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RESEARCH PAPER

Prolyl hydroxylase inhibition corrects functional iron deficiency and inflammation-induced anaemia in rats

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BACKGROUND AND PURPOSE

Small-molecule inhibitors of prolyl hydroxylase (PHD) enzymes are a novel target for the treatment of anaemia and functional iron deficiency (FID). Other than being orally bioavailable, the differentiation of PHD inhibitors from recombinant human erythropoietin (rhEPO) has not been demonstrated.

EXPERIMENTAL APPROACH

JNJ-42905343 was identified and characterized as a novel inhibitor of PHD and its action was compared with rhEPO in healthy rats and in a rat model of inflammation-induced anaemia and FID [peptidoglycan-polysaccharide (PGPS) model].

KEY RESULTS

Oral administration of JNJ-42905343 to healthy rats increased the gene expression of cytochrome b (DcytB) and divalent metal-ion transporter 1 (DMT1) in the duodenum, and increased plasma EPO. Repeated administration of JNJ-42905343 for 28 days increased blood haemoglobin, mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV). The serum iron concentration was increased with low doses (0.3 $mg \cdot kg^{-1}$) but reduced at high doses (6 $mg \cdot kg^{-1}$). In PGPS-treated rats, administration of JNJ-42905343 for 28 days corrected FID and anaemia, as reflected by increased blood haemoglobin, MCH and MCV. Increased expression of *DcytB* and *DMT1* genes in the duodenum resulting in increased iron availability was defined as the mechanism for these effects. rhEPO did not affect DcytB and DMT1 and was not effective in PGPS-treated rats.

CONCLUSIONS AND IMPLICATIONS

PHD inhibition has a beneficial effect on iron metabolism in addition to stimulating the release of EPO. Small-molecule inhibitors of PHD such as JNJ-42905343 represent a mechanism distinct from rhEPO to treat anaemia and FID.

Abbreviations

2-OG, 2-oxoglutarate; ACD, anaemia of chronic disease; DMT1, divalent metal-ion transporter 1; DcytB, duodenal cytochrome b; EPO, erythropoietin; FIH, factor inhibiting HIF; FID, functional iron deficiency; HIF, hypoxia-inducible transcription factors; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; PGPS, peptidoglycan-polysaccharide; PHD, prolyl hydroxylase; rhEPO, recombinant human erythropoietin; VHL, von Hippel–Lindau

Tables of Links

TARGETS	
Transporters[a]	**Enzymes**[b]
DMT1	PHD1
	PHD2
	PHD3

LIGANDS	
2-oxoglutarate (2-OG)	IL-6
Ascorbate	Succinate
Erythropoietin (EPO)	TNF-α
IFN-γ	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Hypoxia-inducible transcription factors (HIF-α) participate in the body's adaptive response to hypoxia. Cellular HIF-α increases rapidly under hypoxic conditions and, after translocation to the nucleus, HIF-α forms a heterodimer with HIF-β to enhance transcription of a large number of genes with a hypoxia response element in their promoter region (Semenza, 2011). The genes regulated by HIF are involved in angiogenesis, anaerobic metabolism, hypoxia tolerance, iron metabolism and transport, and erythropoiesis. Cellular HIF-α content and transcriptional activity are tightly controlled by a family of enzymes called prolyl hydroxylases (PHDs; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001) and factor inhibiting HIF (FIH; Lando *et al.*, 2002). PHD enzymes introduce a hydroxyl group into specific prolyl residues on the HIF-α molecule. Hydroxylated HIF-α is a high-affinity substrate for von Hippel–Lindau (VHL) E3 ubiquitin ligase, which binds to and directs HIF-α to the 26S proteasome where it undergoes poly-ubiquitination by the E3-ubiquitin-ligase complex and is degraded. FIH hydroxylates a C-terminal asparagyl residue in the HIF-α family that is essential for interaction with the co-activator p300 and suppresses HIF-α's transcriptional activity (Lando *et al.*, 2002). PHDs and FIH contain a non-haem iron bound to their active site. They require HIF-α, 2-oxoglutarate (2-OG) and molecular oxygen as substrates and ascorbate as a co-factor for activity (Bruick and McKnight, 2001; Epstein *et al.*, 2001).

HIF-2α is an integral part of the feedback loop that regulates blood haemoglobin and small-molecule inhibitors of PHD represent an attractive prospect for the treatment of anaemia. PHD loss of function mutations and HIF-2α gain-of-function mutants are associated with polycythaemia in humans (McMullin, 2010) and small-molecule PHD inhibitors have been shown to increase blood haemoglobin in subjects with chronic kidney disease (Besarab *et al.*, 2010). Taken together, these observations demonstrate that PHD inhibitors will increase blood haemoglobin. In the present work, we describe the pharmacological characterization of a novel, potent and selective PHD inhibitor, JNJ-42905343 (Figure 1), and investigate the mechanisms whereby it corrects anaemia in a rat model of inflammation-induced anaemia.

Functional iron deficiency (FID) is a major component of the anaemia of chronic disease (ACD) seen in subjects with infectious, inflammatory and malignant diseases (Thomas *et al.*, 2013). FID occurs when there is insufficient iron incorporation into red blood cells despite apparently normal levels of iron in the bone marrow and normal serum ferritin values. FID can also be induced by erythropoietin (EPO) and FID might explain why some subjects are refractory to EPO (Mittman *et al.*, 1997; Johnson *et al.*, 2007; Elliott *et al.*, 2009).

HIF-2α was found to up-regulate duodenal cytochrome b (DcytB) and divalent metal-ion transporter 1 (DMT1) in mice (Mastrogiannaki *et al.*, 2009; Shah *et al.*, 2009). DcytB is an ascorbate-dependent ferric enzyme that mediates the reduction of Fe^{3+} to Fe^{2+} at the brush border membrane of the enterocyte. DMT1 is a divalent metal-ion transporter whose

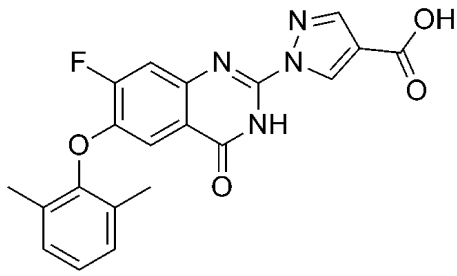

Figure 1
Structure of 1-(6-(2,6-dimethylphenoxy)-7-fluoro-4-oxo-3,4-dihydroquinazolin-2-yl)-1H-pyrazole-4-carboxylic acid (JNJ-42905343).

main physiological role is to transport Fe^{2+} into the enterocyte. Thus, stabilization of HIF-2α in the local environment of the enterocyte up-regulates the expression of DcytB and DMT1, leading to an increase in iron uptake. To evaluate this potential difference between the mechanisms, the effect of the novel PHD inhibitor JNJ-42905343 and recombinant human erythropoietin (rhEPO) on haematological parameters and the gene expression of DcytB and DMT1 in the duodenum of normal rats and rats with inflammation-induced anaemia were examined.

Methods

In vitro *characterization of JNJ-42905343 as a potent and selective PHD inhibitor*

The expression, purification and enzymatic assay of full-length human PHD1, PHD2 and PHD3 as well as the catalytic domain of PHD2 containing amino acids 181 to 417 ($PHD2_{181–417}$) have been described previously (Kanelakis *et al.*, 2009; Barrett *et al.*, 2011) as the methods to characterize the molecular pharmacology of PHD inhibitors. Briefly, HIF-1α peptide residues 547–581 [KNPFSTGDTDLDLEMLAPYIPMDDDFQLRSFDQLS] (10 μM; California Peptide Research Inc., Napa, CA, USA) and [5-^{14}C]-2-oxoglutarate (50 mCi·mmol^{-1}; Moravek Chemicals, Brea, CA, USA) were incubated in reaction buffer (40 mM Tris–HCl, pH 7.5, 0.4 mg·mL^{-1} catalase, 0.5 mM DTT, 1 mM ascorbate). The [1-^{14}C]-succinate formed from the enzymatic reaction was quantified as previously described. The sensitivity to ambient Fe^{2+} concentration was assessed by inclusion of 50 μM $Fe(NH_4SO_4)_2$ in the $PHD2_{181–417}$ assay. Competition with 2-OG was assessed using the $PHD2_{181–417}$ catalytic domain testing a range of JNJ-42905343 and 2-OG concentrations. To test whether the inhibition was reversible, JNJ-42905343 was pre-incubated for 30 min with the enzyme, after which the reaction was started by addition of a 10-fold excess of the reaction mixture containing all necessary co-factors.

The potency of JNJ-42905343 to stabilize HIF-1α in HeLa cells and stimulate the release of EPO into the media bathing Hep3B cells was assessed as previously described (Barrett *et al.*, 2011). HeLa cells were treated with JNJ-42905343 for 6 h in DMEM growth media and then lysed, and HIF-1α content was assessed according to the manufacturer's specifications (Meso-Scale Discovery, Gaithersburg, MD, USA). Hep3B cells were cultured in DMEM/high glucose (Life Technologies, Carlsbad, CA, USA) supplemented with 10% foetal calf serum and 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 50 IU·mL^{-1} of penicillin and 50 μg·mL^{-1} of streptomycin.

Selectivity of JNJ-42905343 for PHD over FIH

The potency of JNJ-42905343 for inhibition of the structurally related enzyme FIH was assessed as previously described (Kanelakis *et al.*, 2009; Barrett *et al.*, 2011). JNJ-42905343 was pre-incubated for 30 min, followed by a 10 min incubation with 1 μM [5-^{14}C]-2-oxoglutarate, in the presence of 10 μM $Fe(NH_4SO_4)_2$.

Animals

All animal studies were performed in USDA-registered, fully accredited AAALAC animal care and use programme. The programme follows Johnson and Johnson Corporate Guidelines for Humane Care and Use of Animals (2013) and all study procedures were reviewed and approved by the Institutional Animal Care and Use Committee. The results are reported here in a format consistent with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Details of these studies related to the design, conduct and animals care programme are included in the Supporting Information.

Acute effects of JNJ-42905343 on gene expression in rats

The acute effects of JNJ-42905343 on the expression of selected genes in the liver, kidney and duodenum were assessed in female Lewis rats (200–225 g) after administration of a single oral dose of 1 or 3 mg·kg^{-1} and compared with vehicle control or 50 μg·kg^{-1} rhEPO given i.p. Two hours post-dose, animals were killed; samples were collected and stored in RNA stabilization reagent, RNAlater (Life Technologies). This time point was selected to exclude the possibility that effects on gene expression were secondary to stimulation of haematopoiesis. Gene expression quantification was carried out using the TaqMan® Gene Expression Assay (Applied Biosystems, Carlsbad, CA, USA) for the following gene products: EPO (Rn01481376_m1), duodenal cytochrome b (DcytB, Rn01484657_m1) and divalent metal transporter-1 (DMT-1, Rn00565927_m1). β-Actin was used to normalize gene expression. The same methods were used to quantify DMT1 and DcytB in peptidoglycan-polysaccharide (PGPS)-treated rats included in the anaemia model (see the following discussion).

Haematological effects of JNJ-42905343 in normal rats

A detailed analysis of the pharmacokinetic–pharmacodynamic relationship for JNJ-42905343 was conducted in normal and PGPS-treated female Lewis rats after administration of a single oral dose. Doses of 0.3, 1, 3 and 6 mg·kg^{-1} were selected to produce effects on plasma EPO ranging from sub-threshold effects to very high elevations of plasma EPO expected to be associated with complete correction of anaemia in the PGPS inflammation-induced anaemia model. Animals in the vehicle control group were administered the same 10 mL·kg^{-1} volume of 0.5% hydroxypropyl-β-methyl cellulose. Serial plasma samples were collected and assayed for determination of JNJ-42905343 and EPO levels before and up to 24 h after administration of JNJ-42905343 or vehicle control. Rat EPO was assayed in accordance with the manufacture's specifications (Meso Scale Discovery, Gaithersburg, MD, USA).

The haematological effects of daily administration of JNJ-42905343 to female Lewis rats (180–200 g) were assessed over 28 days. JNJ-42905343 was administered orally at doses of 0.3, 1, 3 and 6 mg·kg^{-1} and the effects were compared to once weekly administration of rhEPO (50 μg·kg^{-1}, i.p.). Animals in the rhEPO group received vehicle control orally every day, while animals in the other treatment group received the corresponding vehicle for rhEPO i.p. once a week (in PBS).

Haematological effects were assessed on days 14, 21 and 28 (Advia 120 haematology analyser; Siemens, Deerfield, IL, USA) from 0.5 mL samples of EDTA anti-coagulated blood. Plasma samples were collected on day 28 for analysis of selected plasma biomarkers including serum iron.

Haematological effects of JNJ-42905343 in rats with inflammation-induced anaemia

The effects of JNJ-42905343 were assessed in an inflammation-induced anaemia model as described previously (Barrett *et al.*, 2011, based on Sartor *et al.*, 1989). Briefly, 15 μg·kg^{-1} PGPS (Becton Dickinson, Lee Laboratories, Grayson, GA, USA) was administered i.p. to female Lewis rats (180–200 g) to induce chronic systemic inflammation and protracted, moderate anaemia. The resulting anaemia was assessed 14 days after administration of PGPS from EDTA anti-coagulated blood. These data were used to sort animals into treatment groups with a similar mean and SD for blood haemoglobin before starting treatment. Thereafter, animals were treated daily by the oral route with either, vehicle control or JNJ-42905343 at doses of 0.3, 1 or 3 mg·kg^{-1}. rhEPO was administered once a week at a dose of 50 μg·kg^{-1}, i.p. Animals in the rhEPO group received vehicle control for JNJ-42905343 orally every day, while animals in the oral dosing groups received vehicle for rhEPO i.p. once per week. The study was not blinded. Treatment was continued for 28 days with serial haematological assessment on days 14, 21 and 28. Serum iron, plasma IL-6, IFN-γ and TNF-α protein concentrations as well as duodenal RNA expression of DcytB and DMT1 were assessed. Samples were collected between 24 and 36 h after the last dose of JNJ-42905343.

On day 28, bone from the femur was collected for histological evaluation. The bones were fixed in 10% formalin, decaled in 5% formic acid, embedded in paraffin, sectioned at 5 μm thickness and stained with haematoxylin and eosin (H&E). The bone marrow images were captured by QImaging camera (Surrey, BC, Canada) imported into QCapture software (for MAC OS 9 & X V2.68) mounted on Nikon Eclipse E800 microscopy (Melville, NY, USA) and the objective lens was set at 40×/0.75. Sections were scored for PGPS-induced damage according to the following scoring system. Representative bone marrow sections were scored by a trained pathologist from 0 to 4, with 4 as most necrotic and a second score (0–4) for decreased red blood cell precursors and increased white blood cell precursors in the field of view at 200× magnification. The two scores were then added together to represent the overall severity of the lesion with the range of possible values being 0–8.

Data analysis

The data were analysed using GraphPad Prism, version 4 or 5 (GraphPad Software, San Diego, CA, USA) and presented as mean ± SEM. Differences between groups were analysed using ANOVA and either Dunnett's or Tukey's test for differences. For haematology experiments in animals, a sample size of 12 was selected to obtain statistically significant increases in blood haemoglobin except for rhEPO where samples size was 8. The smaller sample size was utilized for rhEPO due to the limited available of rhEPO. In some cases, the *n* values are reported as a range because blood clots or insufficient sample volume prevented haematological analysis. For plasma EPO kinetics, a sample size of 3 was selected as the smallest size from which a representative result could be obtained.

Results

In vitro *pharmacological characterization of JNJ-42905343*

JNJ-42905343 was a potent, reversible, non-iron-dependent 'mixed' inhibitor of isolated and purified human PHD enzymes *in vitro* (Figure 2A–D). JNJ-42905343 was a potent inhibitor of all three full-length PHD isozymes (Figure 2A). The pK_I values were 8.07 ± 0.05, 7.48 ± 0.03 and 7.27 ± 0.03, calculated via the Cheng–Prusoff method at 1 μM concentration of 2-OG, for PHD1, PHD2 and PHD3 respectively. The molecular mechanism of inhibition for JNJ-42905343 was examined using the catalytic domain of $PHD2_{181–417}$. Inclusion of 50 μM Fe^{2+} in the assay produced only a modest, 1.8-fold decrease in the potency of JNJ-42905343 (Figure 2B). To test whether the inhibition produced by JNJ-42905343 was reversible, the compound was incubated with the catalytic domain of the $PHD2_{181–417}$ enzyme across a range of concentrations for 30 min, after which the reaction was started by addition of a 10-fold excess of the reaction mixture containing all necessary co-factors (Figure 2C). This resulted in a 32-fold rightward shift of the concentration–response curve consistent with reversible inhibition of the enzyme. Competition analysis across a range of 2-OG concentrations showed that the inhibition produced by JNJ-42905343 was best described as 'mixed' because at higher concentrations of JNJ-42905343, additional 2-OG was not able to completely restore enzyme activity (Figure 2D). Nevertheless, fitting the data to a competitive model yields a $pK_I = 7.8$. This is roughly consistent with the estimate obtained using the Cheng–Prusoff estimation for the full-length PHD2 above. Despite the 'mixed' inhibition observed in the enzyme assay, JNJ-42905343 is expected to bind to the active site of PHD enzymes based on analogy to the binding mode for 2-OG (Rosen *et al.*, 2010) and JNJ-42041935 (Barrett *et al.*, 2011), as well as a collection of other undisclosed, structurally related PHD inhibitors observed in co-crystal structures with $PHD2_{181–417}$.

JNJ-42905343 stabilized HIF1-α in a concentration-dependent fashion in HeLa cells and stimulated the release of EPO from Hep3B cells with similar potency. The EC_{50} was 19.7 μM in HeLa cells for stabilization of cellular HIF-1α content (Figure 2E). Fitting the concentration–response curve to the maximum response observed in Hep3B cells (excluding the highest concentration from analysis because the response was bell-shaped) gave an EC_{50} = 20.3 μM for stimulation of EPO secretion (Figure 2F).

JNJ-42905343 had a low potency for inhibition of the structurally related enzyme FIH with an IC_{50} = 3.8 μM, yielding a 240-fold selectivity window over the potency for inhibition of the full-length PHD2.

Haematological effects of JNJ-42905343 in normal rats

Acute administration of JNJ-42905343 resulted in time- and dose-dependent increases in plasma EPO in normal female

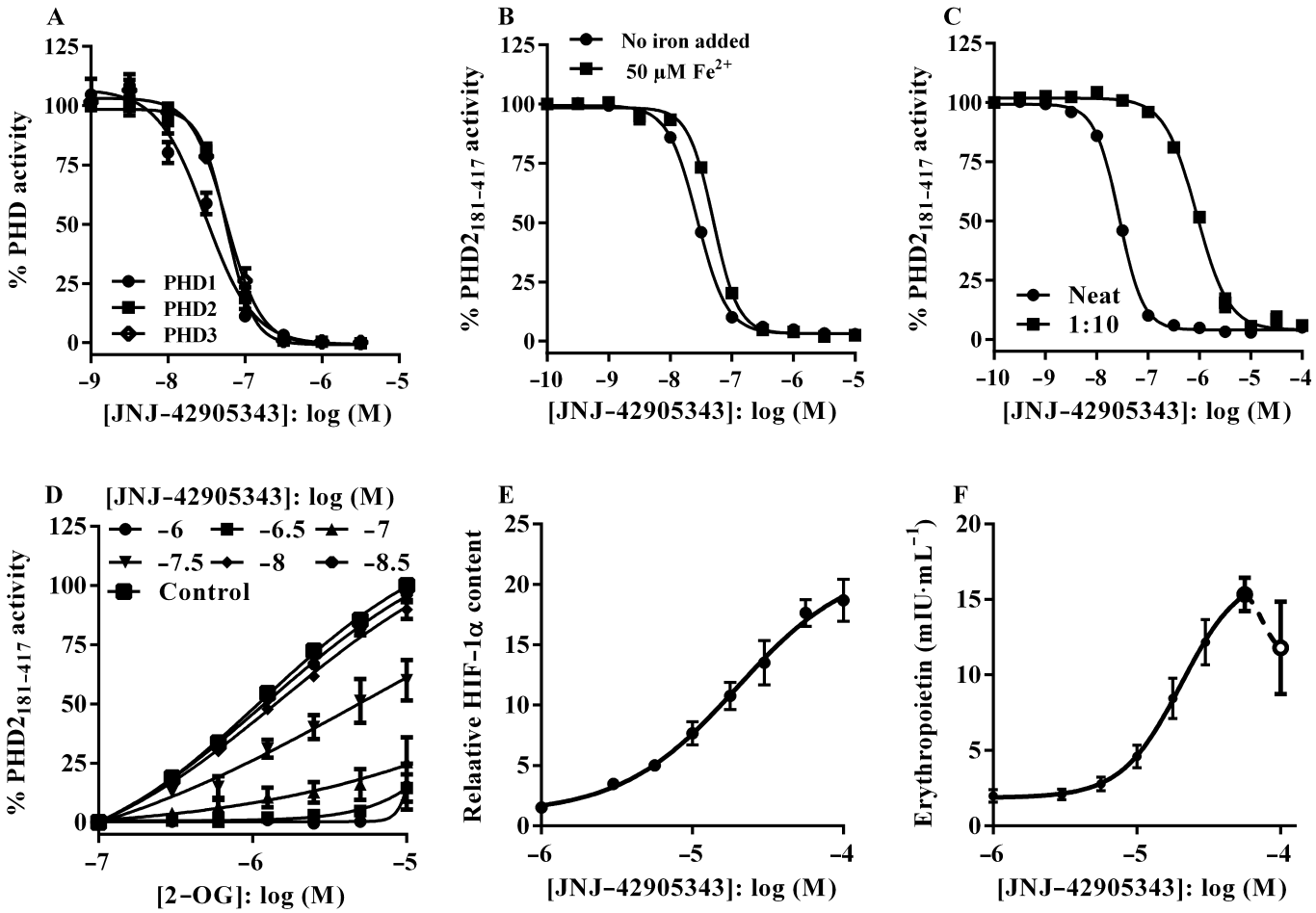

Figure 2

In vitro characterization of JNJ-42905343 as an inhibitor of human PHD enzymes. (A) Concentration-dependent inhibition of the full-length human PHD isotypes by JNJ-42905343. (B) Inhibition of $PHD2_{181–417}$ by JNJ-42905343 is not affected by 50 µM iron. (C) Inhibition of $PHD2_{181–417}$ by JNJ-42905343 is reversible. JNJ-42905343 was pre-incubated for 30 min, after which the reaction was started by the addition of a 10-fold excess of the reaction mixture containing all necessary co-factors. (D) Competition analysis for the inhibition of $PHD2_{181–417}$ by JNJ-42905343. The concentrations of JNJ-42905343 tested are shown across the top and the concentrations of 2-OG tested are shown on the *x*-axis. Global curve fit analysis yields a $pK_i = 7.8$. (E) Concentration-dependent stabilization of HIF-1α in HeLa cells after a 6 h incubation. (F) Concentration-dependent release of EPO from Hep3B cells after a 24 incubation. All values are the mean ± SEM of three replicates in duplicate for isolated enzyme experiment (A–D) and $n = 6$ for cellular data (E–F).

Lewis rats (Figure 3A). The 0.3 $mg \cdot kg^{-1}$ dose was without effect and defines the 1 $mg \cdot kg^{-1}$ dose as the threshold dose for a systemic effect. There was a close relationship between the plasma EPO kinetics and the plasma kinetics of JNJ-42905343 (see Supporting Information).

The effects of a single dose of JNJ-42905343 or rhEPO on the expression of DcytB and DMT1 in the duodenum and EPO expression in the kidney and liver were examined 2 h after administration in normal rats (Figures 3B and 4). The 1 $mg \cdot kg^{-1}$ dose tended to increase DcytB, while the 3 $mg \cdot kg^{-1}$ produced a statistically significant increase in the expression of this iron reductase. Both the 1 and 3 $mg \cdot kg^{-1}$ doses of JNJ-42905343 increased the expression of DMT1. rhEPO did not affect the RNA expression of either DcytB or DMT1 at the dose tested.

Daily oral administration of JNJ-42905343 to normal Lewis rats resulted in time- and dose-dependent increases in blood haemoglobin, mean corpuscular haemoglobin (MCH) and mean corpuscular volume (MCV) over a 28 day period (Figure 3C–E). Blood haemoglobin was increased from baseline values of 143 ± 2 $g \cdot L^{-1}$ to a maximum of 230 ± 3 $g \cdot L^{-1}$ for the 6 $mg \cdot kg^{-1}$ dose group on day 28 (Figure 3C). Administration of rhEPO once a week at a dose of 50 $\mu g \cdot kg^{-1}$ (i.p.) was highly effective in normal rats and increased blood haemoglobin to 195 ± 5 $g \cdot L^{-1}$ on day 28 (Figure 3C). JNJ-42905343 increased MCH and MCV in a dose-dependent fashion on day 28 of the study. The dose of rhEPO (50 $\mu g \cdot kg^{-1}$, i.p.) modestly increased MCH and MCV but to a lesser degree than the maximum effects observed with JNJ-42095343 (Figure 3C–F). The effects on MCH and MCV are

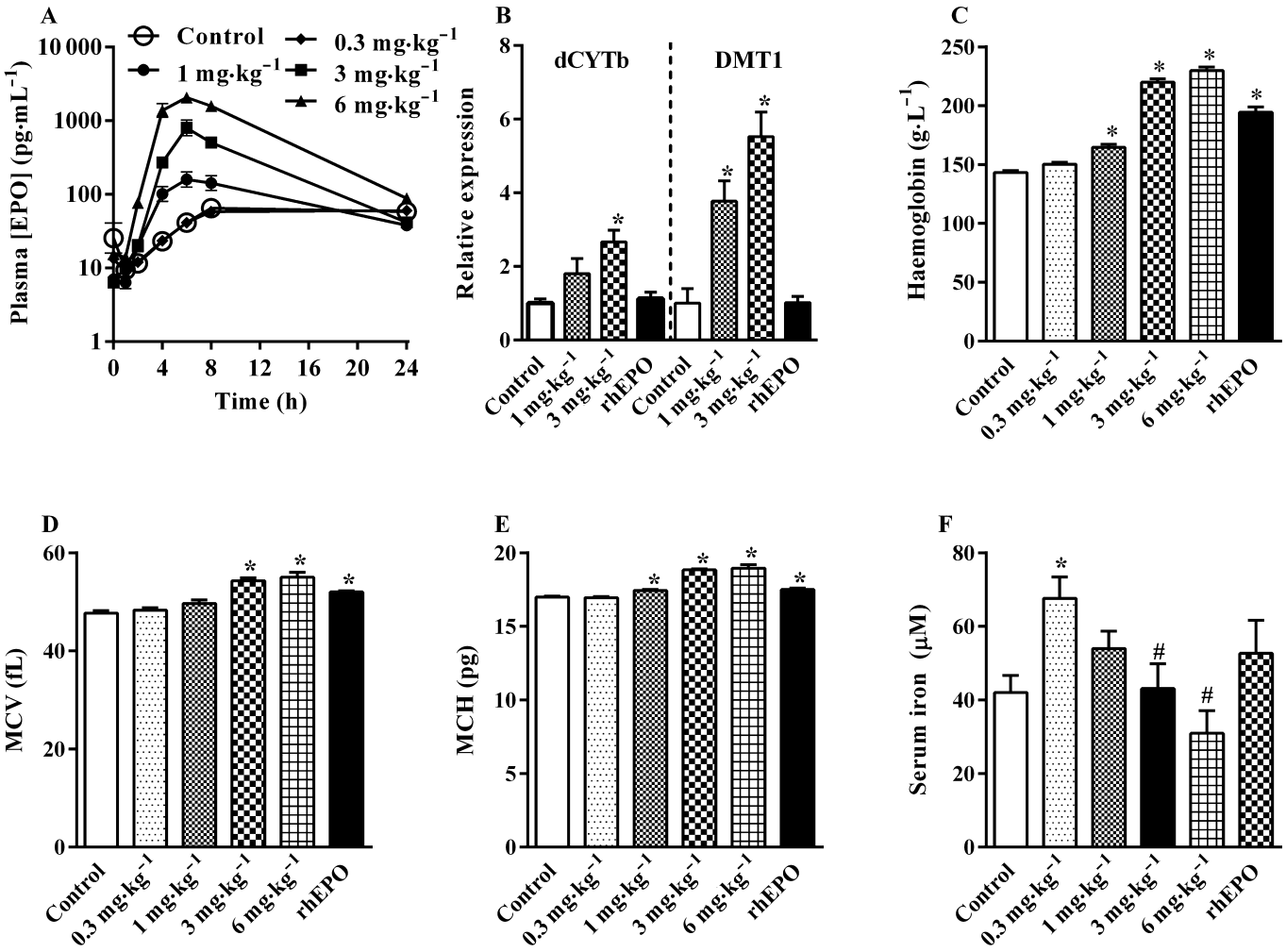

Figure 3

Effect of JNJ-42905343 or rhEPO on haematological and related parameters in normal female Lewis rats. (A) Dose- and time-dependent increases in plasma EPO after oral administration of JNJ-42905343. Values are mean ± SEM, $n = 3$. (B) Effect of JNJ-42905343 on the duodenal gene expression of the iron reductase DcytB and iron transporter protein DMT1. Values are mean ± SEM, $n = 6$. (C-F) Dose-related changes in blood haemoglobin, MCV, MCH and serum iron. Values are mean ± SEM, $n = 7$–12 after 28 days of treatment. *$P < 0.05$ versus control. #$P < 0.05$ for 0.3 mg·kg^{-1} versus 3 and 6 mg·kg^{-1} JNJ-42905343.

consistent with increased iron availability to the bone marrow.

JNJ-42905343 had a biphasic dose–response relationship for effects on serum iron, while rhEPO had no effect (Figure 3F). At 0.3 mg·kg^{-1}, JNJ-42905343 significantly increased serum iron relative to the vehicle control, whereas at the 6 mg·kg^{-1} dose, serum iron was nominally reduced. In addition, the 0.3 mg·kg^{-1} dose significantly elevated serum iron as compared to the 3 and 6 mg·kg^{-1} doses (Tukey's test,) demonstrating the biphasic nature of the dose–response relationship.

Relative EPO expression in the liver and kidney were increased 2 h after the 3 mg·kg^{-1} dose of JNJ-42905343 but not by exogenous administration of rhEPO (Figure 4). The 1 mg·kg^{-1} dose increased EPO expression in the kidney but not in the liver.

Haematological effects of JNJ-42905343 in PGPS-treated rats

As expected and previously described for the inflammation-induced model of anaemia, administration of PGPS resulted in signs of joint inflammation such that both hind limbs became swollen and mobility was reduced in all treated animals. Consistent with this, the white blood cell count was substantially elevated 14 days after administration of PGPS (see Supporting Information).

Basal plasma EPO was elevated ~10-fold in PGPS-treated rats relative to non-PGPS controls (Figure 3A vs. Figure 5A). JNJ-42905343 caused a time- and dose-dependent increase in plasma EPO. The time to the peak elevation after administration of JNJ-42095343 was delayed relative to non-PGPS-treated controls consistent with the pharmacokinetics of JNJ-42905343 in these animals (Figures 3A and 5A and

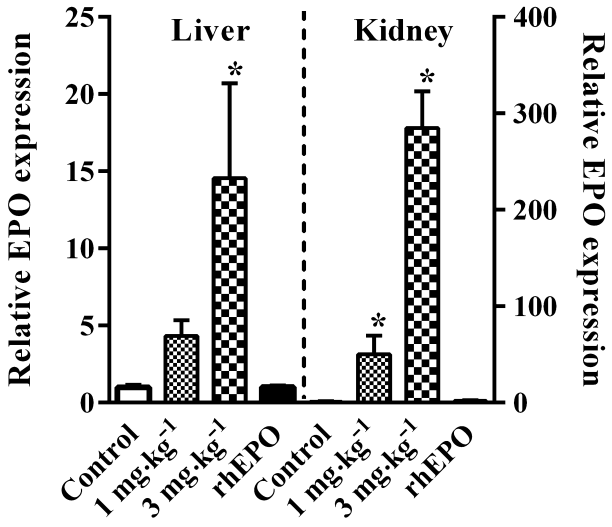

Figure 4

Effect of oral administration of JNJ-42905343 or exogenous rhEPO on EPO gene expression in the liver and kidney of female Lewis rats. Gene expression was assessed 2 h after treatment and is presented as the mean ± SEM, $n = 6$. Values presented were normalized to β-actin expression. Comparisons were made only within a tissue and not between tissues. *$P < 0.01$ versus control.

Supporting Information). Again, there was a close relationship between plasma EPO kinetics and plasma JNJ-42905343 kinetics.

The effects of the highest dose of JNJ-42905343 tested and rhEPO on the expression of DcytB and DMT1 were assessed from duodenal samples collected on day 28 of the chronic PGPS study (Figure 5B). Treatment with PGPS alone did not have a notable effect on the expression of either gene. However, after treatment for 28 days with a daily 3 mg·kg^{-1} dose of JNJ-42905343, a robust up-regulation in the expression of DcytB and DMT1 was observed. Weekly rhEPO administration had no effect on the relative gene expression.

PGPS administration resulted in anaemia with blood haemoglobin values reduced to 106 ± 1 g·L^{-1} relative to 163 ± 1 g·L^{-1} in non-PGPS-treated controls (Figure 5C, $P < 0.05$). JNJ-42905343 caused a dose-dependent increase in blood haemoglobin, haematocrit, red blood cell and reticulocyte number, MCH and MCV after a 28-day treatment period in rats with PGPS-induced anaemia (Figure 5C–F). At the end of the treatment duration, blood haemoglobin was 159 ± 1 g·L^{-1} in vehicle control rats and remained reduced to a value of 101 ± 2 g·L^{-1} in PGPS-treated rats. JNJ-42905343 increased blood haemoglobin in a dose-dependent fashion such that the 3 mg·kg^{-1} dose completely restored blood haemoglobin to values similar to those observed in non-PGPS-treated rats (157 ± 4 g·L^{-1}). Notably, weekly administration of 50 μg·kg^{-1} rhEPO i.p. had no effect on any of the haematological parameters in this model. MCV returned to untreated value at 1 mg·kg^{-1} dose of JNJ-42905343, while it was increased beyond the level observed in non-PGPS-treated rats by the 3 mg·kg^{-1} dose. MCH was restored to values similar to those observed in non-PGPS-treated rats at the 3 mg·kg^{-1} dose of JNJ-42905343. Serum iron was markedly reduced by treatment with PGPS and was not affected by treatment with JNJ-42905343 or rhEPO.

The white blood cell count, serum IL-6 and IFN-γ remained elevated in PGPS-treated rats at the end of the chronic PGPS study and were not different between groups. Serum TNF-α also remained markedly elevated in PGPS-treated rats but was modestly reduced in the JNJ-42905343-treated animals (see Supporting Information).

Administration of PGPS caused inflammation-induced anaemia that was reflected in peripheral red and white cell counts as well as corresponding changes to the morphology of the bone marrow samples (Figure 6). H&E staining showed a marked decrease in red blood cell precursors and increased white blood cell precursors in the PGPS-treated rats (Figure 6E). PGPS treatment increased the damage score to a mean of 5.6 on day 28 from a value of zero in rats not treated with PGPS. JNJ-42905343 caused a dose-dependent reduction such that the 3 mg·kg^{-1} dose reduced the score to a mean value of 0.6. rhEPO did not have a statistically significant effect on the bone marrow morphology score.

Discussion and conclusions

In vitro JNJ-42905343 was a potent inhibitor of the human PHD isozymes. The inhibition produced was reversible and not effected by inclusion of iron. This profile is similar to that described previously for JNJ-42041935 as a 2-OG competitive/active site inhibitor of all three PHD enzymes (Barrett *et al.*, 2011), with JNJ-42905343 being ~30-fold more potent. The behaviour of JNJ-42905343 in terms of competition with 2-OG was not consistent with a competitive model and it was best described as a 'mixed' enzyme inhibitor. This could be explained by JNJ-42905343 having a slow off-rate for dissociation from the enzyme and not allowing 2-OG to bind to the enzyme to restore activity within the time frame of the assay. This is consistent with the higher affinity of JNJ-42905343 for PHD relative to JNJ-42041935 and the relatively short duration of the *in vitro* assay. In HeLa cells, JNJ-42905343 increased the intracellular HIF-1α content and increased EPO secretion from Hep3B cells in a concentration-dependent fashion consistent with inhibition of PHD enzyme in these systems.

In normal female Lewis rats, JNJ-42905343 was a potent stimulator of EPO release and haematopoiesis. We examined the source of the plasma EPO by quantifying the gene expression in the kidney and liver. The kidney was the most sensitive to the actions of JNJ-42905343; however, the liver also showed significant increases in the gene expression. This is consistent with the results obtained with the liver selective triple knockout of PHD1-3 (Minamishima and Kaelin, 2010). It is interesting to note that knockout of all three PHD isozymes was required to produce this effect and single knockout or knockout of any combination of two PHD isozymes had no effect on hepatic expression of the EPO gene. Our results with the PHD1-3 inhibitor JNJ-42905343 and those recently obtained with another PHD inhibitor are consistent with this observation (Flamme *et al.*, 2014).

Our previous paper describing JNJ-42041935 as a PHD inhibitor demonstrated that the characteristics of the FID and

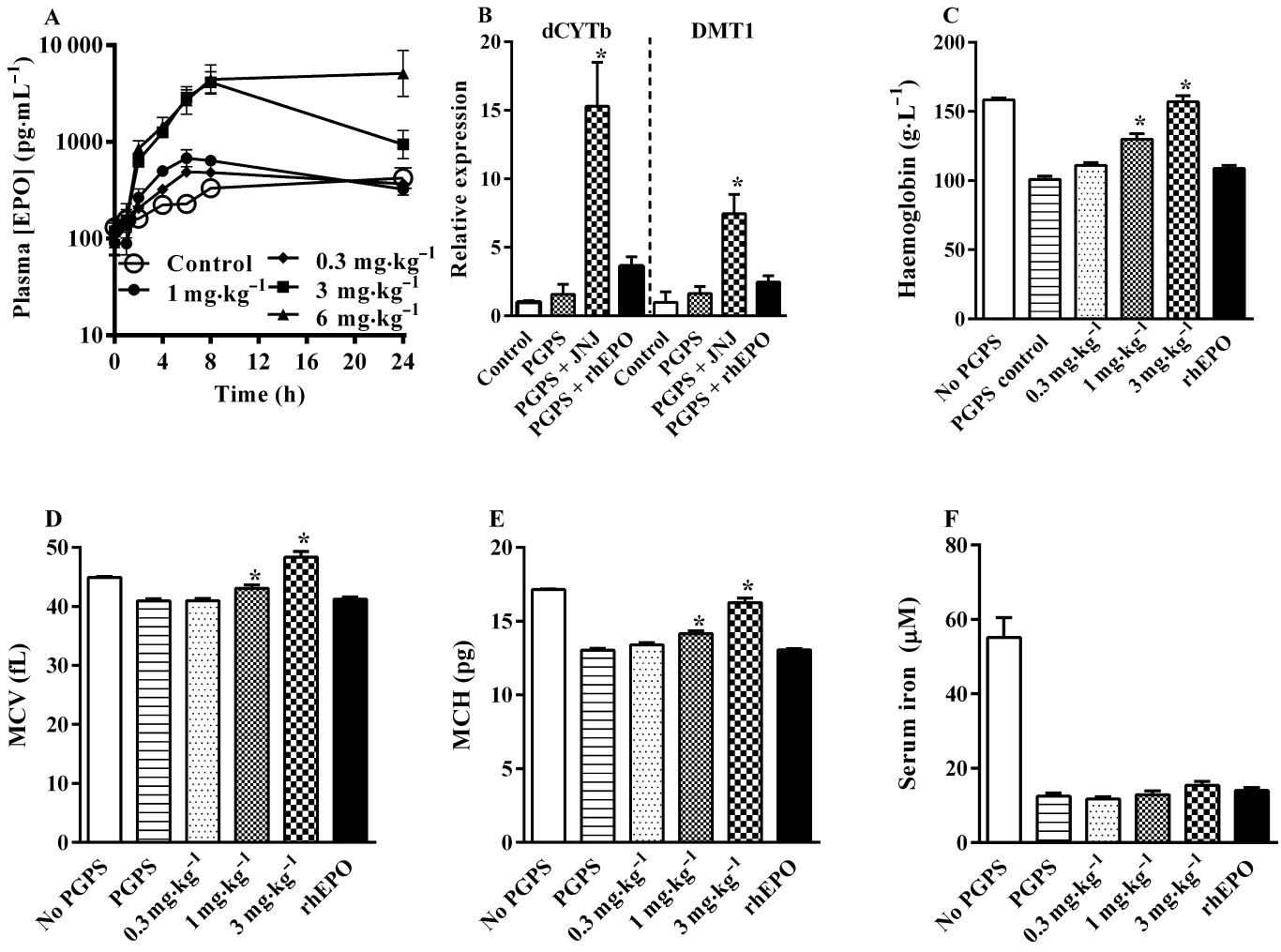

Figure 5

Effect of 28 days of daily treatment with JNJ-42905343 on inflammation-induced anaemia in female Lewis rats. (A) Dose- and time-dependent elevation of plasma EPO are shown after a single oral dose of JNJ-42905343. Values are mean ± SEM, $n = 3$. (B) Expressions of the iron reductase DcytB and iron transporter protein DMT1 in the duodenum are summarized as the mean ± SEM, $n = 5–7$. Treatments shown are no PGPS, PGPS control, 3 mg·kg^{-1} JNJ-42905343 + PGPS and rhEPO + PGPS. (C-F) Dose-dependent changes in blood haemoglobin, MCV, MCH and serum iron concentration. The daily dose of JNJ-42905343 is shown on the *x*-axis. The dose of rhEPO was 50 μg·kg^{-1} (i.p.) once per week. All drug-treated groups were pretreated with PGPS in this study. Values are the mean ± SEM, $n = 8–12$. (C–F) *$P < 0.05$ versus control.

inflammation-induced anaemia could be studied in the PGPS model and that a PHD inhibitor could beneficially influence the hallmark effects of these disorders in red blood cells, more specifically JNJ-42041935-corrected MCV and MCH towards normal values (Barrett *et al.*, 2011). EPO receptor agonists can be effective in this model (Coccia *et al.*, 2001) and it was not clear from the previous work if PHD inhibitors were just 'oral EPO' or if other mechanisms were involved. Another group recently examined the effects of a PHD inhibitor in this model (Flamme *et al.*, 2014) but did not examine the mechanism(s) whereby PHD inhibitors have such effects. The current work excludes anti-inflammatory mechanisms, demonstrates that the release of EPO is not sufficient to explain the robust haematological effects of PHD inhibitors and defines iron absorption in the duodenum via DcytB and DMT1 as one locus where PHD inhibitors act to correct FID and inflammation-induced anaemia. The morphological effects on bone marrow suggest that it might be another locus of action, but further studies would be required to define this.

One possible explanation for the robust haematological effects of PHD inhibitors in the PGPS model is that PHD inhibitors have anti-inflammatory actions. In the current work, JNJ-42095343 had a marginal effect on TNF-α, no effect on IL-6 or IFN-γ, and white blood cell count remained elevated in PGPS-treated rats given JNJ-42095343. This effectively excludes a possible anti-inflammatory mechanism to explain the robust haematological changes in the rat.

PHD inhibitors might be considered 'oral EPO' if the mechanism for the haematopoietic effect was solely to stimulate the release endogenous EPO. JNJ-42905343 and other PHD inhibitors elevate plasma EPO in a dose-dependent fashion. However, it is not clear if this is the only mechanism

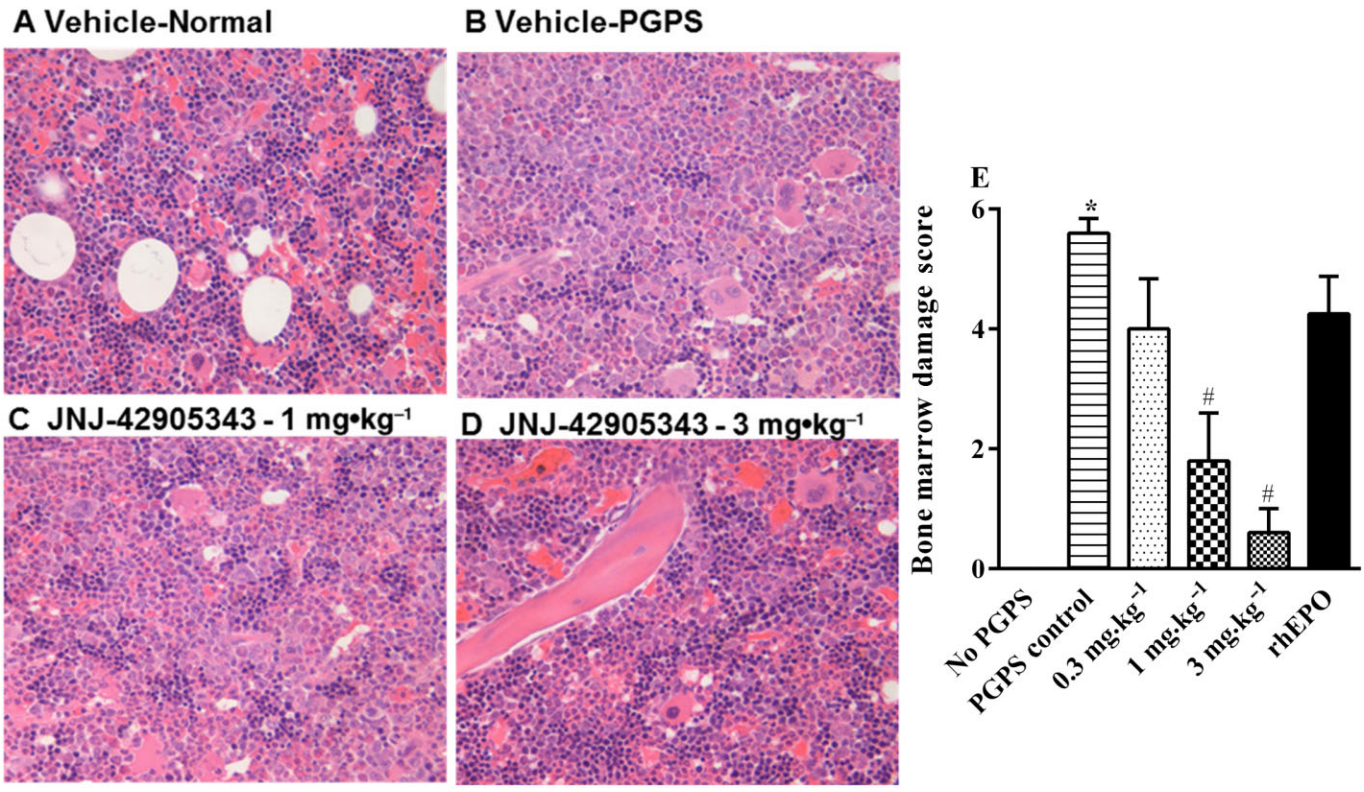

Figure 6

Effect of chronic oral administration of JNJ-42905343 on the morphology of bone marrow in female Lewis rats with PGPS inflammation-induced anaemia. The histology section shows an H&E stain of bone marrow at 400× magnification for the following groups: vehicle control, vehicle-PGPS-treated, 1 mg·kg^{-1} JNJ-42905343-PGPS-treated and 3 mg·kg^{-1} JNJ-42905343-PGPS-treated bone marrow sections (A–D). Note the decreased red blood cell precursors and increased white blood cell precursors in the vehicle-PGPS-treated rats and markedly increased red blood cell precursors in the JNJ-42905343-treated rats. (E) Summary of PGPS bone damage score. *P < 0.001 for the increase in bone marrow damage induced by PGPS and #P < 0.05 for reversal of the bone marrow damage relative to PGPS control. Values are mean ± SEM, n = 4–5.

whereby haematopoiesis is stimulated by PHD inhibitors. The current study replicates our previous finding (Barrett *et al.*, 2011) that a dose of rhEPO that is very effective in normal rats is ineffective in PGPS-treated rats. Notably in the current study, the effects of rhEPO on MCV and MCH were markedly less than for JNJ-42905343 in PGPS-treated rats and rhEPO had no effect on blood haemoglobin at all. The relative lack of effect of rhEPO on MCH in the current study is consistent with that described for darbepoetin alfa in the PGPS model (Coccia *et al.*, 2001). An ~10-fold elevation of plasma EPO was observed in all PGPS-treated animals, indicating that the anaemia was being sensed by the hypoxia regulatory feedback loop. The elevation of plasma EPO was apparently insufficient to correct inflammation-induced anaemia and FID. Taken together, these observations demonstrate that other mechanisms contribute to the haematopoietic response to PHD inhibitors.

Up-regulation of DcytB and DMT1 in the duodenum by JNJ-42905343 was found to be both rapid and durable even in the face of a potent inflammatory stimulus. A corresponding increase in serum iron was observed in normal rats at the lowest dose of JNJ-42905343, but this response was not apparent at higher doses. The biphase response of serum iron can be explained by the inter-relationship between increased iron absorption and incorporation into haemoglobin. The increase in DcytB and DMT1 gene expression can be expected to result in increased absorption capacity for iron and increased serum iron. This is observed directly at the lowest dose of JNJ-429045343 (0.3 mg·kg^{-1}). At higher doses, PHD inhibition continues to increase DcytB and DMT1 expression and in addition acts systemically to increase the release of EPO. With the increased level of haematopoiesis, iron is incorporated into haemoglobin and serum iron is reduced. This is observed as a reduction in serum iron at higher doses relative to the 0.3 mg·kg^{-1} dose. In the case of PGPS-treated rats, iron is sequestered to the reticuloendothelial system and serum iron remains low despite the large increases in haematopoiesis and blood haemoglobin (Weiss, 2009). The relationship between increased iron uptake via DcytB and DMT1 in the duodenum to changes in haematological parameters such as blood haemoglobin, MCH and MCV is complex and complicated by the kinetics of iron absorption and red blood cell formation such that a direct correspondence is not expected between drug effects on one parameter versus another.

These results obtained with a small-molecule PHD inhibitor for DcytB and DMT1 are consistent with those obtained in genetic mouse models. Selective knockout of HIF-2α in enterocytes down-regulated DcytB and DMT1 in the duode-

num of mice and resulted in a reduction in serum iron (Mastrogiannaki *et al.*, 2009; Shah *et al.*, 2009). Stabilization of HIF-2α in the local environment of the enterocyte results in up-regulation of DcytB and DMT1 expression, leading to an increase in iron uptake that could correct FID. Interestingly, FID may be an important factor limiting the effectiveness of rhEPO in haemodialysis patients (Mittman *et al.*, 1997; Johnson *et al.*, 2007; Elliott *et al.*, 2009). Thus, our results define duodenal uptake of iron as a target to treat FID and inflammation-induced anaemia. Oral administration of a low dose of a PHD inhibitor might be an effective treatment for FID and ACD without the requirement for systemic exposure to the compound. As the local concentration of the PHD inhibitor that surrounds the enterocyte of the duodenum is higher after oral administered than that achieved after systemic absorption, a selective inhibition of PHD enzymes in the enterocytes is achieved. In this regard, a PHD inhibitor that has limited systemic absorption and/or one with a short half-life might offer a unique treatment option for FID and ACD.

We can only speculate on the mechanism(s) for the dose-dependent changes in bone morphology produced by JNJ-42905343. IL-6, IFN-γ and TNF-α are known to suppress iron absorption and act directly on the bone marrow to suppress haematopoiesis in part by rendering EPO a less potent haematopoietic stimulus (Weiss, 2009). IL-6, IFN-γ and TNF-α plasma concentrations were markedly elevated in PGPS-treated anaemic animals and they were not substantially reduced by PHD inhibition. This excludes an anti-inflammatory effect as a mechanism for the effects on bone marrow. It is not clear from these studies whether the increased expression of iron transporters in the duodenum and the resulting increased iron flux are sufficient to explain the changes in the bone marrow.

Taken together, these results speak to a broad and coordinated haematological response produced by PHD inhibition that influences not only EPO release but also iron metabolism. PHD inhibition represents a novel treatment mechanism for FID and ACD.

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Author contributions

T. D. B and H. V. participated in research design, conducted experiments, contributed new reagents or analytical tools, performed data analysis and wrote the manuscript. N. P. S. and M. H. R. participated in research design and contributed new reagents or analytical tools. H. L. P., T. I. B., K. C. K., K. P. M., F. S. and M. D. R. participated in research design, conducted experiments, contributed new reagents or analytical tools, and performed data analysis. X. W., W. Y., J. Y. M., J. S., J. G. and D.-T. T. conducted experiments and performed data analysis.

Conflict of interest

All authors were employees of Janssen Pharmaceutical Companies of Johnson & Johnson at the time when the work was done.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13188

Figure S1 Plasma concentrations of JNJ-42905343. Dose and time-dependent increases in plasma JNJ-42905343 after oral administration of JNJ-42905343. (A) Normal female Lewis rats. (B) Female Lewis rats administered PGPS. Values are mean ± SEM, n = 2–3. Note that the 1 mg·kg–1 dose in normal rats has only n = 2 at the 24 h time point and this distorts impression of the terminal elimination phase.

Figure S2 Plasma concentrations of IL-6, TNF-a and interferon-g in female Lewis rats. All drug-treated groups were pretreated with PGPS in this study. Values are the mean ± SEM, n = 8–12 for plasma IL-6 (A), TNF-a (B) and interferion-g (C). *P < 0.05 versus PGPS control group.

Figure S3 White blood cell count in female Lewis rats before starting treatment and after 28 days of treatment with either JNJ-42905343 or rhEPO at the doses indicated. All drug-treated animals as well as the PGPS group were treated with PGPS 14 days prior the first assessment (defined as day 0). Values are mean ± SEM, n = 8–12 on day 0 of the study (A) and after 28 days of treatment (B). All PGPS-treated rats have elevated white blood cells relative to non-PGPS-treated rats (P < 0.05).

Figure S4 Kinetics of blood haemoglobin in normal and PGPS-pretreated female Lewis rats. Dose groups for JNJ-42095343 are indicated on the bottom of each panel for normal rats (A) and PGPS-treated rats (B). Values are the mean ± SEM, n = 8–12.

Figure S5 Evaluation of the potential impact of JNJ-42905343 on serum toxicology markers. (A) Serum ALT, (B) AST, (C) blood urea nitrogen (BUN) and (D) creatinine. Values are mean ± SEM, n = 9–12.