Enasidenib drives human erythroid differentiation independently of isocitrate dehydrogenase 2

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Cancer-related anemia is present in more than 60% of newly diagnosed cancer patients and is associated with substantial morbidity and high medical costs. Drugs that enhance erythropoiesis are urgently required to decrease transfusion rates and improve quality of life. Clinical studies have observed an unexpected improvement in hemoglobin and RBC transfusion-independence in patients with acute myeloid leukemia (AML) treated with the isocitrate dehydrogenase 2 (IDH2) mutant-specific inhibitor enasidenib, leading to improved quality of life without a reduction in AML disease burden. Here, we demonstrate that enasidenib enhanced human erythroid differentiation of hematopoietic progenitors. The phenomenon was not observed with other IDH1/2 inhibitors and occurred in IDH2-deficient CRISPR-engineered progenitors independently of D-2-hydroxyglutarate. The effect of enasidenib on hematopoietic progenitors was mediated by protoporphyrin accumulation, driving heme production and erythroid differentiation in committed CD71+ progenitors rather than hematopoietic stem cells. Our results position enasidenib as a promising therapeutic agent for improvement of anemia and provide the basis for a clinical trial using enasidenib to decrease transfusion dependence in a wide array of clinical contexts.
Figure 1. Enasidenib augments erythroid differentiation. (A) Proportion of CD71+GPA+ (%CD71+GPA+) cells after 8 days culture of CB-CD34+ cells in EDC with DMSO or 10 μM enasidenib (Ena) (left; n = 24 independent CB specimens). Fold change (FC) of percentage of CD71+GPA+ cells (DMSO = 1) cells with baseline differentiation capacity (%CD71+GPA+) of less than 40% (right; n = 14) or greater than 40% (middle; n = 10). (B) Number of CB-derived CD71+GPA+ cells at day 8 of EDC (n = 4). (C) Dose response of enasidenib, represented as FC of percentage of CD71+GPA+ cells (DMSO = 1) at day 8 of EDC (n = 4). (D) Proportion of CD71+GPA+ cells at day 8 of EDC of CD34+ cells from normal bone marrow (BM) (left; n = 3). FC of percentage of CD71+GPA+ cells (DMSO = 1) (right; n = 3). (E) qPCR detection of relative RNA expression of erythroid and myeloid transcription factors with enasidenib treatment compared with DMSO of CB-CD34+ cells at day 8 of EDC (DMSO = 1) (n = 3). (F) FC of hemoglobin in a colorimetric assay after 14 days in EDC (DMSO=1) (n = 3). (G) Representative cell pellets from normal BM (top panel) and CB (bottom panel) after 14 days in EDC (n = 3). (H) Representative image at day 8 of CB-CD34+ cells in EDC treated with DMSO or 10 μM enasidenib (n = 3) and stained with benzidine. (I) Representative image at day 8 of CB-CD34+ cells in EDC treated with DMSO or 10 μM enasidenib (n = 3) and stained with Wright-Giemsa. Arrows indicate maturing erythrocytes. Graphs represent mean ± SD. Statistical significance was calculated using unpaired 2-tailed t tests. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
istic of maturing erythrocytes (8), with increased expression of GATA1, EPOR, and KLF1, and decreased SPI1 and GATA2 (Figure 1E and Supplemental Figure 4). Enasidenib-treated progenitor cells further demonstrated increased hemoglobin production (Figure 1, F–H) and morphologic characteristics of increased erythroid maturation, including decreased cell size and nuclear condensation (Figure 11). Given the important role of HIF1α in erythropoiesis and IDH1/2 mutant signaling pathways (9–11), we performed differentiation studies in hypoxic conditions and found that enasidenib also drives erythroid differentiation at lowered oxygen tension (Supplemental Figure 5).

The capacity to increase erythroid differentiation was unique to enasidenib in the class of IDH inhibitors, as AG-120 (a mutant IDH1 inhibitor), AGI-6780 (a mutant IDH2 inhibitor), and AG-881 (a dual mutant IDH1 and IDH2 inhibitor), did not exhibit the same effects at a range of doses from 1–10 μM (Figure 2A). As expected, enasidenib, AGI-6780, and AG-881 completely suppressed D-2-HG in a THP-1 cell line overexpressing mutant IDH2-R140Q (Supplemental Figure 6, A and B). To explore whether the effect of enasidenib on erythroid differentiation was mediated through D-2-HG, we measured D-2-HG levels in the differentiating erythroid progenitors. As expected for normal HSPCs, D-2-HG was not present at detectable levels in either the DMSO or enasidenib-treated conditions (Figure 2B). Furthermore, addition of a cell-permeable derivative of D-2-HG (2R-octyl-α-hydroxyglutarate) at either 50 or 200 μM did not affect the ability of enasidenib to increase the proportion of CD71+GPA+ cells (Figure 2C).

Because enasidenib has been demonstrated to show moderate inhibitory activity against WT IDH2 at high doses (3), we sought to determine whether the action of enasidenib on erythroid precursors was due to decreased activity of WT IDH2. IDH2 normally functions in the TCA cycle, converting isocitrate into α-ketoglutarate. Treatment of CD71+ and CD71+ erythroid precursors with enasidenib did not significantly alter levels of TCA cycle metabolites or affect flux through the TCA cycle, as measured by liquid chromatography–mass spectrometry (LC-MS) and isotope labeling (Supplemental Figure 7). This indicates that WT IDH2 activity was likely not inhibited at the concentration of enasidenib able to drive erythroid differentiation. To further probe the role of IDH2 in this activity of enasidenib, we used CRISPR-Cas9/AAV6 to knockout IDH2 in CB-CD34+ cells (Figure 2D and Supplemental Figure 8) (12). Genomic and protein-level disruption of IDH2 were confirmed by PCR detection of the AAV vector in the endogenous IDH2 locus and Western blot analysis, respectively (Figure 2, E and F). IDH2-KO CB-CD34+ cells exhibited decreased baseline erythroid differentiation compared with control AAVS1-edited cells (Figure 2G). However, similar to WT CB-CD34+ cells, treatment of IDH2-KO CB-CD34+ cells with enasidenib still demonstrated a 3.4-fold increase in percentage of CD71+GPA+ erythroid cells compared with DMSO treatment (Figure 2H). These results indicate that enasidenib augments erythroid differentiation independently of both mutant and WT IDH2.

We next sought to identify the progenitor population that enasidenib acts on to drive erythroid maturation. Enasidenib did not increase the number of BFU-E or CFU-E colonies in colony-forming assays (Figure 3A and Supplemental Figure 9), nor did it increase the percentage of BFU-E and CFU-E progenitors in liquid culture after 4 days (Figure 3B and Supplemental Figure 10A) (13). In contrast, enasidenib increased late-stage erythroid differentiation after 8 days (14), with increased percentage of GPA (3.7-fold) and increased percentage of Band-3+ (2.7-fold) (Figure 3C and Supplemental Figure 10B). As erythroid cells differentiate, they gain CD71 expression (CFU-E stage) and subsequently acquire GPA (erythroblast stage) (15–18). Consistent with the observation that enasidenib augments differentiation by acting on more mature erythroid progenitors, enasidenib treatment did not affect the percentage of CD71+GPA+ cells in the first 4 days of culture, but instead increased the percentage of CD71+GPA+ cells from day 5 onward (Figure 3D). Furthermore, at least 72 hours of treatment was required for increased differentiation, as wash-out of the drug before that time point resulted in no increase in erythroid differentiation (Figure 3E). Seventy-two hours of erythroid culture corresponds to the time point when CD71 expression is gained in differentiating erythroid cells in our assay, providing additional evidence that enasidenib does not have an effect before the acquisition of CD71 (Figure 3F). Treating sorted mature CD71+ erythroid progenitors with enasidenib increased erythroid differentiation compared with DMSO controls, whereas enasidenib treatment of CD71+ mid/low early erythroid progenitors showed no effect (Figure 3G and Supplemental Figure 1A). Inhibition of CD71, the transferrin receptor, by a small molecule inhibitor (ferrostatin II) caused a dose-dependent decrease in enasidenib-mediated differentiation (Supplemental Figure 11). Together, these observations provide strong evidence that enasidenib acts on CD71+ erythroid progenitors to increase late-stage differentiation.

To further elucidate the mechanism of enasidenib-induced erythroid differentiation, we considered whether enasidenib might rapidly induce transcriptional changes in CD71+ cells. We conducted RNA-sequencing of sorted CD71+ erythroid progenitors treated with enasidenib or DMSO for 24 hours, a time point at which there were no observable erythroid progenitor differences between DMSO- and enasidenib-treated cells (Supplemental Figure 12A). RNA-seq analysis revealed limited transcriptional changes with early enasidenib treatment (Supplemental Figure 12, B and C), suggesting that enasidenib does not directly drive major transcriptional programs.

Given that CD71 is the transferrin receptor and allows for iron uptake into erythroid precursor cells, we hypothesized that enasidenib may affect erythroid differentiation through modulation of the heme biosynthesis pathway, as iron is a critical component of heme synthesis. Enasidenib is known to inhibit ATP binding cassette subfamily G member 2 (ABC2G2) (19), a transporter highly expressed in erythroid progenitors that is responsible for efflux of protoporphyrin IX (PPIX), the direct precursor of heme, from the mitochondrion and the cytosol (20–22). Inhibition of ABC2G2 by enasidenib might therefore lead to PPIX accumulation, potentially driving increased production of heme and hemoglobin synthesis, leading to increased erythroid differentiation. To explore this hypothesis, we first confirmed expression of ABC2G2 on differentiating erythroid progenitors in our assay and observed no difference in ABC2G2 expression between DMSO- and enasidenib-treated cells (Supplemental Figure 13). Consistent with previous data, enasidenib exhibited dose-dependent inhibition of ABC2G2 activity in CHO cells expressing human ABC2G2 (Figure 4A). Measurement of PPIX fluore-
of excess PPIX and is seen in porphyrias with PPIX accumulation (23). This increased PPIX did not increase reactive oxygen species (Supplemental Figure 14A) or oxidative stress, as measured by the GSH/GSSG ratio (Supplemental Figure 14B). To determine if increased PPIX enhances erythropoiesis, we treated CB-CD34+ cells in EDC with 20 μM Ko143, a potent ABCG2 inhibitor, and observed a similar increase in the percentage of CD71+GPA+ cells and PPIX fluorescence, as seen with enasidenib (Figure 4, F and G).

Figure 2. Enasidenib increases erythroid differentiation independently of IDH2. (A) FC of percentage of CD71+GPA+ (DMSO = 1) in CB-CD34+ -derived cells on day 8 of EDC with AG-120 (n = 4), AGI-6780 (n = 3), and AG-881 (n = 4). (B) D-2-HG measurement in the parental THP-1 cell line, an inducible IDH2 R140Q mutant THP-1 cell line, and CB-CD34+ -derived cells treated with DMSO or enasidenib for 8 days in EDC (n = 3). (C) FC of percentage of CD71+GPA+ (DMSO only = 1) in CB-CD34+ -derived cells on day 8 of EDC with the addition of (2R)-octyl-alpha-2HG at the indicated concentrations (n = 3). (D) Schematic of CRISPR-Cas9 knockout strategy, with disruption of IDH2 in exon 3 and integration of AAV donors with BFP or GFP reporters. RHA/LHA – right/left homology arm (E) PCR with a reverse primer in the AAV donor (SFFV) and forward primer in the genome (IDH2) to confirm site-specific integration of the AAV donor. AAVS1-edited cells (safe harbor locus) were used as control. (F) Western blot showing knockout of IDH2 in 3 independent CB samples, with vinculin as the loading control. (G) FC of percentage of CD71+GPA-high IDH2-KO cells at day 8 of EDC compared with AAVS1 control (AAVS1 = 1) (n = 3). (H) FC of percentage of CD71+GPA-high in AAVS1 and IDH2-KO cells treated with DMSO or enasidenib (AAVS1 DMSO = 1, with statistical comparisons made to each respective DMSO condition) (n = 3). Cells were gated on live, singlet, BFP+GFP+ before gating on CD71/GPA. Graphs represent mean ± SD. Statistical significance was calculated using unpaired 2-tailed t tests. *P < 0.05, **P < 0.01, ***P < 0.001.
Intracellular heme (ferrous PPIX) drives transcriptional and translational programs that promote coordinated erythroid differentiation through multiple mechanisms, including modulation of Bach1 transcriptional activity and heme-regulated eIF2α kinase (HRI) kinase activity (24–26). Because heme is a strong driver of globin chain synthesis (25–27), we measured transcription of globin chain genes following treatment with enasidenib by quantitative PCR (qPCR) and observed a striking upregulation of all globin chains, including HBA1/2 (23-fold), HBB (8-fold), HBD (4-fold), and HBG1/2 (3.5-fold) (Figure 4H). These results support a model in which ABCG2 inhibition by enasidenib drives PPIX accumulation, leading to increased heme and hemoglobin production in erythroid progenitors, thereby driving increased erythroid differentiation (Figure 4I).

Symptomatic anemia remains a major clinical problem in cancer patients regardless of mutational context, and contributes to decreased quality of life and increased morbidity. Anemia is also a significant side effect of cancer treatments, ranging from regimens of platinum-based chemotherapy and radiation to novel agents such as azacitadine and venetoclax (28, 29). Given that transfusion independence in cancer patients is associated with increased survival, drugs that improve normal hematopoiesis have great potential to reduce morbidity and mortality (30). In this study, we show that enasidenib, an FDA-approved therapy for treatment of...
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Relapsed/refractory IDH2-mutant AML, drives erythroid differentiation in CD71+ erythroid precursors independently of IDH2 through modulation of PPIX homeostasis and hemoglobin production. These novel findings provide a mechanism explaining the previous clinical observations that enasidenib promotes increased hemoglobin levels and RBC transfusion independence in patients with AML, even when blast count is unchanged (6, 31). This study is the first to present evidence that enasidenib can potentially be repurposed to treat anemia in any clinical context with functional erythroid precursors. Thus, our results provide the groundwork for a clinical trial using enasidenib for improving anemia and decreasing transfusion burden in a wide range of diseases.

Methods

See the Supplemental Material for the detailed experimental methods.

Study approval and data availability. Cord blood and normal bone marrow samples were obtained according to protocols approved by the Administrative Panel on Human Subjects Research Institutional Review Board/Stem Cell Research Oversight Panel (IRB/SCRO) (Stanford IRB nos. 36560, 6453, 14839, and SCRO-291)
with informed consent. RNA-sequencing data are available through GEO (accession number GSE140108).

**Author contributions**

RD, TYZ, TK, DT, ML, EG, AN, and RM designed experiments. RD, TYZ, TK, DT, ML, EG, MS, SK, YN, and RY optimized and conducted experiments. RD, TK, AA, and RM analyzed data. RD and RM wrote the manuscript.

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