Cancer–related anemia is present in over 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 red blood cell (RBC) transfusion-independence in AML patients 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.
Enasidenib drives human erythroid differentiation independently of isocitrate dehydrogenase 2

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Abstract

Cancer–related anemia is present in over 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 red blood cell (RBC) transfusion-independence in AML patients 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.
**Introduction**

Acute Myeloid Leukemia (AML) remains one of the most difficult cancers to treat, with a low cure rate (~25%) and a 2-year survival rate of 30% (1, 2). High-throughput sequencing of AML patient samples has identified mutations, including FMS-like tyrosine kinase 3 (FLT3) and isocitrate dehydrogenase 1/2 (IDH1/IDH2), for which targeted therapies have been developed. Enasidenib is an FDA-approved, first-in-class agent that preferentially inhibits IDH2-mutant activity and reduces levels of the oncometabolite D-2-Hydroxyglutarate (D-2-HG), allowing for differentiation of IDH2-mutated blasts (3–5). Patients with IDH2-mutant AML demonstrated promising clinical responses with enasidenib leading to its FDA approval in 2017 for IDH2-mutant AML. Notably, patients who did not develop objective responses also derived clinical benefit from enasidenib. Most strikingly, greater than 50% of enasidenib-treated patients who did not demonstrate a decrease in their blast count exhibited improvements in their peripheral blood counts and reached red blood cell (RBC) transfusion independence (6). The mechanism underlying this phenomenon is unknown, but is of great clinical interest given the high transfusion-dependence and anemia-associated complications universally associated with AML and other cancers (7). Thus, we sought to investigate how enasidenib drives normal hematopoiesis independent of its effects on AML blast differentiation to improve quality of life and reduce morbidity in cancer-related anemia.
Results and Discussion

We first investigated the effects of enasidenib on normal erythroid differentiation of CD34+ hematopoietic stem and progenitor cells (HSPCs) isolated from umbilical cord blood (CB-CD34+), and identified enhanced differentiation, indicated by an increase in %CD71+GPA+ cells after culture with EPO, SCF, and IL-3 (Erythroid Differentiation Culture, EDC) for eight days (Figure 1A left panel, Supplementary Figure 1A, 1B). CB-CD34+ cells from different donors had a wide range of baseline differentiation capacity. Enasidenib was especially effective at increasing differentiation in CB-CD34+ cells that had lower baseline differentiation capacity (<40% CD71+GPA+), with a 2.5-fold increase in the proportion of differentiated cells compared to control (Figure 1A, middle and right panels). Enasidenib increased both the proportion and total number of CD71+GPA+ cells (Figure 1A, B) without any decrease in cell viability (Supplementary Figure 2). This increase in erythroid differentiation was dose-dependent and observed at a range of doses from 1-25 µM (Figure 1C). Similar results were observed in CD34+ cells isolated from normal human bone marrow (Figure 1D, Supplementary Figure 1C), as well as in the IDH2-wildtype TF-1 erythroleukemic cell line (Supplementary Figure 1D, Supplementary Figure 4B). No increase in myeloid differentiation (%CD33+CD14+) was observed in treated cells (Supplementary Figure 3), indicating that enasidenib selectively promotes erythroid differentiation. The enasidenib-mediated increase in %CD71+GPA+ cells was dependent on EPO signaling and did not occur in the absence of the erythroid differentiation-promoting cytokines (Supplementary Figure 4). In the presence of EPO, enasidenib induced a gene expression signature characteristic of maturing erythrocytes (8), with increased expression of GATA1, EPOR, and KLF1, and decreased SPI1 and GATA2 (Figure 1E, Supplementary Figure 4). Enasidenib-treated progenitor cells further demonstrated increased hemoglobin production (Figure 1F-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 (Supplementary 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 (Supplementary Figure 6A, 6B). To explore whether the effect of enasidenib on erythroid differentiation was mediated through 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 wildtype IDH2 at high doses (3), we sought to determine whether the action of enasidenib on erythroid precursors was due to decreased activity of wildtype IDH2. IDH2 normally functions in the TCA cycle, converting isocitrate into alpha-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 LC-MS and isotope labeling (Supplementary Figure 7). This indicates that wildtype 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 utilized CRISPR-Cas9/AAV6 to knock out (KO) IDH2 in CB-CD34+ cells (Figure 2D, Supplementary 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 (Figures 2E, 2F). IDH2-KO CB-CD34+ cells exhibited decreased baseline erythroid differentiation compared to control AAVS1-edited cells (Figure 2G). However, similar to wildtype CB-CD34+ cells, treatment of IDH2-KO CB-CD34+ cells with enasidenib still demonstrated a 3.4-fold increase in %CD71+GPA+ erythroid cells compared to DMSO treatment (Figure 2H). These results indicate that enasidenib augments erythroid differentiation independently of both mutant and wildtype 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, Supplementary Figure 9), nor did it increase the percent of BFU-E and CFU-E progenitors in liquid culture after four days (Figure 3B, Supplementary Figure 10A) (13). In contrast, enasidenib increased late-stage erythroid differentiation after eight days (14), with increased %GPA (3.7 fold) and increased %Band-3+ (2.7 fold) (Figure 3C, Supplementary 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 %CD71+GPA- cells in the first 4 days of culture, but instead increased %CD71+GPA+ cells from day 5 onward (Figure 3D). Furthermore, at least 72 hours of treatment were required for increased differentiation, as washout of the drug prior to that time-point resulted in no increase in erythroid differentiation (Figure 3E). 72 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 prior to the acquisition of CD71 (Figure 3F). Indeed, treating sorted mature CD71+ erythroid progenitors with enasidenib increased erythroid differentiation compared to DMSO controls, whereas enasidenib treatment of CD71-mid/low early erythroid progenitors showed no effect (Figure 3G, Supplementary Figure 1A). Inhibition of CD71, the transferrin receptor, by a small molecule inhibitor (ferristatin II) caused a dose-dependent decrease in enasidenib-mediated differentiation (Supplementary 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 and 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 (Supplementary Figure 12A). RNA-seq analysis revealed limited transcriptional changes with early enasidenib treatment (Supplementary Figure 12B and 12C), suggesting that enasidenib does not directly drive major transcriptional programs.

Given that CD71 is the transferrin receptor and allows for iron uptake into erythropoietic precursors, 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 (ABCG2) (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
Inhibition of ABCG2 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 ABCG2 on differentiating erythroid progenitors in our assay and observed no difference in ABCG2 expression between DMSO and enasidenib-treated cells (Supplementary Figure 13). Consistent with previous data, enasidenib exhibited dose-dependent inhibition of ABCG2 activity in CHO cells expressing human ABCG2 (Figure 4A). Measurement of PPIX fluorescence by flow cytometry (Figure 4B) and microscopy (Figure 4C) in CB-CD34+-derived erythroid progenitors revealed a 1.2-fold increase of PPIX fluorescence in enasidenib-treated cells compared to DMSO. Ultra-Performance Liquid Chromatography (UPLC) measurement of PPIX showed a 6.1-fold increase in PPIX levels after enasidenib treatment (Figure 4D), along with increased hemin (FePPIX) and ZnPPIX (Figure 4E), a form of PPIX with incorporated zinc instead of iron. ZnPPIX formation is indicative of excess PPIX and is seen in porphyrias with PPIX accumulation (23). This increased PPIX did not increase reactive oxygen species (Supplementary Figure 14A) or oxidative stress, as measured by the GSH/GSSG ratio (Supplementary 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 %CD71+GPA+ cells and PPIX fluorescence, as seen with enasidenib (Figure 4F and 4G). 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 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 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 AML patients, 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 supplemental materials for detailed experimental methods.

Study Approval and Data Availability

Cord blood and normal bone marrow samples were obtained according to the Administrative Panel on Human Subjects Research Institutional Review Board/Stem Cell Research Oversight Panel (IRB/SCRO) approved protocols (Stanford IRB no. 36560, no. 6453, no. 14839 and SCRO-291) with informed consent. RNA-sequencing data is available through GEO (Accession Number GSE140108).

Author Contributions


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References


Figure 1. Enasidenib Augments Erythroid Differentiation (A) (Left) Proportion of CD71+GPA+ (%CD71+GPA+) cells after 8 days culture of CB-CD34+ cells in erythroid differentiation media (EDC) with DMSO or enasidenib 10 µM (Ena) (n=24 independent CB specimens). Fold change (FC) of %CD71+GPA+ cells (DMSO=1) cells with baseline differentiation capacity (%CD71+GPA+) of <40% (Middle) (n=14) or >40% (Right) (n=10). (B) Number of CB-derived CD71+GPA+ cells at D8 of EDC. (n=4) (C) Dose response of enasidenib, represented as FC of %CD71+GPA+ cells (DMSO=1) at D8 of EDC (n=4). (D) (Left) Proportion of CD71+GPA+ cells at D8 of EDC of CD34+ cells from normal bone marrow (BM) (n=3). (Right) FC of %CD71+GPA+ cells (DMSO=1) (n=3). (E) qPCR detection of relative RNA expression of erythroid and myeloid transcription factors with enasidenib treatment compared to DMSO of CB-CD34+ cells at D8 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 D8 of CB-CD34+ cells in EDC treated with DMSO or enasidenib 10 µM (n=3) and stained with benzidine. (I) Representative image at D8 of CB-CD34+ cells in EDC treated with DMSO or enasidenib 10 µM (n=3) and stained with Wright-Giemsa. Arrows indicate maturing erythrocytes. Graphs represent Mean ± SD. Statistical significance was calculated using unpaired two-tailed t-tests.
Figure 2. Enasidenib Increases Erythroid Differentiation Independently of IDH2

(A) FC of %CD71+GPA+ (DMSO=1) in CB-CD34+-derived cells on D8 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 %CD71+GPA+ (DMSO only=1) in CB-CD34+-derived cells on D8 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 %CD71+GPA-high IDH2-KO cells at D8 of EDC compared to AAVS1 control (AAVS1=1) (n=3). (H) FC 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+ prior to gating on CD71/GPA. Graphs represent Mean ± SD. Statistical significance was calculated using unpaired two-tailed t-tests.
Figure 3. Enasidenib Acts on Mature CD71+ Erythroid Progenitors to Increase Differentiation.

(A) Methylcellulose colony forming assay of CB-CD34+ cells indicating the number of erythroid colonies (BFU-E) and myeloid colonies (GM/M/GEMM) observed with DMSO or enasidenib treatment after 14 days (n=3). (B) FC of %BFU-E (IL3R-CD34+CD36-) (Middle) and %CFU-E (IL3R-CD34-CD36+) (Right) at 4 days (D4) of EDC (DMSO=1) (n=3). (C) (Left) FC of %GPA+ and (Right) %GPA+Band3+ at D8 of EDC (DMSO=1) (n=3). (D) Time course of erythroid differentiation: FC of %CD71+GPA- (Left), %CD71+GPA+ (Middle), and %CD71+GPA-high (Right) relative to untreated cells (not shown) (n=3). (E) FC of %CD71+GPA+ measured at D8 of EDC, with DMSO or enasidenib washed out (w/o) of the culture at the indicated timepoints (DMSO=1) (n=4). (F) Timeline of the gain of CD71 expression (%CD71+GPA+) in 3 untreated CB samples. (G) Cells were sorted into CD71 mid/low, CD71+GPA-, CD71+GPA-low, and CD71+GPA-mid after 6 days of EDC and then treated with DMSO or enasidenib for 4 days. FC of %CD71+GPA-high (DMSO for each population=1) (n=3). Graphs represent Mean ± SD. Statistical significance was calculated using unpaired two-tailed t-tests.
Figure 4. Enasidenib Modulates Heme Biosynthesis Through Accumulation of PPIX (A) Enasidenib-mediated inhibition of ABCG2-mediated Hoechst efflux in CHO cells, measured in duplicate, as percent inhibition relative to DMSO control. (B) FC of MFI measuring PPIX fluorescence by flow cytometry (BV650 channel) in CB-CD34+-derived cells at D8 of EDC (DMSO=1) (n=4). (C) Representative microscopy image of PPIX (HeNe 633 laser, 20X) in CB-CD34+-derived cells at D8 of EDC (n=3). (D) UPLC levels of PPIX after 8 days of enasidenib treatment. For (D) and (E), each point represents 3 independent CB-CD34+ samples that were pooled together before metabolite measurement. (E) UPLC levels of hemin and ZnPPIX after 8 days of enasidenib treatment. (F) FC of %CD71+GPA+ cells at D8 of EDC after treatment with enasidenib or Ko143 (DMSO=1) (n=5). (G) FC of MFI measuring PPIX fluorescence by flow cytometry (BV650 channel) in CB-CD34+-derived cells at D8 of EDC with enasidenib or Ko143 (DMSO=1) (n=4). (H) qPCR determination of relative RNA expression of hemoglobin genes in enasidenib-treated CB-CD34+-derived cells at D8 of EDC (DMSO=1) (n=3). (I) Schematic of proposed model. Graphs represent Mean ± SD. Statistical significance was calculated using unpaired two-tailed t-tests.