobilization.[3] Nonetheless, many donors and patients fail to mobilize adequate HSPC with traditional mobilization regimens, leading to multiple apheresis sessions or transplant failure.[4]

Sickle-cell disease (SCD) is an autosomal recessive blood disorder caused by mutations in the β-globin gene (HBB) that produces deoxygenated hemoglobin S (HbS), resulting in sickle-shaped red blood cells (RBC).[5] SCD causes anemia and various types of crises, potentially leading to severe complications and premature death. Allogeneic transplantation of HSPC from a human leukocyte antigen (HLA)-matched donor is curative[6] but is limited by donor availability, graft rejection, and the risk of graft-*versus*-host disease. Autologous transplantation with genetically modified HSPC circumvents these barriers, and several gene therapy strategies, including lentiviral gene delivery and CRISPR genome-editing approaches, have recently received US Food & Drug Administration (FDA) approval as curative treatments for SCD.[7,8] However, these therapies require the collection of large numbers of HSPC from patients. Since G-CSF can trigger fatal vaso-occlusive crises in SCD patients,[9-11] AMD3100 is used as a sole mobilizer

to collect HSPC from SCD patients. Mobilization with AMD3100 alone is often variable and less effective than G-CSF,[12] highlighting the need for alternative or adjunctive HSPC mobilizing strategies.

To better understand how HSPC mobilization is regulated, we previously used pregnancy in mice as a physiologic model and found that HSPC mobilization is naturally induced during pregnancy to replenish RBC through extramedullary hematopoiesis.[13] These responses are dependent on the levels of 27-hydroxycholesterol (27HC), a cholesterol-derived oxysterol and endogenous estrogen receptor (ER) ligand.[14,15] We demonstrated that the treatment of mice with 27HC induces ERα-dependent HSPC mobilization and that mice deficient for Cyp27a1, a sterol hydroxylase necessary to generate 27HC from cholesterol,[16] display impaired HSPC mobilization and splenic erythropoiesis during pregnancy.[17]

In healthy people and autologous transplant patients, higher low-density lipoprotein or total cholesterol levels are associated with increased HSPC mobilization at baseline or in response to G-CSF.[18,19] High-density lipoprotein cholesterol levels are negatively correlated with mobilization of HSPC and monocytes.[20,21] Similarly, in mice, increased cholesterol levels promote HSPC mobilization.[22-24] Given that 27HC levels increase as cholesterol levels increase,[25] these findings suggest that elevated 27HC levels promote HSPC mobilization in mice and humans.

Here, we demonstrate that exogenous 27HC enhances HSPC mobilization in combination with AMD3100 and G-CSF. Given that 27HC is the most abundant oxysterol in human plasma, we used pharmacological approaches to mimic 27HC supplementation. The cytochromes P450 enzyme Cyp7b1 metabolizes 27HC via its oxysterol 7α-hydroxylase activity, and *Cyp7b1*-deficient mice exhibit elevated 27HC levels in tissues and plasma.[26] We show that clotrimazole, a Cyp7b1-inhibiting antifungal,[27] augments AMD3100-induced mobilization in wild-type (WT), SCD, and humanized mice. Importantly, voriconazole, an FDA-approved systemic antifungal that also binds Cyp7b1,[28] produces similar effects without perturbing steady-state hematopoiesis. Our findings identify Cyp7b1-inhibiting azoles as novel, clinically relevant agents to improve HSPC mobilization, particularly for SCD patients who cannot receive G-CSF but require robust HSPC yields for gene therapy.

Methods

Animal studies

C57BL/6J mice (RRID:IMSR_JAX:000664), Townes SCD mice (B6;129-*Hbb*$^{tm2(HBG1,HBB*)Tow}$/ Hbb$^{tm3(HBG1,HBB)Tow}$ Hba$^{tm1(HBA)Tow}$/J; RRID:IMSR_JAX:013071),[29] and NBSGW mice (NOD.Cg-*Kit*$^{W-41J}$ *Tyr*$^{+}$ *Prkdc*^{scid} *Il2rg*^{tm1Wjl}/ThomJ; RRID:IMSR_JAX:026622)[30] were obtained from The Jackson Laboratory. Young adult mice (2-3 months of age) of both sexes were used, and no sex-specific differences were observed. Mice received the following treatments: 27HC (10 mg/kg/day, Avanti Polar Lipids) in 15% 2-hydroxypropyl-β-cyclodextrin (HPβCD; Sigma) by subcutaneous injection; G-CSF (250 μg/kg/day; filgrastim; Sigma) in phosphate buffered saline without calcium or magnesium (PBS; Corning) by subcutaneous injection; AMD3100 (5 mg/kg; plerixafor; Sigma) in PBS by subcutaneous injection; clotrimazole (50 mg/kg/day; Sigma) in corn oil (Sigma) by intraperitoneal injection; or voriconazole (50 mg/kg/day; MedChemExpress) in 30% HPβCD by intravenous injection. Vehicle controls received matched volumes of the corresponding diluent. All mice were housed in American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited, specific-pathogen-free animal facility at UConn Health. All animal procedures were approved by the UConn Health Institutional Animal Care and Use Committee.

Cell and tissue preparation

Peripheral blood was collected from either submandibular vein, retro-orbital sinus, or caudal vena cava. Complete blood count was performed with the Vetscan HM5 Hematology Analyzer (Zoetis). BM cells were isolated by crushing the femurs and tibias with a mortar and pestle in staining medium (PBS without calcium and magnesium, supplemented with 1% heat-inactivated fetal bovine serum [Lonza] and 1mM EDTA [Fisher Scientific]) and filtered through a 70-μm nylon screen (ELKO Filtering). Spleens were dissociated by crushing, followed by gentle trituration and filtering through a 70-μm cell strainer (Fisher Scientific). BM mononuclear cell number and viability were assessed by acridine orange/propidium iodide (AO/PI) staining (AO from Fisher Scientific; PI from Sigma) and counted by the LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems).

Colony formation assay

Peripheral blood mononuclear cells from 10 uL to 100 uL of the blood, depending on the levels of HSPC mobilization, were isolated by Ficoll-Paque Premium density gradient media (1.085 g/mL density for mice and 1.078 g/mL density for humans, Cytiva) according to the manufacturer's instructions. Isolated cells were seeded in a well of a 6-well plate containing Mouse or Human Methylcellulose Complete Media (R&D Systems) supplemented with 10 ng/mL mouse or human thrombopoietin (BioLegend). Colonies were counted after 12 days.

Flow cytometry for mouse tissues

For isolation of immunophenotypic HSC and progenitors, a mixture of antibodies against CD2, CD3, CD5, CD8α, B220, Gr-1, and Ter119 was used to stain lineage markers as previously described.[31] RBC were lysed with ammonium-chloride-potassium lysing buffer. Non-viable cells were excluded during flow cytometry by staining with 4',6-diamid-

ino-2-phenylindole (DAPI, Tocris Bioscience). The antibodies used in this study are listed in *Online Supplementary Table S1*. Data acquisition and cell sorting were performed using a FACSymphony S6 Cell Sorter, FACSymphony A5 SE Cell Analyzer, or LSR II Flow Cytometer (BD Biosciences) and data were analyzed using FlowJo v10 software (BD). The marker combinations used to identify mouse HSPC populations examined in this study are listed in the *Online Supplementary Methods*.

Results

Exogenous 27HC supplementation enhances hematopoietic stem and progenitor cell mobilization

We previously showed that exogenous 27HC supplementation enhances G-CSF-induced HSPC mobilization.[17] To test whether 27HC supplementation also augments AMD3100-induced HSPC mobilization, WT C57BL/6J mice were treated with 27HC (10 mg/kg/day, subcutaneous) for two days, followed by AMD3100 (5 mg/kg, subcutaneous) one hour before blood collection. Mobilization of functional HSPC was quantified by colony-forming assays using PB mononuclear cells. Two measures were used: colony-forming unit-cell (CFU-C), which represents HSPC capable of generating any hematopoietic colony, and CFU-granulocyte, monocyte, erythroblast and megakaryocyte (CFU-gmEM), which represents the most primitive HSPC subset retaining multilineage differentiation potential to form colonies containing these four lineages. Co-administration of 27HC and AMD3100 significantly increased both CFU-C (2.7-fold) and CFU-gmEM (2.6-fold) in the PB compared with AMD3100 alone (Figure 1A, B).

Because AMD3100 is used clinically when patients and donors fail to respond to G-CSF alone, we next tested whether 27HC supplementation further enhances HSPC mobilization in combination with G-CSF and AMD3100. C57BL/6J mice were treated with 27HC and/or G-CSF (250 μg/kg/day, subcutaneous) for four days, followed by AMD3100 one hour before blood collection (Figure 1C). Supplementation of 27HC significantly increased the numbers of mobilized CFU-C (1.7-fold) and CFU-gmEM (2.1-fold) compared with the combination of G-CSF and AMD3100 alone (Figure 1D, E).

To test whether the mobilization-enhancing effect is specific to 27HC or shared with other oxysterols, we treated C57BL/6J mice with 25-hydroxycholesterol (25HC) at the same dose as 27HC. Both 27HC and 25HC can signal through estrogen receptors and liver X receptors (LXRs).[15] However, unlike 27HC, 25HC did not increase CFU-C or CFU-gmEM under either the AMD3100-only or G-CSF plus AMD3100 regimens (Figure 1F, G). These findings demonstrate that elevated 27HC levels, but not 25HC levels, enhance HSPC mobilization, suggesting a 27HC specific mechanism in regulating mobilization.

Clotrimazole enhances hematopoietic stem and progenitor cell mobilization induced by G-CSF and AMD3100

Given that 27HC is one of the most abundant oxysterols in human plasma,[32] increasing its levels by direct supplementation may be clinically challenging. The enzyme Cyp7b1 metabolizes 27HC into downstream products through its oxysterol 7α-hydroxylase activity, and *Cyp7b1*-deficient mice exhibit elevated 27HC levels in plasma and tissues.[26] Notably, *Cyp7b1* is expressed at higher levels in long-term hematopoietic stem cells (LT-HSC) than in hematopoietic progenitors or differentiated cells, based on data from the mouse ImmGen stem and progenitor cell datasets[33,34] (Figure 2A) and the mouse hematopoiesis model in Gene Expression Commons[35] (Figure 2B). These findings suggest that LT-HSC preferentially metabolize 27HC and thereby limit its intracellular availability.

We used clotrimazole, an antifungal agent known to inhibit Cyp7b1 function,[27] to test whether pharmacologic inhibition of Cyp7b1 enhances HSPC mobilization, potentially through elevating intracellular 27HC levels. In a similar mobilization regimen (Figure 2C), C57BL/6J mice co-treated with clotrimazole (50 mg/kg/day, intraperitoneally for four days) and AMD3100 displayed greater numbers of mobilized CFU-C (3.2-fold) and CFU-gmEM (4.3-fold) compared with AMD3100 alone (P=0.02 and P=0.04, respectively; one-way ANOVA across vehicle, clotrimazole, AMD3100, and co-treatment of clotrimazole and AMD3100 groups) (Figure 2D, E). While clotrimazole combined with G-CSF did not significantly increase mobilized CFU-C or CFU-gmEM compared with G-CSF alone, clotrimazole significantly increased mobilized CFU-C (1.4-fold) and CFU-gmEM (2.0-fold) when co-administered with G-CSF and AMD3100 (Figure 2D, E). Although the combination of G-CSF and AMD3100 significantly increased white blood cell (WBC) counts compared with vehicle control, the addition of clotrimazole did not further increase WBC (Figure 2F). This suggests that clotrimazole preferentially enhances mobilization of immature HSPC rather than mature leukocytes, consistent with the restricted expression of its target, Cyp7b1, in LT-HSC.

We next tested whether shorter dosing regimens were sufficient to enhance HSPC mobilization. C57BL/6J mice were treated with clotrimazole for one hour, one day, two days, or four days, followed by AMD3100 for one hour. While two days of clotrimazole treatment showed a trend toward enhanced mobilization of CFU-C (2.5-fold) and CFU-gmEM (5.7-fold), only the 4-day regimen produced a significant increase in CFU-C (5.3-fold) and CFU-gmEM (11-fold) compared with AMD3100 alone (Figure 2G, H).

Clotrimazole is also known to inhibit the intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca} or Gardos) channel, thereby reducing erythrocyte dehydration in SCD mice and patients.[36,37] To determine whether IK_{Ca} channel inhibition contributed to enhanced HSPC mobilization, we treated mice with TRAM-34, a clotrimazole analog that selectively

blocks IKCa channel without inhibiting cytochrome P450 activity.[38] Unlike clotrimazole, IK_{Ca} channel inhibition by TRAM-34 did not promote AMD3100-induced HSPC mobilization (Figure 2 I, J). Together, these findings indicate

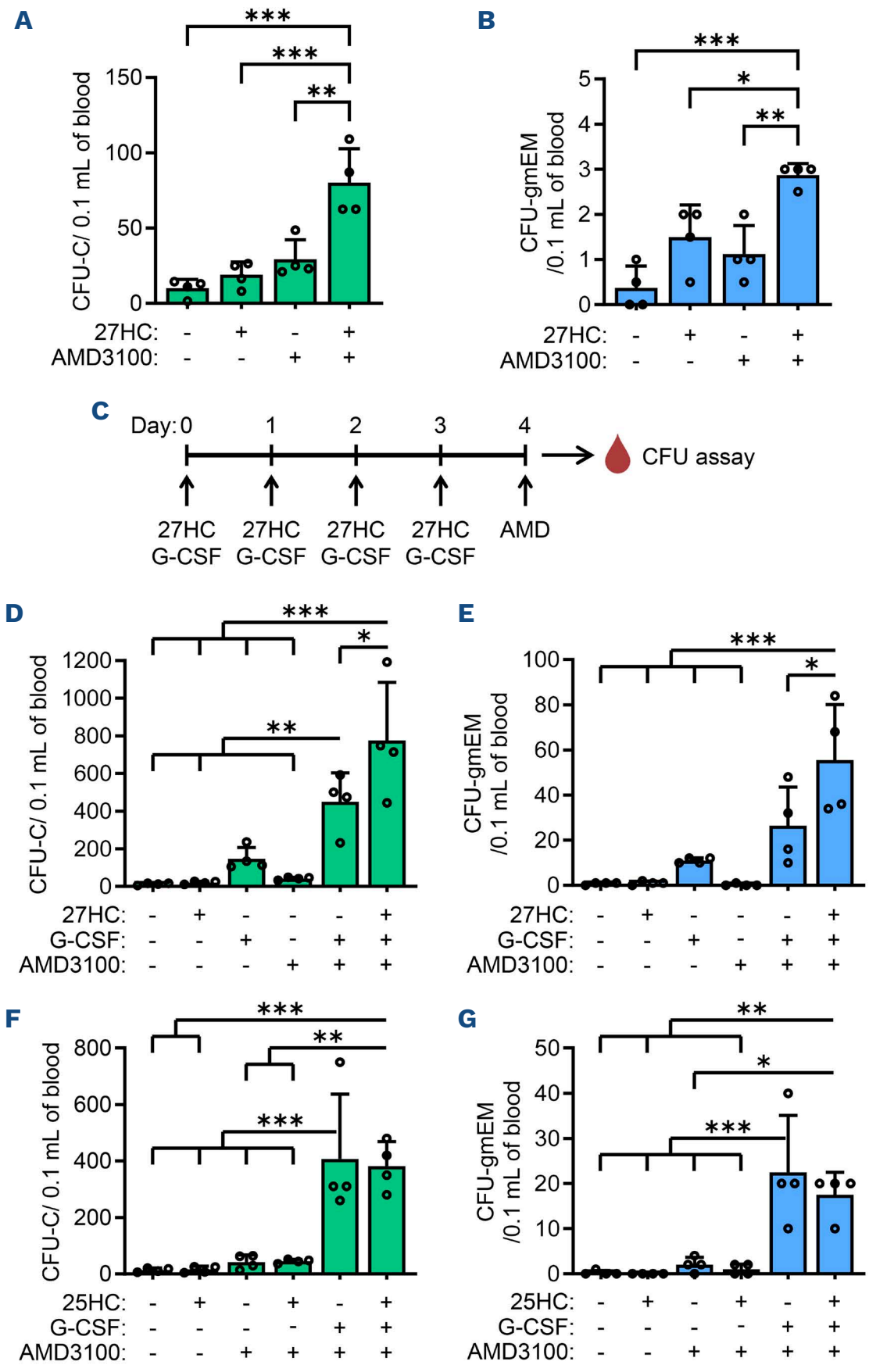
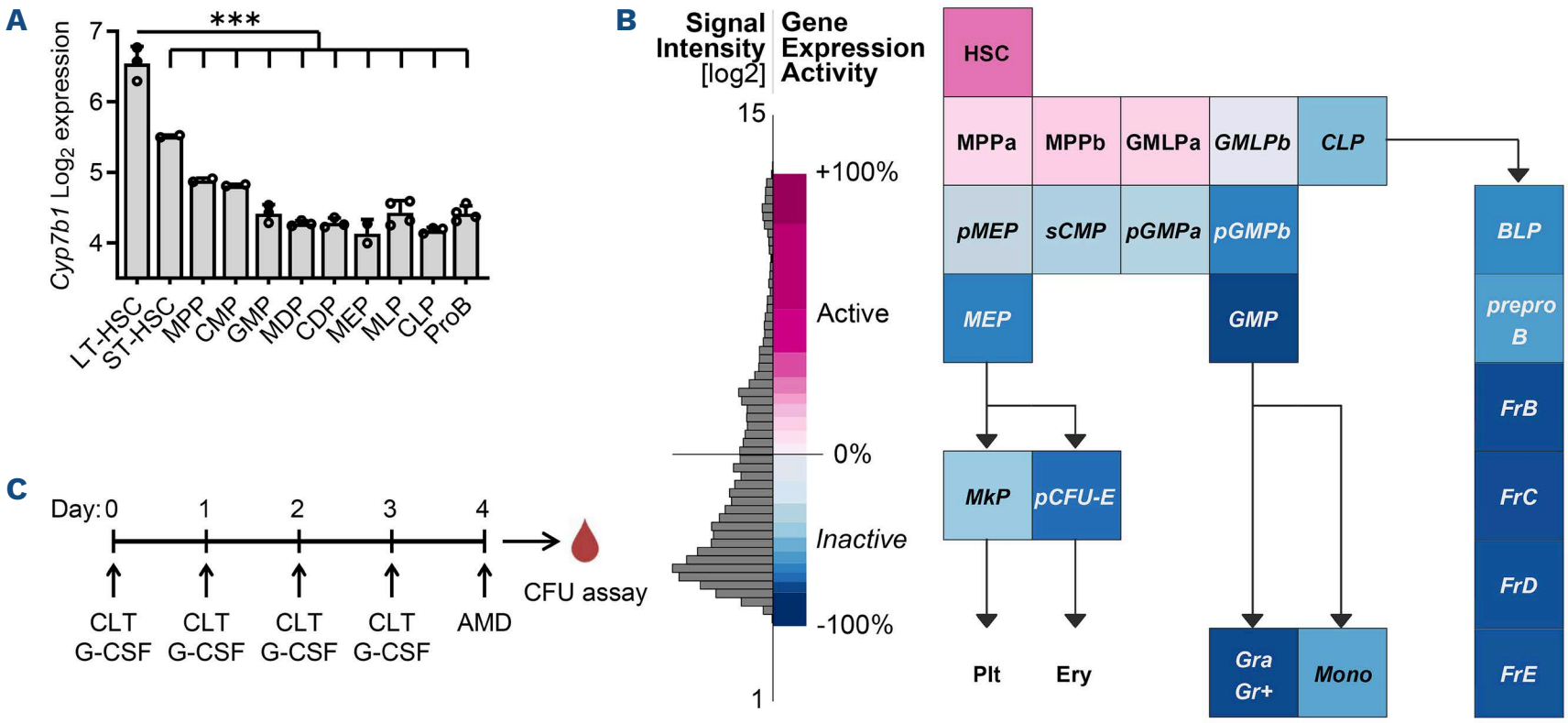


Figure 1. Enhanced hematopoietic stem and progenitor cell mobilization in wild-type mice co-treated with 27-hydroxycholesterol and clinically used hematopoietic stem and progenitor cell mobilizing agents. (A and B) Wild-type C57BL/6J mice were treated with 27-hydroxycholesterol (27HC) or vehicle daily for two days, followed by AMD3100 (plerixafor) or vehicle one hour before peripheral blood collection for CFU assays. Numbers of CFU-C (A) and CFU-gmEM (B) after the indicated treatments (N=4 mice per condition from 3 experiments). (C) Experimental design for hematopoietic stem and progenitor cell (HSPC) mobilization by co-treatment with 27HC, G-CSF, and AMD3100. (D and E) C57BL/6J mice were co-treated with 27HC, G-CSF and/or vehicle daily for four days, followed by AMD3100 or vehicle one hour before peripheral blood collection for CFU assays. Numbers of CFU-C (D) and CFU-gmEM (E) in peripheral blood after the indicated treatments (N=4 mice per condition from 4 experiments). (F and G) C57BL/6J mice were co-treated with 25HC, G-CSF and/or vehicle daily for four days, followed by AMD3100 or vehicle one hour before peripheral blood collection for CFU assays. Numbers of CFU-C (F) and CFU-gmEM (G) in peripheral blood after the indicated treatments (N=4 mice per condition from 3 experiments). Data represent mean ± standard deviation. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test:*P<0.05, **P<0.01, ***P<0.001.



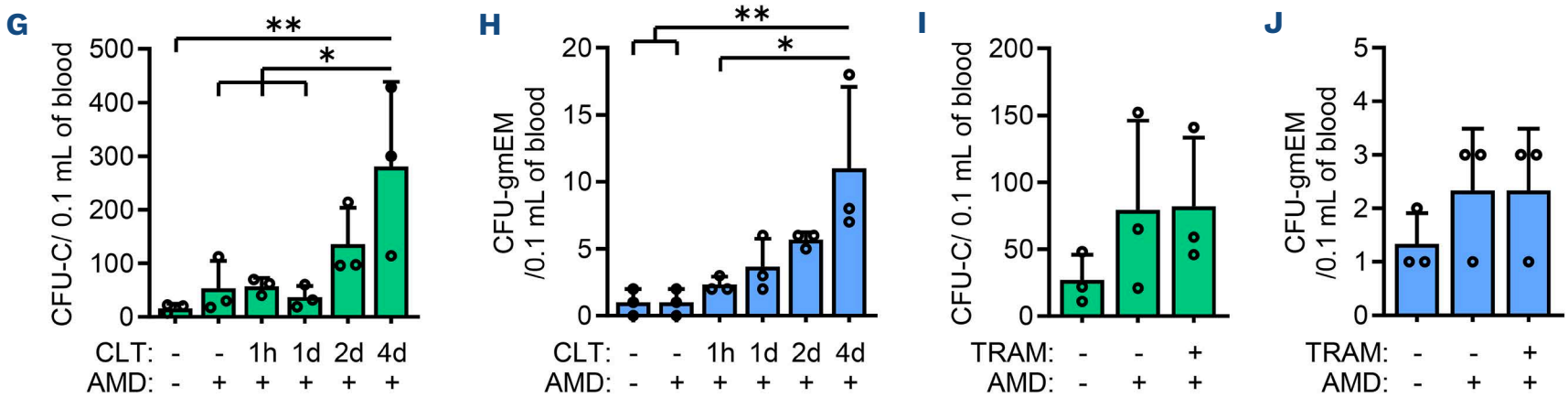


Figure 2. A Cyp7b1 inhibitor, clotrimazole, enhances hematopoietic stem and progenitor cell mobilization in combination with clinically used mobilizing agents. (A) *Cyp7b1* mRNA expression across bone marrow hematopoietic populations. Data were obtained from BloodSpot[33] (mouse ImmGen stem and progenitor cell dataset; N=2-4 per population[34]). LT34F, ST34F, MPP34F, proB.CLP, and proB.FrA labels were converted to long-term hematopoietic stem cells (LT-HSC), short-term hematopoietic stem cells (ST-HSC), multipotent progenitors (MPP), common lymphoid progenitors (CLP), and Pro-B progenitors (ProB), respectively. (B) *Cyp7b1* mRNA expression across bone marrow hematopoietic populations generated using Gene Expression Commons and adapted from the mouse hematopoiesis model (probeset 1421074_at).[35] (C) Experimental design for hematopoietic stem and progenitor cell mobilization by co-treatment with clotrimazole (CLT), granulocyte colony-stimulating factor (G-CSF), and AMD3100. (D-F) C57BL/6J mice were co-treated with CLT, G-CSF and/or vehicle daily for four days, followed by AMD3100 or vehicle one hour before peripheral blood collection for colony-forming unit (CFU) assays. Numbers of CFU-C (D) and CFU-gmEM (E) in peripheral blood and white blood cell (WBC) counts (F) after the indicated treatments (N=4 mice per condition from 4 experiments). (G and H) C57BL/6J mice were treated with CLT or vehicle daily for the indicated times, followed by AMD3100 or vehicle one hour before peripheral blood collection for CFU assays. Numbers of CFU-C (G) and CFU-gmEM (H) in the peripheral blood (N=3 mice per condition from 3 experiments). (I and J) C57BL/6J mice were treated with TRAM-34 (TRAM), an IK_{Ca} channel blocker, or vehicle daily for four days, followed by AMD3100

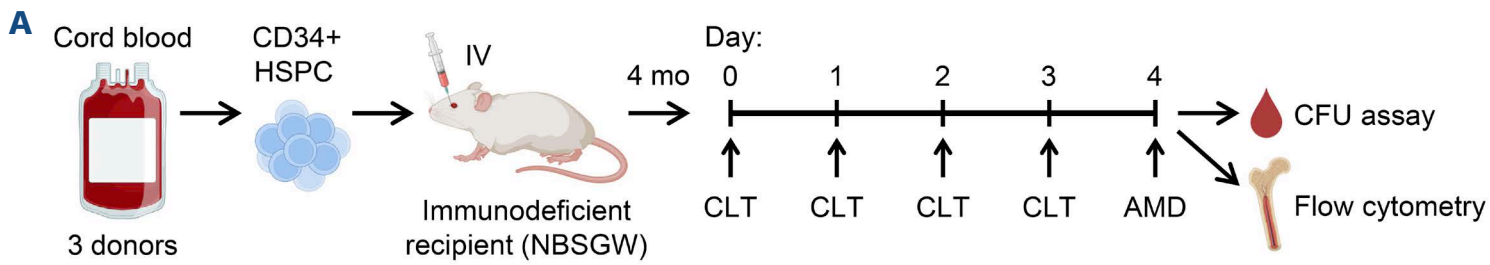
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or vehicle one hour before peripheral blood collection for CFU assays. Numbers of CFU-C (I) and CFU-gmEM (J) in the peripheral blood (N=3 mice per condition from 3 experiments). Data represent mean ± standard deviation. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test (*P<0.05, **P<0.01, ***P<0.001).

that clotrimazole treatment mimics the effects of 27HC supplementation by enhancing HSPC mobilization in conjunction with clinically used HSPC mobilizers, primarily through inhibition of Cyp7b1 rather than blockade of the IK_{Ca} channel.

Clotrimazole enhances human hematopoietic stem and progenitor cell mobilization in humanized mice

To examine the effect of clotrimazole on human HSPC mobilization, we used a humanized mouse model transplanted with cord blood-derived human HSPC. $CD34^+$ HSPC were magnetically enriched from freshly obtained cord blood from 3 independent donors, and cells from each donor were transplanted into unconditioned NBSGW immunodeficient mice.[30] Four months post transplantation, humanized mice were treated with clotrimazole and/or AMD3100, followed by peripheral blood colony assays and flow cytometric analysis of the BM (Figure 3A). For the colony assays, methylcellulose medium supplemented exclusively with human cytokines was used, ensuring selective support of colonies derived from human HSPC. Generated colonies expressed either human CD33 (hCD33, granulocyte/monocyte marker), hCD235a (erythroid marker), or both (Figure 3B). Co-treatment with clotrimazole and



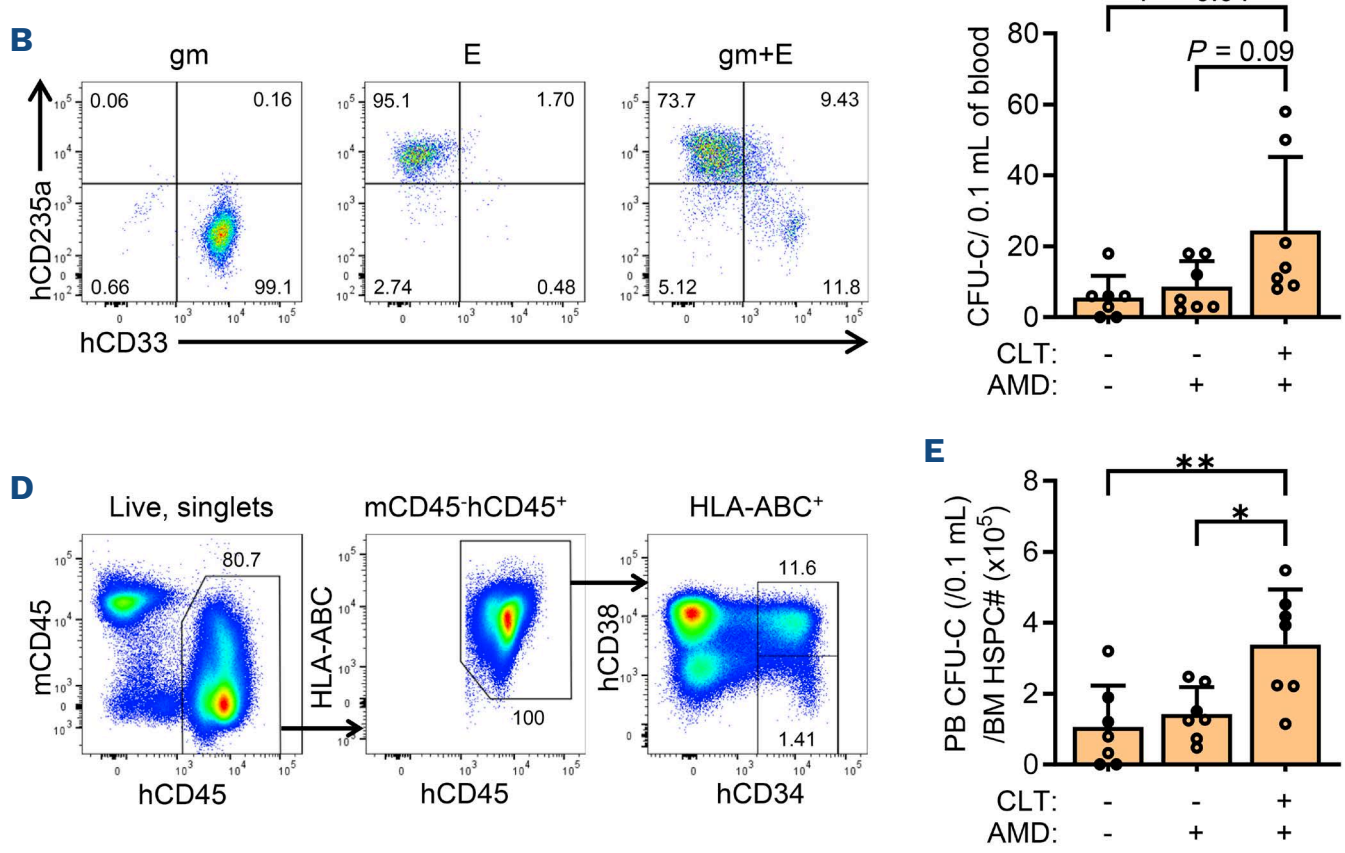


Figure 3. Clotrimazole enhances human hematopoietic stem and progenitor cell mobilization in immune-humanized mice co-treated with AMD3100. (A) Immunodeficient NBSGW mice were transplanted with $CD34^+$ hematopoietic stem and progenitor cells (HSPC) from human cord blood. After four months of engraftment, humanized mice were treated with clotrimazole (CLT) or vehicle daily for four days, followed by AMD3100 or vehicle one hour before analysis. (B) Flow cytometry dot plots of representative colonies derived from peripheral blood (PB) of humanized mice. Human granulocytes/macrophages (gm, $hCD33^+hCD235a^-$), erythroblasts (E, $hCD235a^+hCD33^-$), or mixed colonies (gm+E) are shown. (C) Absolute numbers of mobilized human colony-forming units (CFU)-C in PB of humanized mice after the indicated treatments (N=7 mice per condition from 4 experiments). (D) Representative flow cytometry dot plots of $mCD45^-hCD45^+HLA\text{-}ABC^+CD34^+CD38^-$ human HSPC in bone marrow (BM) of humanized mice. (E) Ratio of mobilized human CFU-C in PB to the number of BM human HSPC in humanized mouse (N=7 mice per condition from 4 experiments). Data represent mean ± standard deviation. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test (*P<0.05, **P<0.01).

AMD3100 significantly increased the number of mobilized human CFU-C in PB compared with vehicle-treated controls (4.4-fold) and showed a strong but non-significant trend toward increased mobilization compared with AMD3100 single treatment (2.8-fold) (Figure 3C). Because human HSPC chimerism varied across recipients, we normalized the levels of humanization by calculating the PB CFU-C number per BM human HSPC, defined as mouse CD45 (mCD45)$^-$hCD45$^+$HLA-ABC$^+$hCD34$^+$hCD38$^-$ (Figure 3D). After adjustment, clotrimazole co-administration significantly enhanced AMD3100-induced mobilization of human HSPC by 2.4-fold (Figure 3E). These results demonstrate that pharmacologic inhibition of Cyp7b1 can augment human HSPC mobilization *in vivo*, highlighting its potential as a translational strategy to improve HSPC collection for clinical transplantation.

Clotrimazole enhances hematopoietic stem and progenitor cell mobilization in sickle cell disease mice

We next examined whether clotrimazole could enhance HSPC mobilization in the "Townes" SCD mouse model.[29] Because G-CSF is contraindicated for HSPC mobilization in SCD patients due to the risk of fatal vaso-occlusive crises,[9-11] we focused on testing the potential synergy between

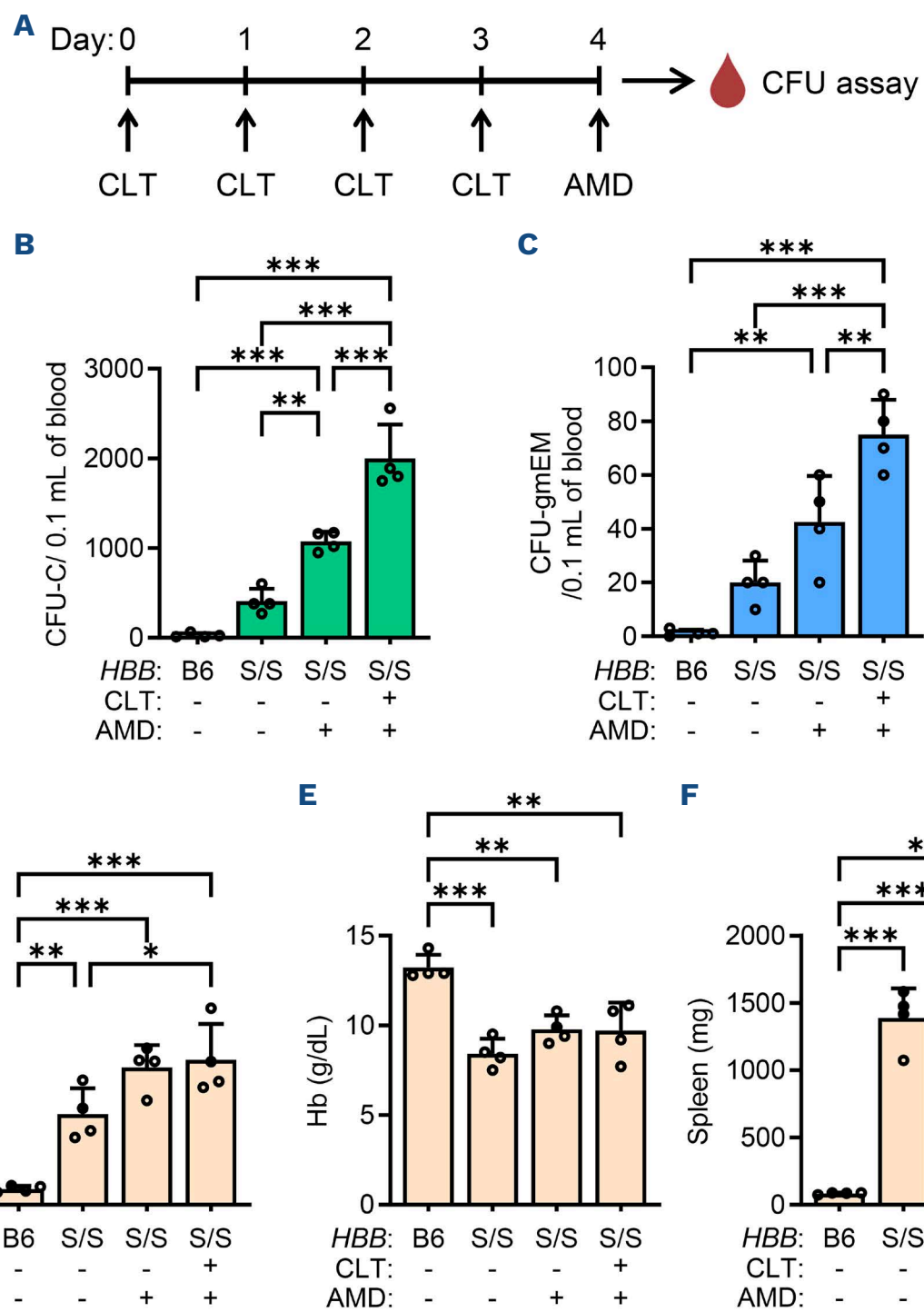


Figure 4. Clotrimazole enhances hematopoietic stem and progenitor cell mobilization in sickle-cell disease mice in combination with AMD3100. (A) Experimental design for hematopoietic stem and progenitor cell (HSPC) mobilization in "Townes" sickle-cell disease (SCD) mice. Mice were treated with clotrimazole (CLT) or vehicle daily for four days, followed by AMD3100 or vehicle one hour before peripheral blood collection for colony-forming units (CFU) assays. (B and C) Numbers of CFU-C (B) and CFU-gmEM (C) in peripheral blood after the indicated treatments (N=4 mice per condition from 4 experiments). B6, wild-type C57BL/6J mice; *HBB* S/S, mice homozygous for human sickle-cell β-globin alleles. (D-F) White blood cell (WBC) counts, hemoglobin (Hb) levels, and splenic weights after the indicated treatments (N=4 mice per condition from 4 experiments). Data represent mean ± standard deviation. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test: *P<0.05, **P<0.01, ***P<0.001.

clotrimazole and AMD3100 (Figure 4A). Vehicle-treated SCD mice homozygous for human HbS knock-in alleles (S/S) exhibited markedly elevated baseline levels of mobilized CFU-C (15-fold, *P*=0.01, unpaired *t* test with Welch's correction) and CFU-gmEM (16-fold, *P*=0.02) in the PB compared with vehicle-treated WT C57BL/6J mice (Figure 4B, C). Although AMD3100 alone had little effect on HSPC mobilization in WT C57BL/6J mice (Figures 1 and 2), in SCD mice it significantly increased mobilized CFU-C (2.6-fold) and showed a non-significant trend toward increased CFU-gmEM (2.1-fold, *P*=0.07) compared with vehicle-treated SCD mice (Figure 4B, C). Importantly, four days of clotrimazole treatment further potentiated AMD3100-induced HSPC mobilization in SCD mice, leading to significantly higher CFU-C (1.9-fold) and CFU-gmEM (1.8-fold) compared with AMD3100 alone (Figure 4B, C).

As expected, SCD mice displayed elevated WBC counts relative to WT C57BL/6J mice (5.7-fold) (Figure 4D). Clotrimazole plus AMD3100 co-treatment significantly increased (1.6-fold) (*P*=0.04) and AMD3100 single treatment showed a non-significant increase (1.5-fold) (*P*=0.09) of WBC counts compared with vehicle-treated SCD mice, but there was no significant difference between AMD3100 alone and co-treatment with clotrimazole (1.1-fold) (*P*=0.97). SCD mice also exhibited reduced hemoglobin levels and splenomegaly compared with WT C57BL/6J mice, consistent with the disease phenotype; however, neither AMD3100 nor clotrimazole treatment altered these parameters (Figure 4E, F). Taken together, these findings indicate that AMD3100 mobilizes HSPC more effectively in SCD mice than in WT mice, and co-administration of clotrimazole further enhances this mobilization without exacerbating anemia or splenomegaly.

Voriconazole enhances hematopoietic stem and progenitor cell mobilization in wild-type and sickle-cell disease mice

While clotrimazole shows promising effects in enhancing HSPC mobilization, it is currently FDA-approved only for topical and lozenge formulations and is not available for systemic use. To identify alternative Cyp7b1 inhibitors suitable for systemic administration, we tested voriconazole, another Cyp7b1-binding azole[28] that is FDA-approved for systemic antifungal therapy via both intravenous and oral routes. Importantly, voriconazole is already widely used in patients after HSPC transplantation as antifungal therapy,[39] minimizing safety concerns regarding its potential repurposing for HSPC mobilization pre-transplant. In WT C57BL/6J mice, four days of intravenous voriconazole treatment significantly enhanced AMD3100-induced mobilization of CFU-C (1.5-fold) and CFU-gmEM (1.8-fold) into the PB (Figure 5A-C). Similarly, in SCD mice, voriconazole co-treat-

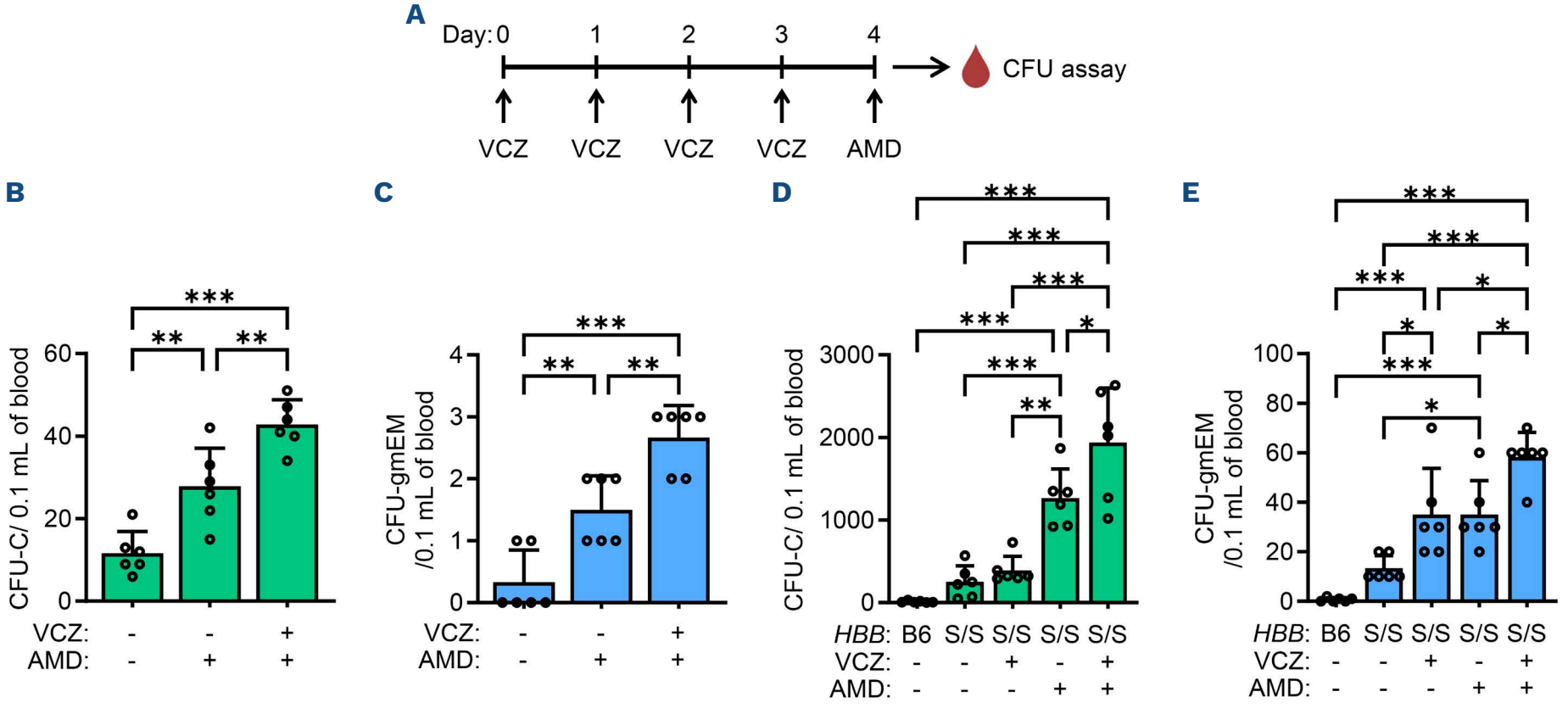


Figure 5. Voriconazole intravenous treatment enhances hematopoietic stem and progenitor cell mobilization in wild-type and sickle-cell disease mice in combination with AMD3100. (A) Experimental design for hematopoietic stem and progenitor cell (HSPC) mobilization. Wild-type (WT) C57BL/6J or Townes sickle cell disease (SCD) mice were treated with voriconazole (VCZ) or vehicle intravenously daily for four days, followed by AMD3100 or vehicle one hour before peripheral blood collection for colony-forming units (CFU) assays. (B and C) Numbers of CFU-C (B) and CFU-gmEM (C) in peripheral blood of C57BL/6J mice after the indicated treatments (N=6 mice per condition from 2 experiments). (D and E) The numbers of CFU-C (D) and CFU-gmEM (E) in peripheral blood of WT (B6) and SCD (*HBB* S/S) mice after the indicated treatments (N=6 mice per condition from 5 experiments). Data represent mean ± standard deviation. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test (*P<0.05, **P<0.01, ***P<0.001).

ment augmented AMD3100-induced mobilization, increasing CFU-C (1.5-fold) and CFU-gmEM (1.7-fold) compared with AMD3100 alone (Figure 5D, E). These findings suggest that voriconazole, an FDA-approved systemic antifungal already in use among HSPC transplant recipients, could be rapidly repurposed as a clinically feasible strategy to enhance HSPC mobilization, including in patient populations such as SCD where current mobilization options are limited.

Voriconazole administration does not affect hematopoiesis

We next examined whether voriconazole administration alters hematopoiesis beyond its effect on mobilization. Following four days of intravenous voriconazole treatment in C57BL/6J mice, we quantified hematopoietic populations, including hematopoietic stem cells (HSC), multipotent progenitors (MPP), hematopoietic progenitor cells (HPC-1 and HPC-2), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), megakaryocyte-erythroid progenitors (MEP), granulocytes (Gra), monocytes/macrophages (Mac), erythroid progenitors (Ery), B cells, and T cells, in the BM, spleen, and peripheral blood. Voriconazole treatment did not alter the cell numbers of any of these populations, nor did it affect WBC, RBC, hemoglobin, and platelet counts (Figure 6A-C). Moreover, 5-bromo-2'-deoxyuridine (BrdU) incorporation assay during the last three days of treatment revealed no change in HSC cell-cycle activity in voriconazole-treated mice (Figure 6D). We also evaluated hematopoietic cell populations in SCD mice treated with vehicle, voriconazole alone, AMD3100 alone, or the combination of voriconazole and AMD3100. Aside from the expected increase in WBC counts observed in both AMD3100 alone and voriconazole plus AMD3100 treatment groups, no significant differences were detected in BM hematopoietic populations (Figure 6E), whole BM (WBM) cellularity and spleen weight (Figure 6F), or peripheral blood hematopoietic populations and complete blood count (CBC) parameters (Figure 6G) across treatment groups. Together, these findings indicate that the effects of voriconazole treatment are restricted to enhancing HSPC mobilization, without perturbing HSC cell-cycle dynamics, lineage differentiation, or PB composition. This selective activity underscores the safety of voriconazole treatment and supports its potential as a clinically viable mobilization agent in combination with AMD3100.

Voriconazole augments but does not prolong hematopoietic stem and progenitor cell mobilization

To determine whether the mobilization effect of voriconazole is transient or persistent, we examined the kinetics of mobilized HSPC following co-treatment with voriconazole and AMD3100 in Townes SCD mice (Figure 7A).

As expected, AMD3100 alone induced a significant increase in mobilized CFU-C one hour after injection, with levels returning to baseline within one day, consistent with prior reports (Figure 7B).[40] Co-treatment with voriconazole and AMD3100 further augmented CFU-C mobilization at one hour after AMD3100 injection compared with AMD3100 alone, but this effect was no longer evident after one day, when mobilized CFU-C numbers were comparable across vehicle-, AMD3100-, and combination-treated mice (Figure 7B). Mobilization kinetics of CFU-gmEM followed a similar pattern; however, a modest but non-significant increase persisted at one-day post-AMD3100 treatment, suggesting that voriconazole may exert a slightly more durable effect on immature subsets of HSPC (Figure 7C).

Bone marrow hematopoietic populations remained comparable across treatment groups at four days post treatment (Figure 7D). A non-significant trend toward increased splenic HSC was observed in both AMD3100 alone (2-fold, P=0.32 by one-way ANOVA) and voriconazole plus AMD3100 (1.6-fold, P=0.61) cohorts, consistent with transient HSPC distribution to extramedullary sites (Figure 7E). No significant differences were observed in WBC counts, hemoglobin levels, or platelet counts between AMD3100 single and voriconazole co-treatment groups (Figure 7F). Together, these findings indicate that voriconazole enhances AMD3100-induced HSPC mobilization in a transient manner without perturbing steady-state hematopoiesis, further supporting its safety profile for potential clinical HSPC mobilization.

Discussion

In this study, we identify a novel strategy to enhance HSPC mobilization by targeting oxysterol metabolism using 27HC or Cyp7b1 inhibiting azoles (clotrimazole and voriconazole), in combination with clinically established HSPC mobilizers G-CSF and/or AMD3100 (Figure 8). Notably, this strategy was effective in both WT and SCD mice, a clinically significant finding given the limited mobilization options available for SCD. Current FDA-approved gene therapies for SCD require substantially larger HSPC collections than standard autologous HSPC transplantation,[7,8] yet safe and efficient mobilization remains a major barrier. We did not observe adverse effects of voriconazole on steady-state hematopoiesis in either WT or SCD mice. Voriconazole is already FDA-approved for systemic antifungal therapy and has been administered to patients following HSPC transplantation,[39] supporting its translational feasibility. The mobilizing effect observed here was modest and transient, suggesting that Cyp7b1 inhibition is best positioned as an adjunct rather than a standalone mobilizer. Optimization of dosing, scheduling relative to HSPC collection, and pharmacodynamic monitoring will be essential to maximize its impact. It will also be important to evaluate potential synergy with emerging mobilizers such as motixafortide,[41] tGro-β,[42] and VLA4 antagonists,[43] particularly in poor mobilizers.

Beyond transplantation, enhanced mobilization by Cyp7b1 inhibitors may have broad applications, such as *in*

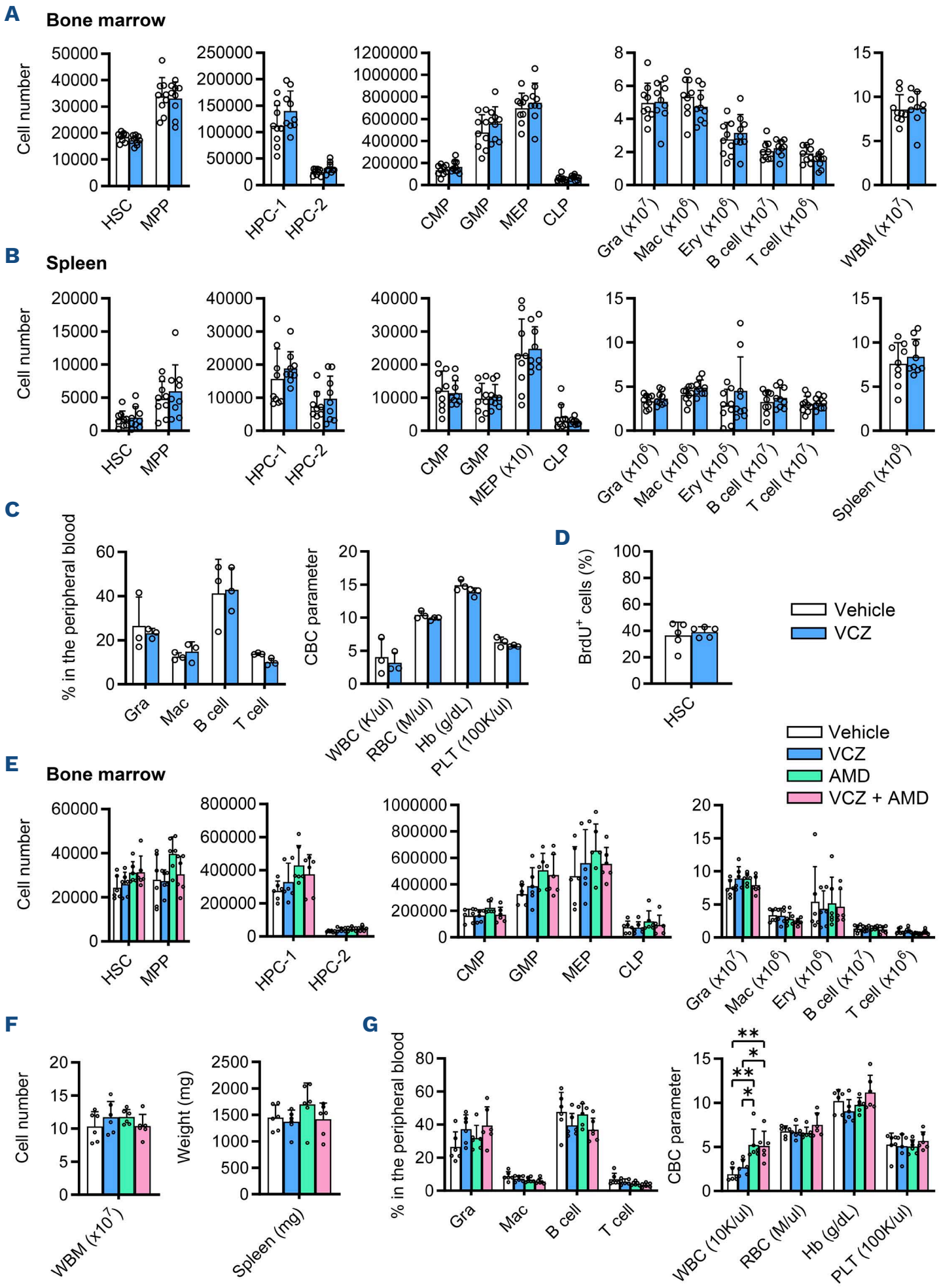


Figure 6. Voriconazole administration does not affect hematopoiesis. (A-D) Wild-type C57BL/6 mice treated with voriconazole (VCZ) or vehicle intravenously daily for four days with BrdU incorporation during the last three days. (A and B) Flow cytometric quantification of hematopoietic populations in bone marrow (A) and spleen (B) (N=9 mice per condition from 3 experiments). (C) Frequencies of peripheral blood cell types (left) and complete blood count (CBC) parameters (right: Hb, hemoglobin; PLT, platelets) (N=3 mice per condition from one experiment). (D) Frequencies of $BrdU^+$ cells within the hematopoietic stem cell (HSC) population (N=5 mice per condition from 2 experiments). (E-G) Townes SCD mice were treated with VCZ or vehicle intravenously daily for four days, followed by AMD3100 or vehicle one hour before analysis (N=6 mice per condition from 5 experiments). (E) Flow cytometric quantification of hematopoietic populations in bone marrow. (F) Bone marrow cellularity (left) and spleen weights (right). (G) Frequencies of peripheral blood cell type (left) and CBC parameters (right). Data represent mean ± standard deviation. Statistical significance was determined by two-tailed unpaired Student *t* tests (A-D) or one-way ANOVA with Tukey's multiple comparisons test (E-G): **P*<0.05, ***P*<0.01.

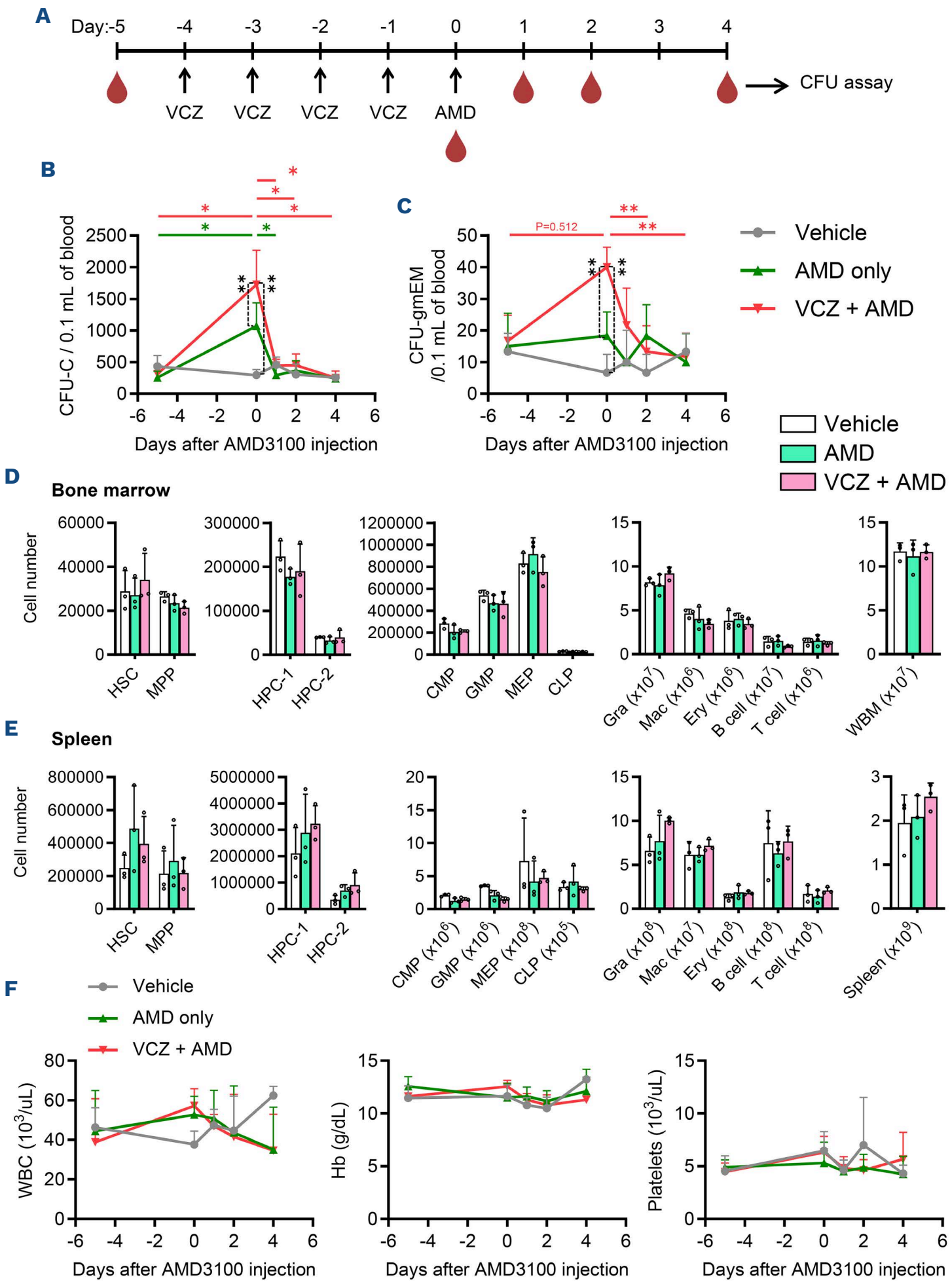


Figure 7. Voriconazole enhances hematopoietic stem and progenitor cell mobilization transiently but not persistently. (A) Experimental design to study the kinetics of hematopoietic stem and progenitor cell (HSPC) mobilization. Townes SCD mice were treated with voriconazole (VCZ) or vehicle intravenously daily for four days, followed by AMD3100 or vehicle. Peripheral blood was collected for colony-forming units (CFU) assays and complete blood count (CBC) on day -5, at one hour, and days 1, 2, and 4 post AMD3100 treatment. Bone marrow and spleen were harvested on day 4. (B and C) Numbers of CFU-C (B) and CFU-gmEM (C) in peripheral blood with the indicated treatments (vehicle: N=3 mice; AMD and VCZ+AMD: N=6 mice per condition from 2 experiments). (D and E) Flow cytometric quantification of hematopoietic populations in bone marrow (D) and spleen (E) (N=3 mice per condition from one experiment). (F) Kinetics of CBC parameters (vehicle: N=3 mice; AMD and VCZ+AMD: N=6 mice per condition from 2 experiments). Data represent mean ± standard deviation. Statistical significance was determined by mixed-effects analysis with Šídák's multiple comparisons test (B, C and F) or one-way ANOVA with Tukey's multiple comparisons test (D and E): **P*<0.05, ***P*<0.01.

vivo gene therapies. The current gene therapies FDA-approved for treating SCD involve *ex vivo* manipulation of HSPC.[7,8] While this is a promising approach for treating several monogenic disorders, including immunodeficiencies, metabolic disorders, and osteopetrosis, it requires robust HSPC collection, maintaining HSPC *ex vivo* during genetic modification, and patient conditioning for transplantation.[44] Importantly, these procedures can promote positive selection of mutant clones harboring potential driver mutations associated with clonal hematopoiesis or myeloid neoplasms during *ex vivo* culture.[45] *In vivo* gene therapy has the possibility to overcome these obstacles, and HSPC mobilization can enhance HSPC gene transfer in mouse models.[46] Even transient, short-term increases in circulating HSPC could improve the efficiency of *in vivo* targeting strategies.

Mobilization strategies must also consider patient populations beyond SCD. For example, autologous HSPC transplantation remains a standard therapy for multiple myeloma,[47] where chemotherapy-based HSPC mobilization has been used to provide both HSPC mobilization and anti-tumor activity. It will be important to determine whether elevated Cyp7b1 inhibition can further enhance chemotherapy-based HSPC mobilization while maintaining tolerability.

Mechanistically, our findings suggest a model in which Cyp7b1 functions as a metabolic checkpoint in LT-HSC. We previously demonstrated that 27HC enhances HSPC mobilization in an ERα-dependent manner.[17] Because Cyp7b1 metabolizes 27HC, high expression of Cyp7b1 in LT-HSC may restrict intracellular 27HC accumulation and thereby limit mobilization potential. Pharmacologic inhibition of Cyp7b1 could transiently release this brake, creating a mobilization-permissive state. The transient kinetics observed *in vivo* are consistent with a reversible metabolic mechanism. However, the link between Cyp7b1 inhibition, intracellular 27HC accumulation, and mobilization remains inferential. We were unable to reliably quantify intracellular 27HC levels in purified HSPC following azole treatment, due to limited cell numbers. Future studies directly measuring intracellular 27HC levels and defining pharmacodynamic relationships will be required to establish this mechanism more definitively. While our previous study implicates ERα signaling as a key mediator,[17] additional work will be needed to determine whether Cyp7b1 inhibition intersects with other pathways. Cyp7b1 catalyzes the hydroxylation of 27HC and 25HC into their downstream metabolites, 7α,27-dihydroxycholesterol (7α,27HC) and 7α,25-dihydroxycholesterol (7α,25HC), respectively.[16] These metabolites have distinct signaling functions: 7α,27HC acts as an agonist of RAR-related orphan receptor gamma t (RORγt),[48] while 7α,25HC serves as a ligand for the GPR183 receptor (also known as EBI2).[49,50] We have not ruled out that altered oxysterol signaling through these receptors impacts HSPC mobilization. However, although both 27HC and 25HC can signal through estrogen receptors and LXR,[15] our results show that only 27HC enhances HSPC mobilization, whereas 25HC does not. This indicates that HSPC mobilization is mediated specifically by 27HC signaling, likely through ERα as shown in our

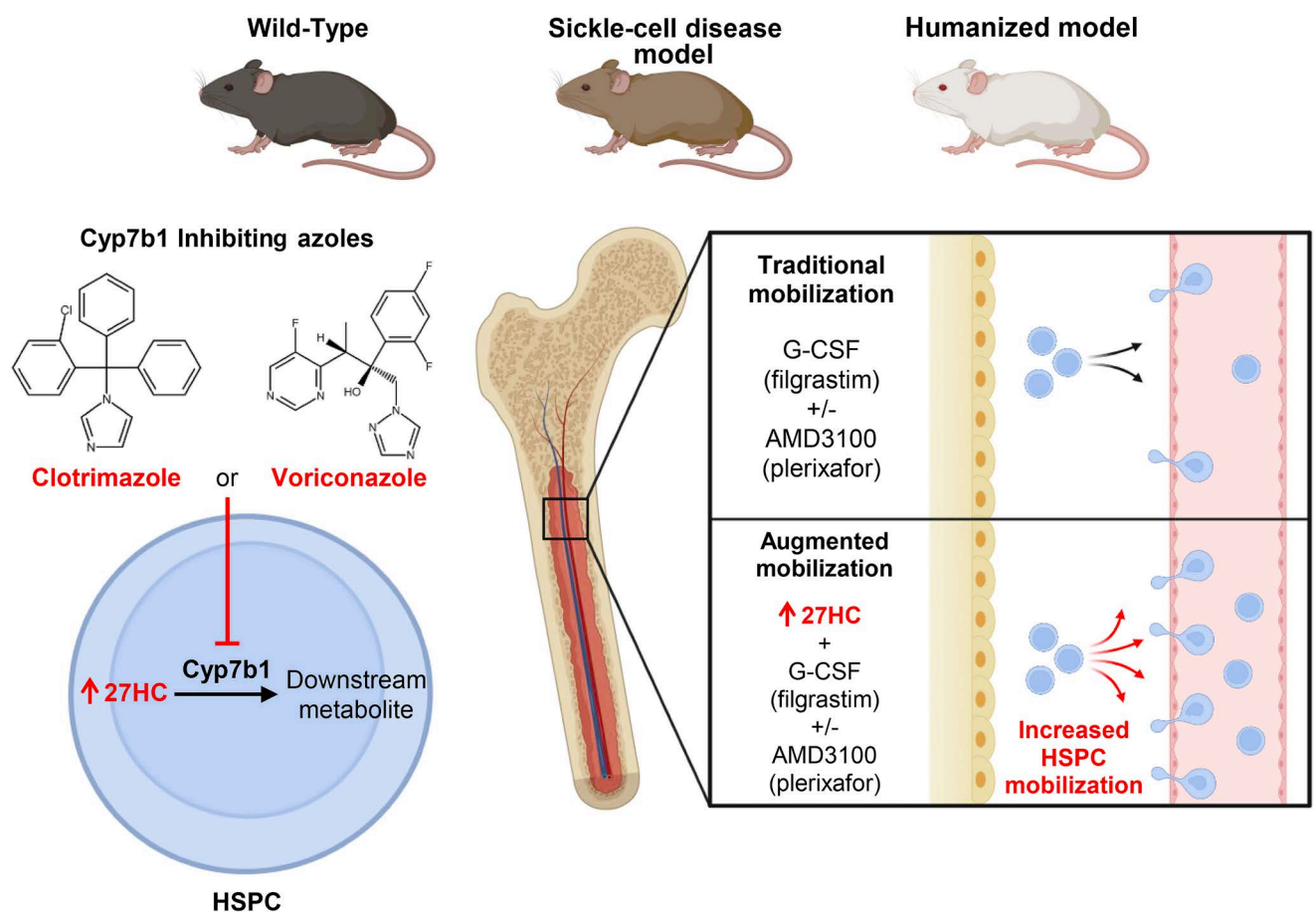


Figure 8. Cyp7b1 inhibition offers a safe, clinically translatable strategy to enhance hematopoietic stem and progenitor cell mobilization in normal and sickle-cell disease contexts. Schematic model summarizing how Cyp7b1 inhibition promotes hematopoietic stem and progenitor cell (HSPC) mobilization. G-CSF: granulocyte colony-stimulating factor.

previous study,[17] rather than by 25HC-derived pathways. Moreover, because 27HC and related oxysterols may act on both HSPC and their niche microenvironment, delineating cell-intrinsic *versus* extrinsic contributions will be an important next step.

A limitation of this study is that our primary functional endpoints evaluate colony-forming HSPC, and we did not directly assess long-term multilineage engraftment potential of mobilized cells through competitive transplantation assays. Therefore, whether Cyp7b1 inhibition enhances the mobilization of bona fide long-term repopulating HSC, rather than predominantly progenitor populations, remains to be formally determined.

Finally, these results establish oxysterol metabolism as a novel regulator of HSPC mobilization. By targeting Cyp7b1 with clinically relevant agents such as voriconazole, it may be possible to develop mobilization strategies that are mechanistically distinct from, yet complementary to, current approaches. While the mobilizing effect is modest and short-term, its reproducibility across models and compatibility with established regimens support further exploration as an adjunctive strategy. Such an approach may be particularly valuable in clinical contexts where mobilization options are constrained, including SCD, and more broadly where incremental improvements in collection efficiency can meaningfully reduce procedural burden. Together, these findings position Cyp7b1 inhibition as a promising and mechanistically novel strategy for enhancing HSPC mobilization.

Disclosures

No conflicts of interest to disclose.

Contributions

HO conceived the study and planned experiments; BLV and TJR performed most of the experiments with help from JKK, ALK, ET and HO; BLV, TJR and HO analyzed data and wrote the manuscript; HO acquired funding and supervised the study. All authors reviewed and approved the final manuscript for publication.

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Data-sharing statement

For original data, please contact Hideyuki Oguro (oguro@uchc.edu).

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