Pharmacological characterization of 1-(5-chloro-6-(trifluoromethoxy)-1H-benzoimidazol-2-yl)-1H-pyrazole-4-carboxylic acid (JNJ-42041935), a potent and selective HIF prolyl hydroxylase (PHD) inhibitor

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Running title: JNJ-42041935, a potent PHD inhibitor

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Non-standard abbreviations: HIF, Hypoxia Inducible Factor; PHD, prolyl-4-hydroxylase; 2-OG, 2-oxoglutarate; HRE, hypoxia response element; pVHL, von Hippel Lindau protein; DTT, dithiothreitol; EPO erythropoietin.

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ABSTRACT

The HIF prolyl hydroxylase (PHD) enzymes represent novel targets for the treatment of anemia, ulcerative colitis, ischemic and metabolic disease *inter alia*. We have identified a novel small molecule inhibitor of PHD (JNJ-42041935) through structure-based drug design methods. The pharmacology of JNJ-42041935 was investigated in enzyme, cellular and whole animal systems and was compared to other compounds described in the literature as PHD inhibitors. JNJ-42041935, was a potent (pK_I=7.3 to 7.9), 2-oxoglutarate competitive, reversible and selective inhibitor of PHD enzymes. In addition, JNJ-42401935 was used to compare the effect of selective inhibition of PHD to intermittent, high doses (50 µg/kg i.p.) of an exogenous erythropoietin receptor agonist in an inflammation-induced anemia model in rats. JNJ-42041935 (100 µmol/kg, once a day for 14 days) was effective in reversing inflammation-induced anemia whereas erythropoietin had no effect. The results demonstrate that JNJ-42041935 is a new pharmacological tool which can be used to investigate PHD inhibition and demonstrate that PHD inhibitors offer great promise for the treatment of inflammation-induced anemia.

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INTRODUCTION

Hypoxia-inducible factor-α (HIF-α) mediates the cells transcriptional response to hypoxia (Semenza & Wang, 1992). HIF-1α was shown to be increased in concentration in cells exposed to low oxygen and to bind to the promoter of the erythropoietin gene. HIF-1α forms a heterodimeric protein complex that includes HIF-1β and p300 and then binds to the hypoxia response element (HRE) consensus sequences in the promoter region of hypoxia responsive genes and up-regulates their expression (Semenza, 2003). HIF-1α target genes encode proteins involved in a wide range of processes including erythropoiesis, angiogenesis, vasodilation and glycolysis.

The mechanism for the hypoxia responsive nature of cellular HIF-1α content was not described until 2001 when two groups independently described the role of prolyl hydroxylase (PHD) enzymes in the process (Ivan et al., 2001; Jaakkola et al., 2001). Soon after the description of the mechanism, the enzymes responsible for oxygen sensitivity were cloned and characterized (Epstein et al., 2001; Bruick & McKnight, 2001). HIF-PHD enzymes are non-heme, iron containing enzymes that require molecular oxygen, 2-oxoglutarate (2-OG), ascorbic acid and a protein substrate containing the consensus sequence for prolyl hydroxylation for catalytic activity. Once HIF is hydroxylated it binds to von Hippel Lindau protein (pVHL) and is subsequently poly-ubiquitinylated and degraded by the proteasome. This process is very efficient and cellular HIF-1α has a half life of only ~5 min under normoxic conditions. As the affinity of the PHD enzymes for oxygen is within the physiological range, changes in cellular

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oxygen concentration reduce catalytic activity of PHD enzymes and allow HIF to accumulate in cells and initiate the bodies transcriptional response to hypoxia.

Three PHD isozymes have been described in humans and the role of these different isozymes in determining cellular HIF content has been evaluated by various means. The general consensus is that PHD2 plays a dominant role in determining the cellular HIF-1α content with PHD1 and PHD3 playing modulatory roles (Berra et al., 2003). In addition, loss of function mutations in the PHD2 gene have been found in cases of familial polycythemia (Percy et al., 2006 & 2007, Al-Sheikh et al. 2007; Pappalardi et al., 2008). Studies in knockout mice also corroborate the central role of PHD2 in determining cellular HIF content as PHD2 knock-out mice die during embryonic development due to defects in hematopoiesis and formation of the vasculature (Takeda et al. 2006, Takeda et al., 2007). In contrast PHD1 and PHD3 knock-out mice are viable and have more subtle phenotypes (Takeda et al., 2008, Minamishima and Kaelin, 2010).

The possibility of mimicking the body's coordinated response to hypoxia with a small molecule PHD inhibitor offers promise in treating a range of oxygen-deprivation-related disorders such as anemia, ulcerative colitis, myocardial ischemia, stroke and metabolic disorders. Competition with necessary co-factors for PHD activity is one way to achieve this pharmacologically. Metal ions, in particular cobalt, compete with iron in the active site of the enzyme and have even been used to treat anemia but not without significant side effects. Chelation of iron with compounds such as desferrioxamine is an effective

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means to inhibit PHD enzymes (Ivan et al., 2001; Jaakkola et al., 2001). This mechanism has been used extensively *in vitro* but appears to have little effect *in vivo*. Mimetics of 2-OG such as dimethyloxalylglycine (DMOG) and N-oxalylglycine (N-OG) inhibit PHD but are not potent or have poor cellular activity (Ivan et al., 2001; Jaakkola et al., 2001). A collection of other compounds including inhibitors of the related dioxygenase enzyme procollagen prolyl 4-hydroxylase such as 3,4-dihydroxybenzoate (Warnecke et al., 2003), clioquinol (Choi et al., 2006) and ciclopirox (Linden et al., 2003) have also been used to inhibit PHD enzymes. Unfortunately these compounds are low affinity inhibitors of PHD, are not selective for PHD enzymes relative to other dioxygenases and other targets and they tend to have poor activity in cellular and *in vivo* systems. Furthermore they appear to act by different and ill-defined mechanisms.

The novel PHD inhibitor described here, JNJ-42041935, is the best characterized compound resulting from a medicinal chemistry program aimed at identifying novel inhibitors of PHD2 (Rosen et al., 2010). JNJ-42041935 is a potent, 2-OG competitive, reversible and selective inhibitor of all three PHD isozymes. In an inflammation-induced anemia model, JNJ-42041935 was more effective than intermittent, high doses of an exogenous erythropoietin receptor agonist. The results highlight JNJ-42041935 as a new tool to investigate PHD inhibition and demonstrate that PHD inhibitors offer great promise for the treatment of a range of anemic conditions.

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MATERIALS AND METHODS

Compounds

JNJ-42041935 (1-(5-chloro-6-(trifluoromethoxy)-1H-benzo[d]imidazol-2-yl)-1H-pyrazole-4-carboxylic acid; see Figure 1) was synthesized in house at Johnson & Johnson Pharmaceutical Research and Development, L.L.C. DMOG (dimethyloxalylglycine), 2-OG (2-oxoglutarate), 3,4-EDHB, ciclopirox, clioquinol and desferrioxamine were purchased from Sigma-Aldrich (St. Louis, MO).

Expression, purification and potency for inhibition of PHDs

The expression, purification and enzymatic reaction of a human PHD2 construct containing amino acids 181 to 417 ($PHD2_{181-417}$) and a construct containing the full length sequence for factor inhibiting HIF (FIH) were previously described (Kanelakis et al., 2009). Full length sequences of human PHD1, PHD2 and PHD3 were also cloned, expressed and purified by Emerald Biostructures (Bainbridge Island, WA) using similar methods. Briefly, PHD1 was cloned into baculovirus with N-terminal TEV-cleavable His-tag. A Ni-IMAC (HiTrap column, GE Healthcare, Piscataway, NJ) column was used to purify PHD1 protein. After elution the PHD1 protein was digested with TEV protease (Invitrogen, Carlsbad, CA) to remove the His-tag. PHD2 was cloned into baculovirus with an N-terminal His-tag and was purified on a Ni^{2+} HiTrap Column. For the full length PHD2 the protein, the His-tag was not removed as attempts to do so resulted in inactive protein. This is consistent with the experience of others (McDonough et al., 2006, Dao et al., 2008). PHD3 was cloned into baculovirus with an N-terminal His-tag and was purified on a Ni2+ HiTrap column.

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Purification of all PHD proteins was performed with 25 μM ammonium Fe^{2+} sulfate with 1 tablet of protease inhibitor (Roche COMPLETE without EDTA, Roche, Boulder, CO). The optimization of the reaction conditions for $PHD2_{181-417}$ have previously been described and were used for all PHD enzymatic assays (see Kanelakis et al., 2009). The protein concentration was selected to give a good signal-to-noise ratio (~10-fold increase in signal above background) and the K_M for 2-OG was determined by examining enzymatic activity over a range of 2-OG concentrations (0.1-20 μM). HIF-1α peptide residues 547-581 [KNPFSTGDTDLDLEMLAPYIPMDDDFQLRSFDQLS] (10 μM, California Peptide Research Inc., Napa, CA) (Koivunen et al., 2006), and [5-^{14}C]-2-oxoglutarate (50 mCi/mmol, Moravek Chemicals, Brea, CA) were incubated in a final volume of 0.5 ml reaction buffer (40 mM Tris-HCl, pH 7.5, 0.4 mg/ml catalase, 0.5 mM DTT, 1 mM ascorbate). The [1-^{14}C]-succinate formed from the enzymatic reaction was separated from [5-^{14}C]-2-oxoglutarate by incubating the reaction mixture with 100 μl of 0.16 M DNPH prepared in 30% perchloric acid and separated by centrifugation (Kanelakis et al., 2009).

Sensitivity of inhibition to iron

The effect of iron(II) on the ability of compounds to inhibit $PHD2_{181-417}$ was assessed by constructing a concentration-response curve for compounds in the absence and presence of 10 μM $Fe(NH_4SO_4)_2$.

Competition with 2-OG

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To assess whether compounds were competitive with 2-OG, concentration-response curves were constructed across a range of 2-OG concentrations (0.25-10 μM) and a range of inhibitor concentrations.

Assessment of the reversibility of inhibition of PHD2$_{181-417}$

In order to assess whether the inhibition of PHD2$_{181-417}$ was reversible, the compound was pre-incubated for 30 min with the enzyme after which time the reaction was started by addition of a 10-fold excess of the reaction mixture containing all necessary co-factors (an undiluted reaction was included as a control). Thus if the compounds bound to the enzyme in a reversible fashion they would be diluted to a lower, less active concentration when the reaction mixture was diluted and the concentration-response curve would be shifted by a factor commensurate with the degree of dilution. To facilitate sufficient signal to noise to allow analysis the reaction was allowed to proceed to 40 min before terminating it as described above.

Co-crystals of JNJ-42041935 and PHD2$_{181-417}$

Co-crystals of JNJ-42041935 were generated by vapor diffusion against 200 mM Na Acetate pH4.6, 2 M NaCl, 200 mM LiCl by Emerald BioStructures (Bainbridge Islands, WA).

Selectivity of JNJ-42041935 for PHD over FIH and other pharmacological loci

The potency of JNJ-42041935, DMOG and desferrioxamine for inhibition of the structurally related enzyme FIH was assessed by similar methods to those described for

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PHD2. In brief, activity of FIH was determined using purified glutathione S-transferase (GST)–tagged full-length FIH amino acids 1-350 purchased from Novus Biologicals (Littleton, CO) and a synthetic HIF-1α peptide corresponding to residues Asp788-Leu822 [DESGLPQLT SYDCEVNAPIQGSRNLQGELRAL] (10 μM, California Peptide Research). Compounds were pre-incubated with 17.1 nM FIH for 30 minutes, followed by a 10 minute incubation with 1 μM 2-^{14}C-2-oxoglutarate, in the presence of 10 μM $FeNH_4SO_4$ in reaction buffer. All other enzymatic conditions were identical to those described for $PHD2_{181-417}$ activity. The selectivity of JNJ-42041935 for inhibition of a range of other targets available for testing in commercial assays was also assessed at concentrations of 1 and 10 μM (Cerep receptor panel, CEREP, Celle L'Evescault, France and non-kinase enzyme panel & Upstate kinase panel, Millipore, Billerica, MA).

Iron binding in a protein free solution

Compounds were tested for their iron binding potential in protein free solution using calcein and $(NH_4)_2Fe(SO_4)_2.6H_2O$ (FAS) as an indicator of iron chelation (Breuer et al., 1995). A range of concentrations were tested in the presence of 0.2 μM calcein (Invitrogen, Carlsbad, CA), 0.2 μM FAS (Sigma Chemical, St. Louis, MO), 1 mM ascorbate in 20 mM HEPES, 150 mM NaCl, pH 7.2 at room temperature in the dark for an incubation period of 2.5 h. Prior kinetic analysis demonstrated that the reaction had reached equilibrium at this time. Calcein fluorescence (excitation 485, emission 530 nm) was then measured in a FL600 microplate fluorescence reader (BioTek, Winooski, Vermont USA). The dequenched fluorescence detected after the incubation with

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compound was normalized to the fluorescence measured in the presence of the prototypical iron binding compound 8-hydroxyquinoline at a concentration of 1 mM.

HIF-1α elevation and erythropoietin release from Hep3B cells

The potency of compounds for stabilization of intracellular HIF-1α and release of erythropoietin into the media was assessed in the human hepatoma cell line, Hep3B. Cells were cultured in Dulbecco's DMEM/High Glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 2mM L-Glutamine, 1% Non-Essential Amino Acids, 1mM Sodium Pyruvate, 50 IU/mL of penicillin and 50 ug/mL of streptomycin. A range of concentrations were incubated in 96-well plates with 30,000 cells/well for 24 h. Incubation media was collected and cells were subsequently lysed for assessment of cellular HIF-1α content and erythropoietin concentration (Meso-Scale Discovery, Gaithersburg, Maryland).

Hypoxia response element-driven luciferase expression in the mouse

To demonstrate that JNJ-42041935 elevated cellular HIFα content *in vivo,* the mouse model described by Safran et al. (2006) was used. In this model, luciferase bioluminescent reporter consisting of firefly luciferase fused to a region of HIF that is sufficient for oxygen-dependent degradation. Thus bioluminescence is increased when the breakdown of this HIF mimetic peptide is reduced. JNJ-42041935 or vehicle control was administered orally 1 h before administration of 50 mg/kg luciferin i.p. Mice were anesthetized using isofluorane (4% induction, 2-3% maintenance) and images were collected 15 min later by IVIS camera (Xenogen, Alameda, CA) with an exposure period

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of 5 to 20 s. Bioluminescence was quantified in the peritoneal region a total of 2 h after administration of JNJ-42041935. These studies were conducted by Jackson Laboratories (Sacramento, CA) and approved by their Institutional Animal Care and Use Committee.

Erythropoietin release in mice and hematological effects of JNJ-42041935

All procedures and experiments were performed according to internationally accepted guidelines for the care and use of laboratory animals in research and were approved by the local Institutional Animal Care and Use Committee.

Compounds were administered orally in a hydroxypropyl methyl cellulose suspension, at a dose of 300 µmol/kg to Balb/C mice (n=4/group). JNJ-42041935 was administered at doses of 30, 100 and 300 µmol/kg. For these studies plasma was collected 6 h after the dose. Plasma erythropoietin concentration was measured in accordance with the manufacturer's specifications (Mesoscale Discovery). The hematological effects of JNJ-42041935 were assessed by administering the 100 µmol/kg dose on 5 consecutive days and collecting blood anti-coagulated with EDTA on day 8 (3 days after the last dose). Hematological variables were assessed using an Advia 120 (Siemens, Deerfield, IL).

Effect of JNJ-42041935 in an inflammation-induced anemia model; comparison to erythropoietin

The effect of a once weekly dose of recombinant human erythropoietin (rhEPO) administered i.p. was characterized in normal rats prior to using this dose in an

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inflammation-induced anemia model. This dosing schedule was selected from the literature to mimic the use of this agent in the clinical setting. A 50 µg/kg rhEPO (i.p) was administered once per week for two weeks and hematological effects were evaluated after two weeks of dosing (i.e., the effects were measured a week after the 2nd dose). The pharmacokinetics and plasma erythropoietin after either a single oral dose of 10 or 100 µmol/kg JNJ-42041935 were also characterized in this gender and strain of rat.

The effects of a daily 100 µmol/kg oral dose of JNJ-42041935 were compared to 50 µg/kg i.p. rhEPO administered once-weekly in an inflammation-induced anemia model. A single i.p. injection of peptidoglycan-polysaccharide polymers (PGPS, Becton Dickinson, Lee Laboratories, Grayson, GA) derived from group A streptococci caused chronic systemic inflammation, granuloma formation, spontaneously relapsing arthritis and protracted, moderately severe anemia. Anesthetized female Lewis rats weighing 180-200 g were injected with 15 µg/kg PGPS i.p. The severity of the resulting anemia was assessed after 14 days by collecting blood, anti-coagulated with EDTA, from the tail vein and assessing hematological variables (Advia 120, Siemens, Deerfield, IL). Due to inherent variability, animals were sorted into treatment groups to give groups with similar mean and standard deviation for blood hemoglobin before starting treatment. On Day 15 (or Day 0 for treatment administration), dosing for the following treatment groups began: non-PGPS treated control, PGPS control, 100 µmol/kg JNJ-42041935 dosed orally once a day and 50 µg/kg recombinant human erythropoietin (rhEPO) dosed i.p. once a week. All animals received two oral doses of 5 ml/kg of 0.5%

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HPMC and an intraperitoneal injection of PBS to prevent dehydration and reduce the variance in hematological variables.

Hematological end-points were assessed after 7 and 14 days of treatment. On day 7, assessment of plasma erythropoietin concentration was made 2 h after the dose of rhEPO and 6 h after the oral dose JNJ-42041935 to capture the peak concentrations of human and rat erythropoietin, respectively, in these studies. All other hematological endpoints were measured on day 15 (24 hours after the final administration of test compounds).

Data analysis

The data were plotted using GraphPad Prism, version 4 or 5 (Graph Pad Software, San Diego, CA) and presented as mean±s.e.mean. For the enzyme data the average background count (cpm) was considered to be the carry over amount of [5-^{14}C]-2-oxoglutarate in the supernatant and was subtracted from each data point. For compounds that behaved as competitive inhibitors, the K_I was calculated by fitting of the data at various 2-OG concentration (Equation 1) and compared to the value obtained via the Cheng-Prussoff equation (Equation 2).

Equation 1 $$\left[\alpha(i) = \frac{\alpha_{\max}}{1+10^{((\log IC_{50} - [L])\cdot nH)}}\right]$$

where, $\alpha(i)$ is the response to the inhibitor ; α_{max} is the maximal response of an inhibitor when PHD2s activity is inhibited 100%; [L] is logarithm of compound concentration and nH is Hill slope. The pK_I was also estimated using the Cheng-Prussoff correction. A

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correction factor of 0.35 was calculated from the K_M=0.8 μM relative to the 1 μM concentration of 2-OG used to determine the pIC_{50} (Kanelakis et al., 2009).

Equation 2: $$\left[Ki = \frac{IC_{50}}{1+[S]/Km} \right]$$

where, K_i is the inhibition constant, expressed in molar concentration; IC_{50} is the concentration of inhibitor that reduces the enzyme activity to half; [S] is the substrate concentration in molar concentration; and K_M is the Michaelis-Menten constant, expressed in molar concentration.

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RESULTS

Inhibition of PHD enzymes by JNJ-42041935

JNJ-42041935 was the most potent inhibitor of $PHD2_{181-417}$ with a pIC_{50} value of 7.0±0.03 (Table 1, Figure 2A). The rank order for potency of inhibition of $PHD2_{181-417}$ was JNJ-42041935 > 3,4-EDHB > desferrioxamine = clioquinol > ciclopirox > DMOG (Table 1).

JNJ-42041935 also inhibited full length PHD1, PHD2 and PHD3 enzymes (pK_I values = 7.91±0.04, 7.29±0.05 and 7.65±0.09, respectively; Figure 2B). The Km values for these isotypes for 2-OG were 0.80, 0.50 and 0.82 μM, respectively (data not shown).

To determine if the compounds bound reversibly to the enzyme, the inhibition of $PHD2_{181-417}$ was also assessed with and without a 10-fold dilution of the assay after the incubation period. For JNJ-42041935 and DMOG, dilution of the assay resulted in an approximate 1 log unit shift in the inhibition curve. Conversely, no shift in the inhibition curve was observed for desferrioxamine (Figure 2E). Thus JNJ-42041935 is a potent and reversible inhibitor of PHD.

In vitro iron binding

Inclusion of 10 μM iron had no effect on the potencies of JNJ-42041935, 3,4-EDHB (data not shown) and DMOG for inhibition of $PHD2_{181-417}$ (see Figure 2D and Table 1). In contrast, desferrioxamine, clioquinol and ciclopirox (data not shown for the last two) were markedly less potent when iron was included in the assay (see Figure 2D and

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Table 1). The degree of iron-binding was also assessed using a protein-free fluorescence assay. Desferrioxamine, clioquinol and cyclopirox dequenched the iron-calcein fluorescence in a concentration dependent manner and reached the same maximum produced by the prototypical iron binding compound 8-hyodroxyquinoline (Figure 2F). In contrast the concentration-response curve produced by JNJ-42041935 was shallow and only reached 67% of the maximum response at a concentration of 1 mM. 3,4-EDHB had a similarly low potency for binding iron in solution. The potency ratio between desferrioxamine and JNJ-42041935 for binding iron in solution was >650-fold. These results suggest that desferrioxamine, clioquinol and ciclopirox inhibit PHD by chelating iron while JNJ-42041935, 3,4-EDHB and DMOG do not.

Selectivity of JNJ-42041935 for PHD over other pharmacological loci

JNJ-42041935 was highly selective for PHD relative to FIH (pIC_{50} ~4, Figure 2C). DMOG and desferrioxamine inhibited FIH with similar potencies to those estimated for inhibition of $PHD2_{181-417}$. In addition, JNJ-42041935 was found to be >100 fold selective across a range of commercially available assays (Cerep & Upstate enzyme panels, data not shown).

2-OG competition studies

JNJ-42041935 and the other reference PHD inhibitors were also characterized in the presence of increasing concentrations of 2-OG (Figure 3). From these studies, it appeared that JNJ-42041935, clioquinol and DMOG behaved in a simple competitive manner. Thus, it was possible to globally analyse these data to generate estimated pK_I

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values (Table 1). Desferrioxamine and ciclopirox exhibited non-competitive enzyme kinetics whereas 3,4-EDHB appeared to behave in a 'mixed' fashion (Figure 3). The inhibition constant estimated for JNJ-42041935 was not significantly different from that estimated by correcting the pIC_{50} value to account for the concentration of 2-OG used in the assay (pK_I = 7.4 using the Cheng-Prussoff correction). The results show that JNJ-42041935, DMOG and clioquinol behave as 2OG competitive inhibitors of PHD while the other compounds included in this study do not.

Co-crystals of JNJ-42041935 and $PHD2_{181-417}$

JNJ-42041935 co-crystallized in the active site of $PHD2_{181-417}$ at a resolution of 2.10°A. (Figure 4). The co-crystal structure demonstrated that the acidic group present in JNJ-42041935 formed a salt bridge with Arg383. The lone pair of electrons on the nitrogen atom of the pyrazole and the benzimidizole bound to iron in the active site in a bi-dentate fashion. The other benzimidizole NH formed a hydrogen bond with a conserved water molecule that also participated in a hydrogen bond with Y303 of the protein. The co-crystal data corroborate the 2-OG competitive mechanism of action for JNJ-42041935.

Potency of JNJ-42041935 in cell based assays: HIF-1α accumulation and erythropoietin secretion in Hep3B cells

All compounds except 3,4-EDHB caused a concentration dependent increase in HIF-1α in Hep3B cells measured after 24 h incubation (Figure 5). DMOG and 3,4-EDHB expressed the lowest potency in the erythropoietin secretion assay (DMOG pA_{50}=4.02±0.07 and 3,4-EDHB pA_{50}=3.84±0.44). JNJ-42041935 and desferrioxamine

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were approximately equipotent in the HIF-1α accumulation and erythropoietin secretion assays in Hep3B cells (see Table 1). Ciclopirox was 10-fold more potent than JNJ-42041935 in these cell based assays, however, the response was biphasic (Figure 5F) and, therefore, only the data for the concentration range 1-10μM were analyzed. The data for clioquinol could not be analyzed using non-linear regression due to the steep concentration-response curves generated (Figure 5D). The maximal responses for JNJ-42041935, desferrioxamine and ciclopirox in the HIF-1α accumulation and erythropoietin secretion assays were not significantly different (Table 1). Thus JNJ-42041935 was a robust tool to elevate HIF-1α and stimulate erythropoietin secretion in Hep3B cells.

Hypoxia response element-driven luciferase expression in the mouse

JNJ-42041935 was evaluated in the HIF-driven luciferase mouse model (Safran et al., 2006). Two hours after oral administration of 300 μmol/kg of JNJ-42041935 the bioluminescence over the peritoneal area was increased by 2.2±0.3-fold relative to luciferase treated vehicle controls (1.0±0.3, Figure 6).

Hematological effects of JNJ-42041935 in mice

Six hours after administration of test compounds, only JNJ-42041935 stimulated erythropoietin secretion *in vivo* (Figure 7A). Thus, plasma erythropoietin was elevated by 55 and 304-fold after oral doses of 100 and 300 μmol/kg JNJ-42041935. Furthermore, administration of JNJ-42041935 (100 μmol/kg po) for five consecutive days resulted in a 2-fold increase in reticulocytes, an increase in hemoglobin by 2.3

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g/dL and an increase in the hematocrit of 9% (Figure 7B-D). JNJ-42041935 was the only compound tested that performed well *in vivo*.

Hematological effects of recombinant human erythropoietin in normal rats

In a separate study, the hematological effects of a 50 µg/kg, once weekly dose of rhEPO was confirmed in normal female Lewis rats. Blood hemoglobin and hematocrit were increased from baseline values of 16.0±0.1 g/dL and 43.7±0.3% to 19.1±0.6 g/dL and 52.4±1.7% (both $p<0.05$), respectively, on the final day of the study (Day 15). The percentage of reticulocytes in the blood was reduced from 1.9±0.1 to 1.1±0.2 % by treatment with rhEPO. These results demonstrate that the 50 µg/kg i.p. dose of rhEPO is highly effective in normal female Lewis rats.

Effects of JNJ-42041935 in an inflammation-induced anemia model

Fourteen days after administration of PGPS, the rats showed signs of severe inflammation, such that both hind limbs became swollen and mobility was reduced in all animals and serum IL-6 and TNFα concentrations were elevated from below the limit of detection (40 and 10 pg/ml, respectively) to 466±42 and 35.9±2.9 pg/ml, respectively. Consistent with the inflammation produced by PGPS administration, white blood cells were markedly elevated from 10.7±0.3x10^3 cells/µL in non-PGPS treated animals to 49.0±2.3x10^3 cells/µL in PGPS treated animals. Animals became severely anemic as demonstrated by the decrease in blood hemoglobin from 16.3±0.1 g/dL in non-PGPS treated animals to 10.8±0.2 g/dL in PGPS treated animals. Animals were randomized

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to treatment such that the mean and standard deviation was similar between PGPS treated groups at the start of the study.

Plasma erythropoietin was elevated from a value of 9.8±0.5 pg/ml in vehicle controls to 83±17 pg/ml (~8.4-fold increase) in PGPS treated animals. Following 7 days of JNJ-42041935 administration, plasma erythropoietin was increased to 1917±865 pg/ml (measured six h after the oral dose of JNJ-42041935 to capture peak plasma erythropoietin concentration). Seven days of treatment with human erythropoietin resulted in the plasma erythropoietin concentration of 62,385±10,786 pg/ml (measured 2 h after administration of rhEPO to capture the peak plasma erythropoietin concentation). By day 14 of the study, the plasma erythropoietin concentration in the JNJ-42041935 treated group was 29±5 pg/ml (measured 24 hours after the final dose of JNJ-42041935). In addition, after 14 days of human erythropoietin administration, the concentration of erythropoietin in the plasma was not significantly different from control animals.

After 14 days of treatment JNJ-42049135 increased blood hemoglobin substantially (~2 g/dL) in PGPS treated animals, while exogenous administration of 50 µg/kg rhEPO was ineffective (Figure 8 C). No effect on serum iron concentration was observed for either JNJ-42041935 or rhEPO. Further characterization of the hematological response to these two agents in the setting of inflammation-induced anemia shows that JNJ-42041935 increased the number of circulating reticulocytes relative to PGPS treated controls (Figure 8A), whereas exogenous erythropoietin reduced the number of

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reticulocytes relative to PGPS treated controls. PGPS treatment reduced the number of circulating red blood cells as well as their MCV and MCH, mean corpuscular hemoglobin and mean cell hemoglobin (Figure 8). These changes are consistent with the changes observed in humans with inflammation-induced anemia. Treatment with JNJ-42041935 reversed these changes back towards the values observed in non-PGPS treated rats (Figure 8). A significant increase in red blood cell size (MCV) was observed in PGPS treated rats after treatment with JNJ-42041935 (Figure 8E). Indices of the amount of hemoglobin in individual cells were also beneficially affected by treatment with JNJ-42041935 as demonstrated by the changes in mean cell hemoglobin (MCH) and the cellular hemoglobin content of mature red blood cells (CHm, data not shown). A trend towards an increase in the hemoglobin content of retuculocytes (CHr) was also observed. Thus, JNJ-42049135 addressed the iron limited hematopoiesis that occurred under conditions of severe inflammation.

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DISCUSSION

PHD inhibitors could potentially be used to treat a range of oxygen-deprivation-related disorders such as anemia, myocardial ischemia, stroke and metabolic disorders by stimulating the body's adaptive response to hypoxia without reducing oxygen availability. In addition stimulating the hypoxic response has recently been shown to promote an anti-apoptotic phenotype in intestinal epithelial cells and improve disease indices in mouse models of ulcerative colitis (Cummins et al, 2008; Robinson et al 2008). Here we describe the molecular and *in vivo* pharmacological characterization of a novel PHD enzyme inhibitor, JNJ-42041935. We have demonstrated that JNJ-42041935 is a potent, 2-OG competitive, reversible and selective inhibitor of the three PHD isozymes . We also demonstrate the therapeutic potential of PHD inhibition in an inflammation-induced anemia model.

While a number of compounds that act as inhibitors of PHD enzymes have been previously been described (e.g., Ivan et al., 2001) the mechanism of action is not always apparent. For example, the present work demonstrates that cyclopirox functions as an iron chelator (similar to desferrioxamine) whereas DMOG inhibits PHD enzymes via competition with 2-OG. Warshakoon *et al.* (2006) describe several different series of compounds that may well act via different molecular mechanisms. More recently Tegley (2008) and Dao (2009) described the compounds identified from an HTS campaign which acted in a 2-OG competitive fashion while Smirnova et al. (2010) described hits from a cell based HTS campaign that likely inhibit PHD enzymes via iron

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chelation although this has not been demonstrated. The differences in the mechanism of action can have a dramatic impact on the translation of the pharmacology across different test systems.

JNJ-42041935 was the most potent PHD inhibitor evaluated in this study. In addition, DMOG, 3,4-EDHB and JNJ-42041935 were not sensitive to the inclusion of exogenous iron in the assay. Conversely, cyclopirox and clioquinol were found to behave in a similar fashion to the prototypical iron chelator desferrioxamine. Thus these compounds: 1) inhibited $PHD2_{181-417}$ with an IC_{50}~1 μM, 2) tended to have steep concentration-response curves, 3) inclusion of 10 μM iron in the enzyme assay prevented the inhibition of $PHD2_{181-417}$, 4) all three interacted strongly with iron in protein free solution and 5) none of these compounds elevated plasma erythropoietin in mice (at the doses tested). There were some differences in the profile of inhibition produced by these compounds in that clioquinol behaved in a 2-OG competitive fashion and its actions were partially reversible.

It is clear that depletion of the labile iron pool in cells is an effective mechanism to inhibit PHD. While this is possible in isolated cell based systems this mechanism of action would probably not be viable in intact organisms as the amount of iron that would have to be chelated would be relatively large. Furthermore, chelation of the labile iron pool can be expected to interfere with other cellular process such as electron transport and may inhibit other non-heme, iron containing enzymes. Consistent with this, desferrioxamine inhibited the structurally related enzyme FIH. This iron-binding

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mechanism of action has further implications for PHD inhibitors that act in this way as the potency for inhibition of PHD enzymes appears to be dependent on the affinity of iron at the active site of the enzyme. This observation is supported by our own observations (Kanelakis et al., 2009) as well as the observations of McDonough et al. (2006) and Dao et al. (2009) that find the K_M of PHD2 for iron to be ~1 μM. The iron chelators tested in the current study had a similar potency and had steep concentration-response curves consistent with the inhibition being dependent on the depletion of active enzyme. Together these results imply that iron dissociates from the active site of the PHD enzyme and then binds to the most potent iron chelator available (i.e., desferrioxamine and not PHD) resulting in reduced activity of PHD. Thus it is also possible that iron chelators do not interact directly with the enzyme to inhibit PHD, however, this could be compound or time dependent. Compounds such as clioquinol might briefly interact with the enzyme to facilitate extraction of the iron from the active site and then act irreversibly thereafter. This would explain the apparent competitive behavior of clioquinol with respect to 2-OG and the iron-dependent and partially reversible inhibition of $PHD2_{181-417}$. Given that iron chelators may not directly interact with the enzyme this predicts that no structure activity relationship will be found for inhibition of PHD enzymes other than that for chelating iron and penetration into the cell. This is consistent with our unpublished observations. Perhaps not surprisingly the actions of iron chelating compounds were complicated by the occurrence of bi-phasic concentration-response curves in cell based systems and no response was observed *in vivo*.

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The pharmacology of 3,4-EDHB was complex across the different assays. It did not interact strongly with iron in a protein free solution, however, inclusion of 10 μM iron in the enzyme assay decreased its potency. Furthermore, it displayed complex enzyme kinetics when incubated with 2-OG and its actions were irreversible. 3,4-EDHB did not result in elevation of HIF-1α in Hep3B cells or the increase the release of erythropoietin. Interestingly other groups do not find 3,4-EDHB as a PHD inhibitor even in the isolated enzyme up to a concentration of 100 μM (Ivan et al., 2001). Overall, these observations make it difficult to interpret studies using 3,4-EDHB to inhibit PHD enzymes.

DMOG and JNJ-42041935 were found to be 2-OG competitive inhibitors of $PHD2_{181-417}$ enzyme with JNJ-42041935 having >25-fold higher affinity. The actions of both compounds were reversible, insensitive to inclusion of iron in the enzyme assay and neither interacted strongly with iron in protein free solution. Consistent with the high degree of homology of the active site of the PHD enzymes, JNJ-42041935 had similar potency for inhibition of full length human PHD1, PHD2 and PHD3 to that observed with $PHD2_{181-417}$. We also obtained a co-crystal of JNJ-42041935 in the active site of $PHD2_{181-417}$ that corroborates the competitive nature of the inhibition observed in the functional assay. The binding mode of JNJ-42041935 is similar to that reported for compound A (McDonough et al., 2006) except that the shared water bridge interaction is replaced by a direct hydrogen bound with Y303 for compound A.

JNJ-42041935 caused a concentration dependent elevation of cellular HIF-1α and erythropoietin release in Hep3B cells. In contrast, the effect of DMOG on erythropoietin

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release was bi-phasic and complicated by changes in cell morphology at high concentrations that suggest that DMOG was not tolerated well by cells. Only JNJ-42041935 produced concentration-dependent elevation of plasma erythropoietin after oral administration to mice. DMOG was without effect at the doses tested which is consistent with its relatively low potency for inhibition of the enzyme, poor cellular activity and its unknown but likely poor pharmacokinetics. The ability of JNJ-42041935 to prevent the breakdown of the oxygen sensitive domain of HIFα was confirmed *in vivo* using the mouse model described by Safran et al. (2006). Interestingly this group also reports that desferrioxamine or DMOG are ineffective in this model.

Although the primary aim of our drug discovery program was to identify inhibitors of PHD2, we also characterized the potency of JNJ-42041935 at the closely related PHD1 and PHD3 isozymes. We found that JNJ-42041935 was a not selective for PHD isoforms. This is consistent with the very high degree of homology within the active site of these isozymes and across species. Interestingly a recent study by Minamishima and Kaelin (2010) suggests that this would be a beneficial mechanism of action as they demonstrated that inhibition of all three PHD isozymes is required to re-activate hepatic erythropoietin production. In adults, erythropoietin is produced mainly by the kidney but in fetus and for the first few months after birth the liver is the primary source (Palis and Segel, 1998). These data suggest that a pan-PHD inhibitor may be effective in treating anemia resulting from chronic kidney disease where a PHD2 selective inhibitor might fail.

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The hematopoietic effects of JNJ-42041935 QD po and exogenous erythropoietin administered using a dosing regimen to mimic its clinical use were compared in an inflammation-induced anemia model. A chronic inflammatory state was induced by administration of PGPS to female Lewis rats (Sartor et al., 1989) and the resulting microcytic anemia had many of the characteristics of inflammation-induced anemia in humans (Andrews, 2008). Administration of JNJ-42041935 once daily by the oral route for 14 days partially corrected blood hemoglobin and hematocrit level in PGPS-treated animals. In contrast, once weekly administration of a dose of erythropoietin that was effective in normal animals had no effect on blood hemoglobin and hematocrit in PGPS treated animals. JNJ-42041935, but not exogenous erythropoietin, corrected the microcytic nature of the inflammation-induced anemia as demonstrated by increases in MCV, MCH and CHm. A trend toward an elevation of CHr was also observed for JNJ-42041935. Darbepoetin has been examined in this model and was found to restore hemoglobin values in 2 to 7 weeks (Coccia et al., 2001). PHD inhibitors have also been described as treating anemia in this model (Langsetmo et al., 2004).

JNJ-42041935 is a new tool compound that is potent, 2-OG competitive, reversible and selective inhibitor of PHD enzymes that can be used to investigate the role of this target across a range of biological systems. Comparison of JNJ-42041935 to exogenous erythropoietin in an inflammation-induced anemia model demonstrated that PHD inhibition but not exogenous erythropoietin corrects anemia and reversed the characteristic changes in red blood cell size and hemoglobin content found in this form

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of anemia. The results suggest that PHD inhibition may be an effective means to treat anemia of various origin where current treatments are ineffective or not optimal.

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LEGENDS FOR FIGURES

Figure 1. **Structure of JNJ-42041935.** 1-(5-chloro-6-(trifluoromethoxy)-1H-benzoimidazol-2-yl)-1H-pyrazole-4-carboxylic acid.

Figure 2. ***In vitro* characterization of JNJ-42041935.** (A) Concentration-response curves for inhibition of $PHD2_{181-417}$ by JNJ-42041935 and reference PHD inhibitors in the absence of iron. (B) Inhibition of full length PHD1, 2 & 3 by JNJ-42041935. (C) Inhibition of FIH by JNJ-42041935, DMOG and desferrioxamine. (D) Inhibition of $PHD2_{181-417}$ by JNJ-42041935, DMOG and desferrioxamine in the presence of 10 μM Fe^{2+}. (E) Inhibition of $PHD2_{181-417}$ with (open symbols) and without (filled symbols) 10-fold dilution to assess the reversibility of the inhibition. (F) Comparison of the iron-binding properties of the compounds in protein free solution using an assay which measured the dequenching of the iron-calcein fluorescence signal (data normalized to reference iron chelator 8-hydroxyquinilone).

Figure 3. **Assessment of 2-oxoglutarate competition for inhibition of $PHD2_{181-417}$.** Concentration response curves for PHD inhibitors were conducted in the presence of increasing concentrations of 2-OG. Log molar concentrations of PHD inhibitors are shown as an inset on each graph.

Figure 4. **Representation of the co-crystal of $PHD2_{181-417}$ and JNJ-42041935**. Key interactions made by JNJ-42041935 are noted on the diagram: Bi-dentate ligation of iron, the salt bridge interaction with R383 and the shared water bridge with Y303.

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Figure 5. **HIF-1α accumulation and erythropoietin secretion in response to incubation with PHD inhibitors in Hep3B cells.** Concentration response curves for PHD inhibitors were conducted in Hep3B cells. Erythropoietin (empty squares, left y-axis) and HIF-1α (filled squares, right y-axis) were measured using an electrochemiluminescence based assay from Meso Scale Discovery.

Figure 6. **Effect of JNJ-42041935 on HRE-driven luciferase expression in the mouse.** (A) Representative images of a luciferin treated control mouse and (B) a mouse which was dosed orally with 300 µmol/kg JNJ-42041935 2h before measurement of bioluminescence.

Figure 7. **Effect of selected PHD inhibitors on plasma erythropoietin in mice and hematological effects of JNJ-42041935 in normal mice.** (A) Plasma erythropoietin concentration was measured in mice 6 h after the oral administration of selected PHD inhibitors. In addition, the effects of 5 consecutive daily doses of 100 µmol/kg JNJ-42041935 on the hematological parameters: (B) % blood reticulocytes, (C) hemoglobin concentration and (D) hematocrit were also measured 3 days after the final compound administration.

Figure 8. **Comparison of JNJ-42041935 and recombinant human erythropoietin (rhEPO) in an inflammation-induced anemia model.** Fourteen days after administration of PGPS animals were sorted into groups to obtain similar mean

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hemoglobin values before starting treatment. JNJ-42041935 100 μmol/kg was administered orally once a day for fourteen days while recombinant human erythropoietin (rhEPO, 50 μmol/kg i.p.) was administered once a week in female Lewis rats treated with PGPS. Hematological endpoints were assessed 24 hours after the last dose using an Advia 120 hematology analyzer. (A) reticulocyte number, (B) red blood cell number, (C) blood hemoglobin, (D) blood hemocrit, (E) mean corpuscular volume (MCV) and (F) mean cell hemoglobin (MCH). The star * indicates $p<0.05$ by ANOVA followed by Tukey's test for differences.

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Table 1. Summary of the *in vitro* pharmacological characterization of selected PHD inhibitors. Results from purified enzyme and cell based assays.

Assay	Enzymatic $PHD2_{181-417}$ assay					HEP3B HIF assay	
Result type	pIC_{50}	pK_I	Effect of iron	Mechanism of inhibition	Reversibility of interaction	pA_{50}	Max (MSD counts)
Compound							
JNJ-42041935	7.00±0.05	7.30±0.05	No effect	Competitive	Reversible	4.49±0.08	12108±1
DMOG	5.60±0.07	6.00±0.07	No effect	Competitive	Reversible	3.39±0.07	4350±23
Desferrioxamine	6.00±0.05		↓potency	Non-competitive	Irreversible	4.57±0.19	18424±3
Clioquinol	6.00±0.04	6.30±0.04	↓potency	Competitive	Partial	~4.7**	~10000
3,4-EDHB	6.30±0.06		No effect	Mixed	Irreversible	NR	NR
Ciclopirox	5.80±0.04		↓potency	Non-competitive	Irreversible	5.49±0.05*	14190±1

NR=No response over the concentration range tested.

*Concentration-response curve was bi-phasic, therefore, only 1st 4 points used in non-linear regression

**Non-linear regression of these data to a four-parameter logistic did not converge, therefore, estimated potency and maximum values are given.

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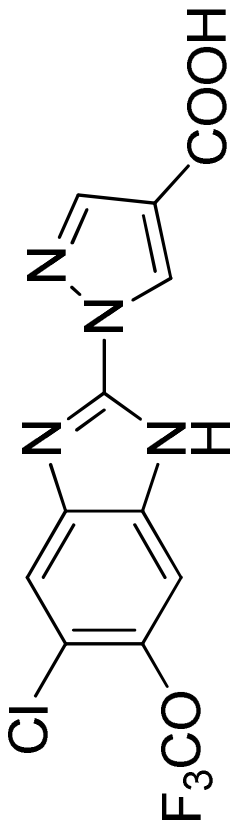

Figure 1

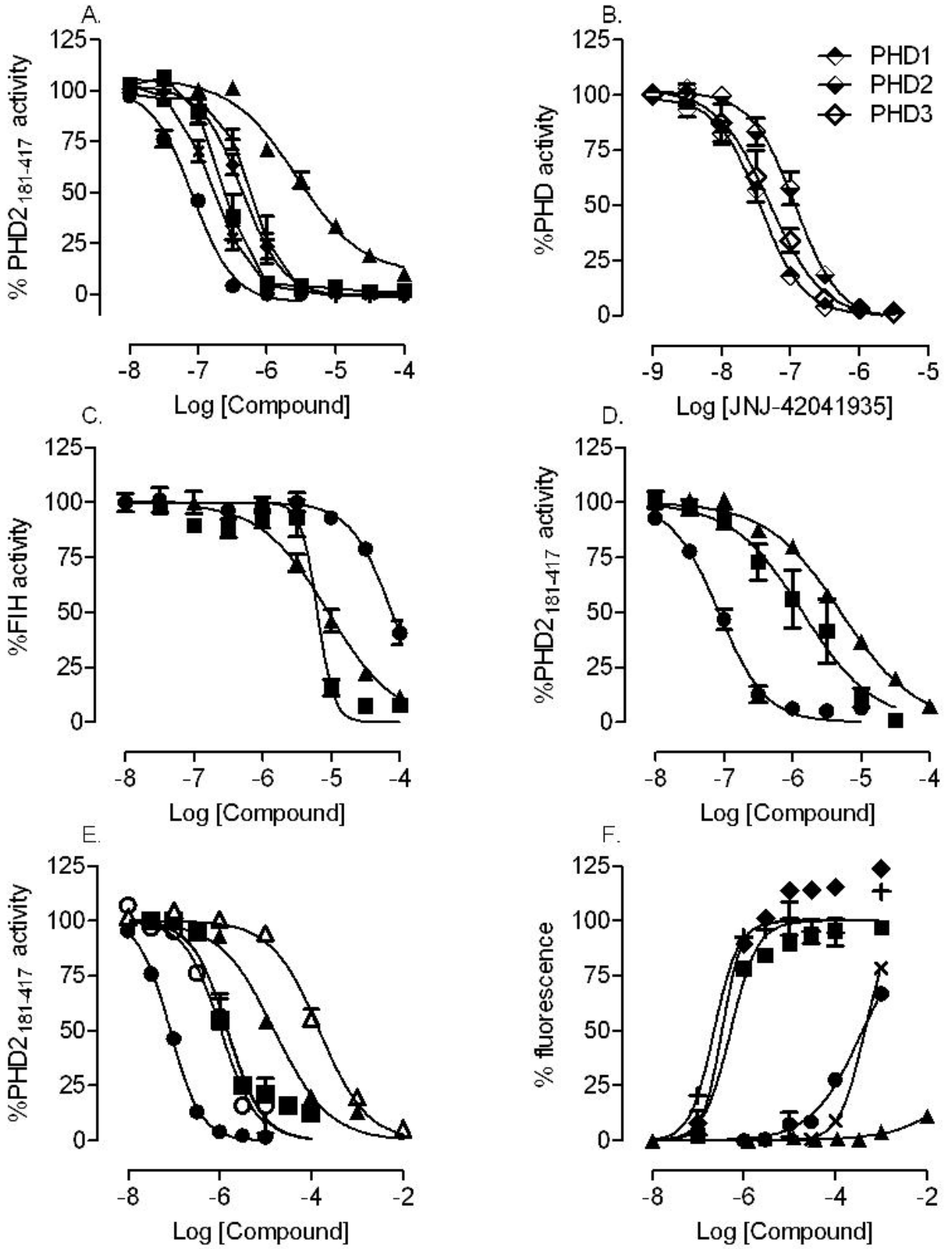
A.
% PHD2181-417 activity
Log [Compound]
B.
PHD1
PHD2
PHD3
%PHD activity
Log [JNJ-42041935]
C.
%FIH activity
Log [Compound]
D.
%PHD2181-417 activity
Log [Compound]
E.
%PHD2181-417 activity
Log [Compound]
F.
% fluorescence
Log [Compound]
JNJ-42041935
DMOG
Desferrioxamine
Clioquinol
3,4-EDHB
Cyclopirox
JNJ-42041935, 1/10 dilution
DMOG, 1/10 dilution
Desferrioxamine, 1/10 dilution

Figure 2

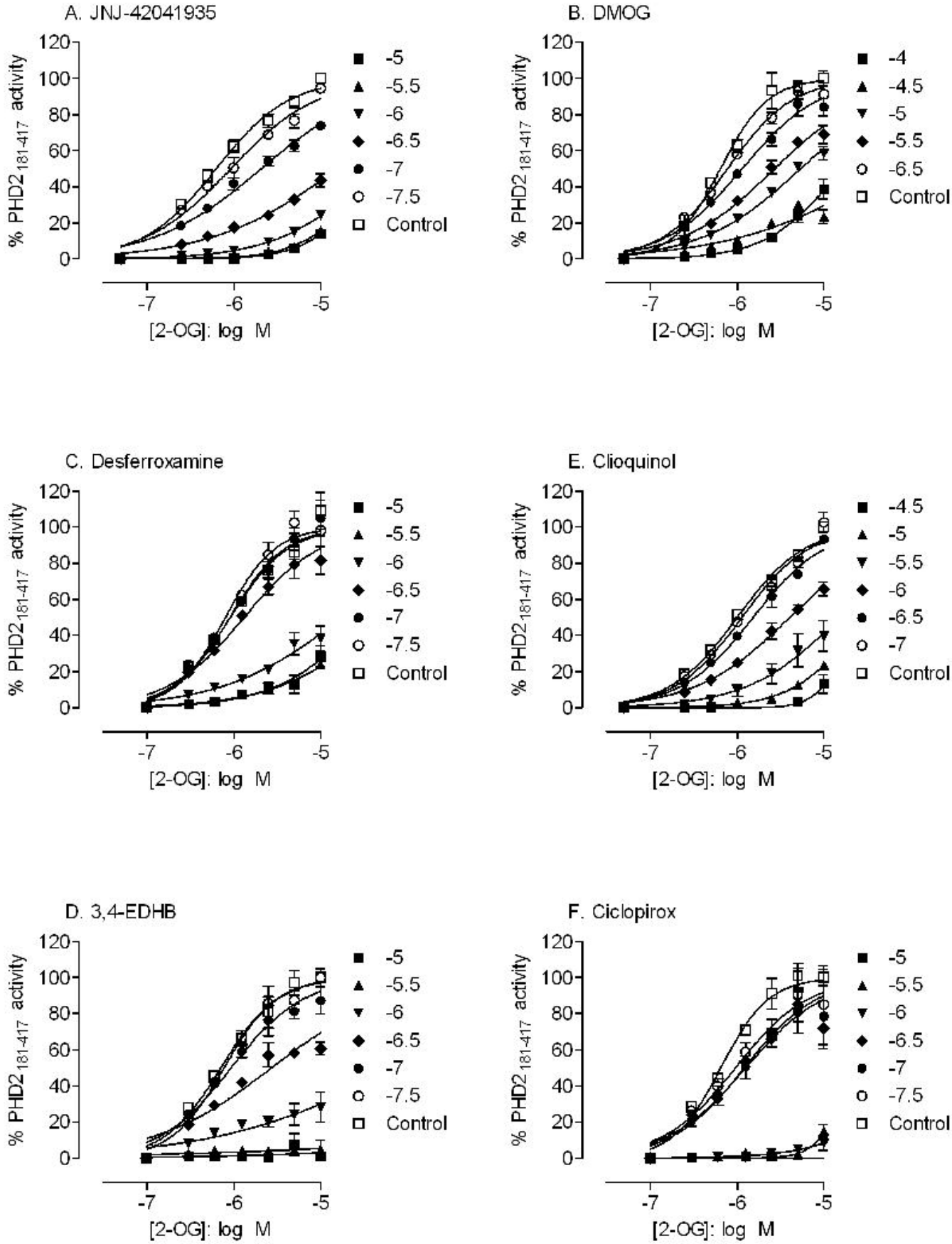
A. JNJ-42041935
B. DMOG
C. Desferroxamine
E. Clioquinol
D. 3,4-EDHB
F. Ciclopirox
% PHD2181-417 activity
[2-OG]: log M
-5
-5.5
-6
-6.5
-7
-7.5
Control
-4
-4.5

Figure 3

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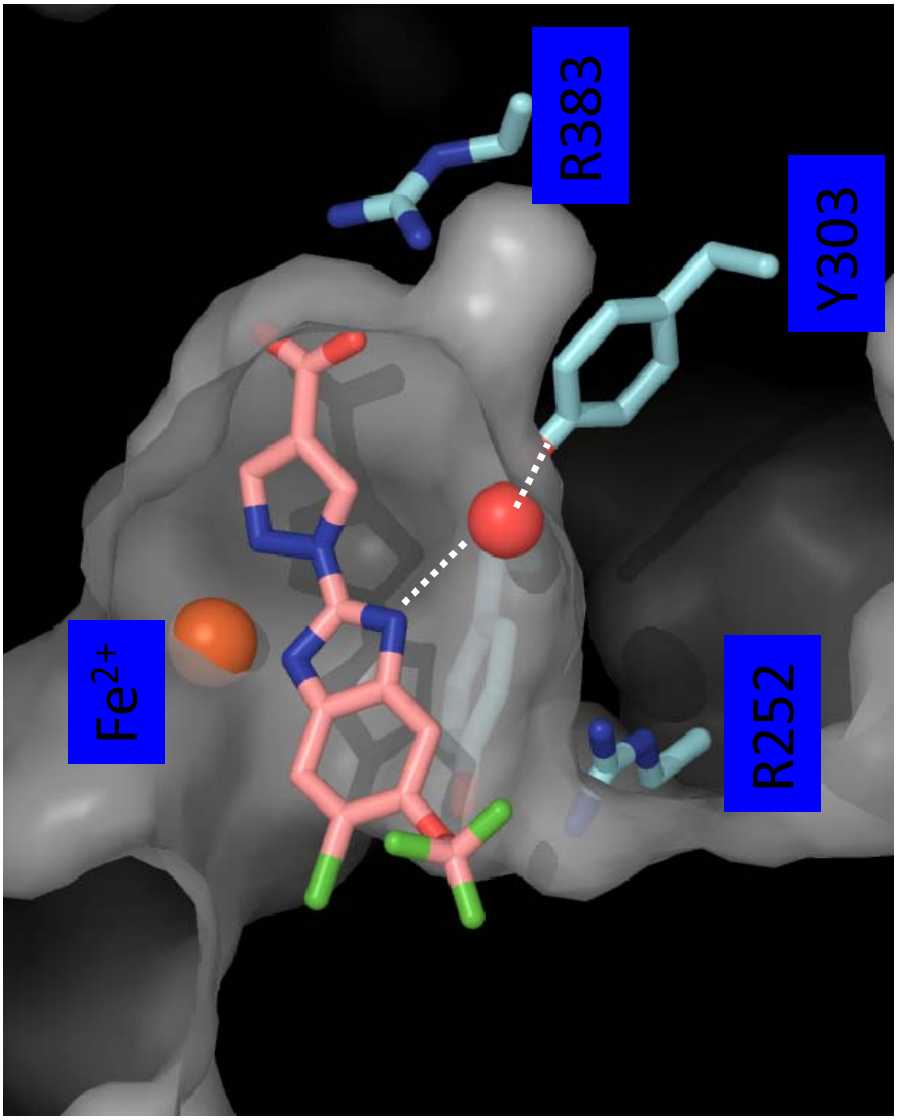

Figure 4

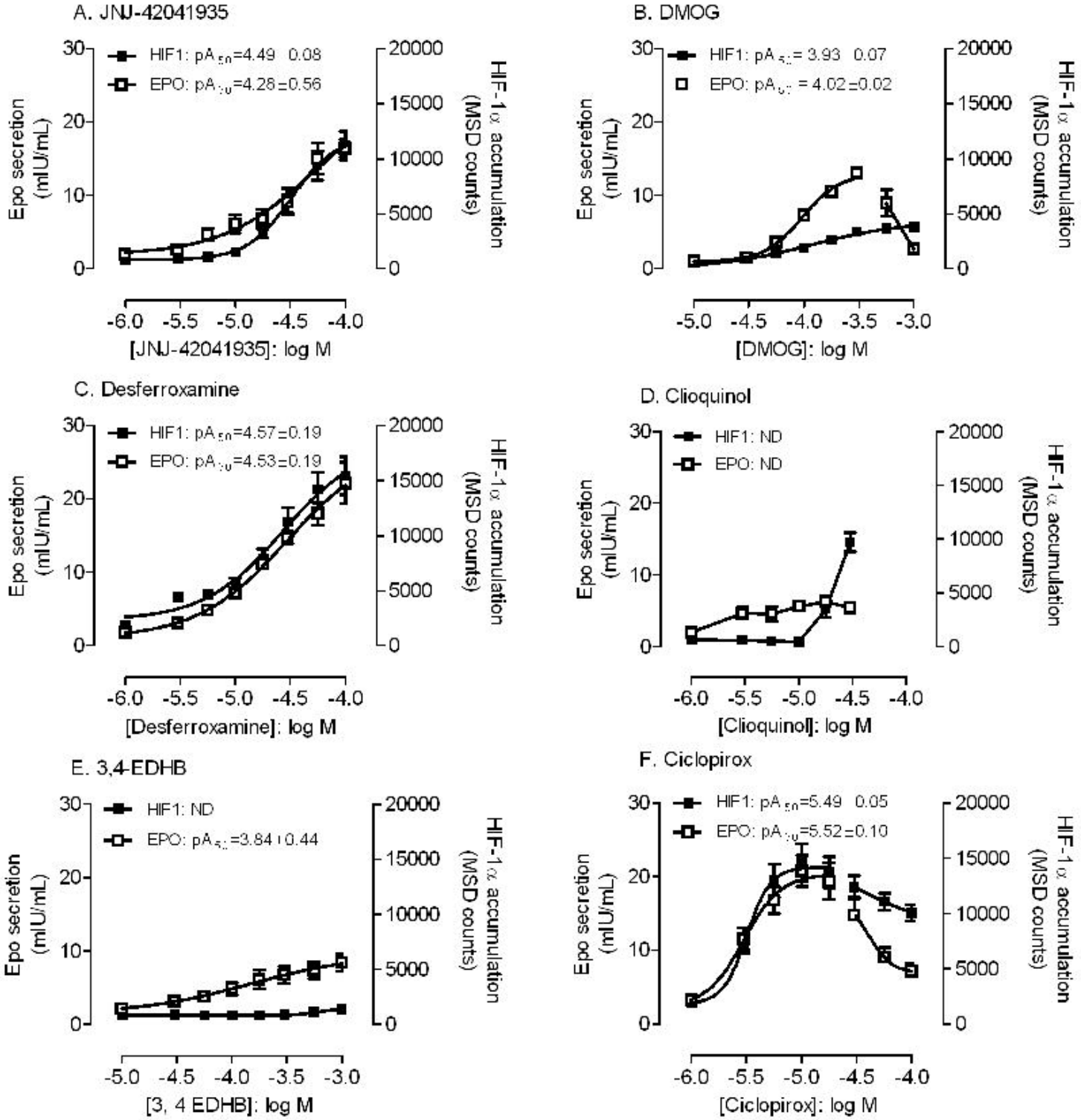
A. JNJ-42041935
HIF1: pA50=4.49 0.08
EPO: pA50=4.28±0.56
Epo secretion (mIU/mL)
HIF-1α accumulation (MSD counts)
[JNJ-42041935]: log M
B. DMOG
HIF1: pA50= 3.93 0.07
EPO: pA50 = 4.02±0.02
[DMOG]: log M
C. Desferroxamine
HIF1: pA50=4.57±0.19
EPO: pA50=4.53±0.19
[Desferroxamine]: log M
D. Clioquinol
HIF1: ND
EPO: ND
[Clioquinol]: log M
E. 3,4-EDHB
HIF1: ND
EPO: pA50=3.84 0.44
[3, 4 EDHB]: log M
F. Ciclopirox
HIF1: pA50=5.49 0.05
EPO: pA50=5.52±0.10
[Ciclopirox]: log M

Figure 5

Figure 6

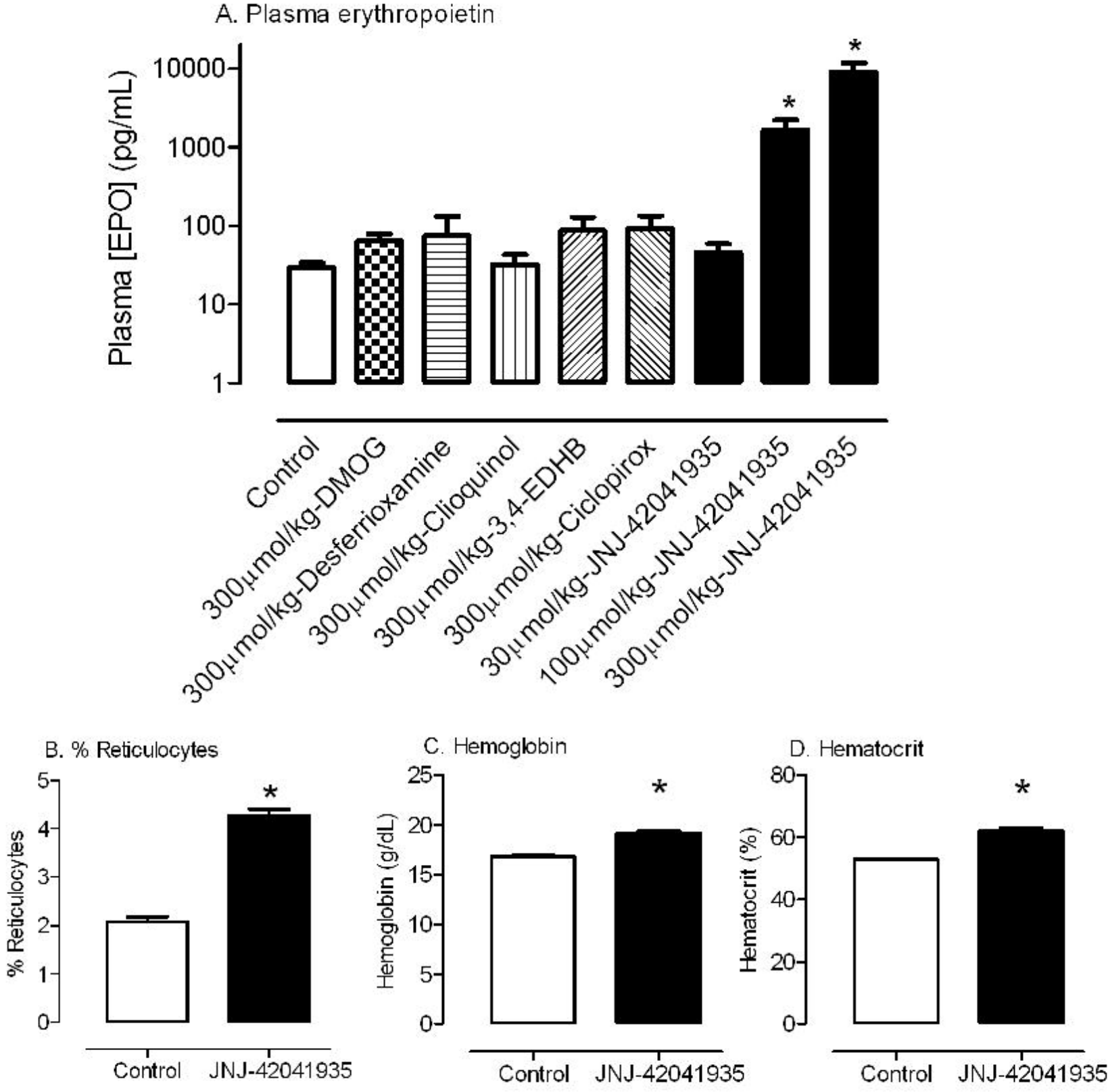
A. Plasma erythropoietin
Plasma [EPO] (pg/mL)
10000
1000
100
10
1
*
*
*
Control
300µmol/kg-DMOG
300µmol/kg-Desferrioxamine
300µmol/kg-Clioquinol
300µmol/kg-3,4-EDHB
300µmol/kg-Ciclopirox
300µmol/kg-JNJ-42041935
30µmol/kg-JNJ-42041935
100µmol/kg-JNJ-42041935
300µmol/kg-JNJ-42041935
B. % Reticulocytes
% Reticulocytes
5
4
3
2
1
0
*
Control
JNJ-42041935
C. Hemoglobin
Hemoglobin (g/dL)
25
20
15
10
5
0
*
Control
JNJ-42041935
D. Hematocrit
Hematocrit (%)
80
60
40
20
0
*
Control
JNJ-42041935

Figure 7

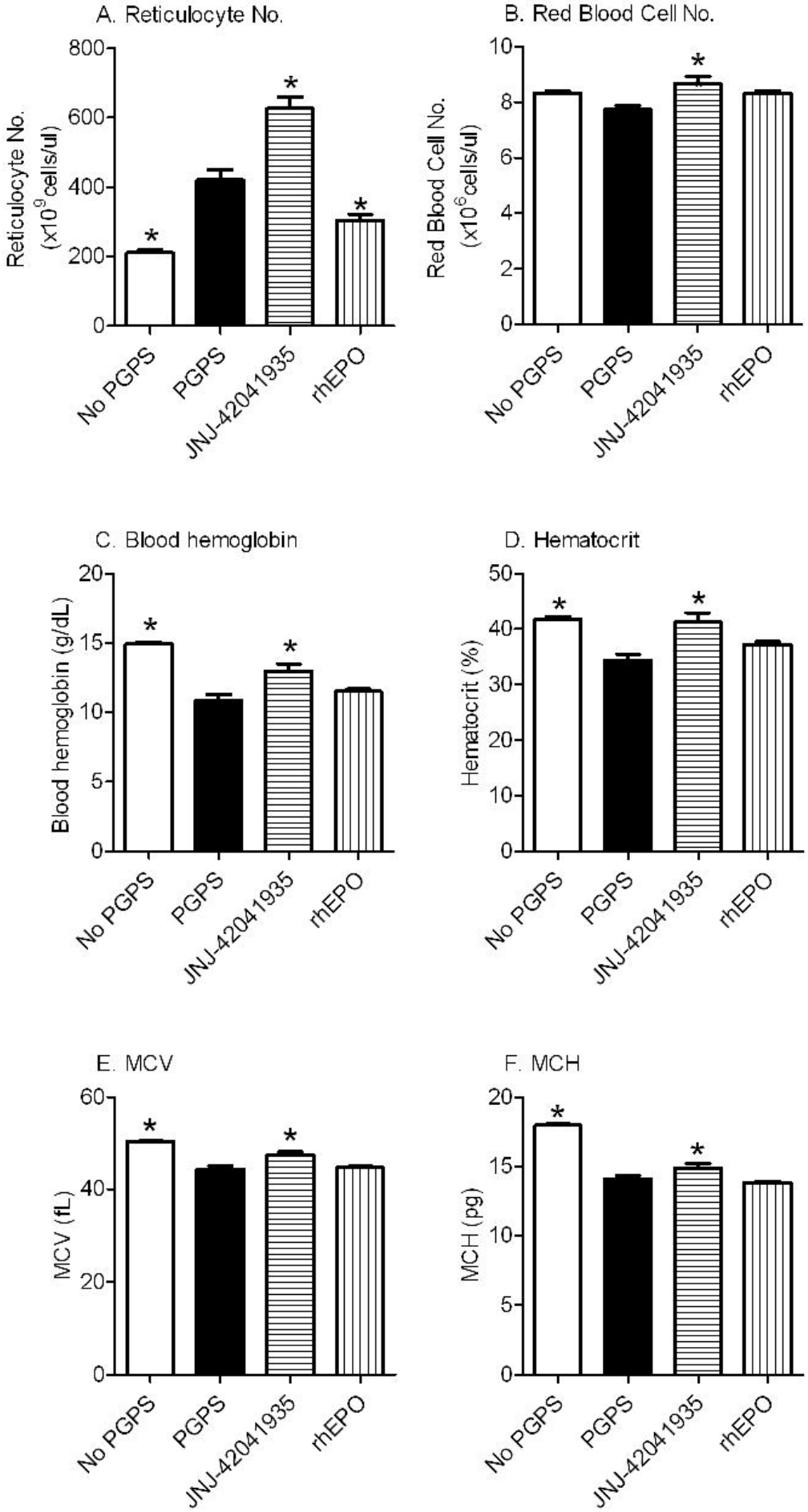
A. Reticulocyte No.
Reticulocyte No. (x10^9 cells/ul)
800
600
400
200
0
No PGPS
PGPS
JNJ-42041935
rhEPO
B. Red Blood Cell No.
Red Blood Cell No. (x10^6 cells/ul)
10
8
6
4
2
0
C. Blood hemoglobin
Blood hemoglobin (g/dL)
20
15
10
5
D. Hematocrit
Hematocrit (%)
50
40
30
E. MCV
MCV (fL)
60
F. MCH
MCH (pg)

Figure 8