Prolyl hydroxylase inhibitor desidustat improves anemia in erythropoietin hyporesponsive state

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ABSTRACT

Many anemic chronic kidney disease (CKD) patients are refractory to erythropoietin (EPO) effects due to inflammation, deranged iron utilization, and generation of EPO antibodies. This work assessed the effect of desidustat, an inhibitor of hypoxia inducible factor (HIF) prolyl hydroxylase (PHD), on EPO-refractory renal anemia. Sprague Dawley rats were made anemic by cisplatin (5 mg/kg, IP, single dose) and turpentine oil (5 mL/kg, SC, once a week). These rats were given recombinant human EPO (rhEPO, 1 μg/kg) and desidustat (15 or 30 mg/kg) for eight weeks. Separately, rhEPO (1–5 μg/kg) was given to anemic rats to sustain the normal hemoglobin levels and desidustat (15 mg/kg) for eight weeks. In another experiment, the anemic rats were treated with rhEPO (5 μg/kg) for two weeks and then desidustat (15 mg/kg) for the next two weeks. Dosing of rhEPO was thrice a week, and for desidustat, it was on alternate days. Desidustat inhibited EPO-resistance caused by rhEPO treatment, decreased hepcidin, IL-6, IL-1β, and increased iron and liver ferroportin. Desidustat reduced EPO requirement and anti-EPO antibodies. Desidustat also maintained normal hemoglobin levels after cessation of rhEPO treatment. Thus, novel prolyl hydroxylase inhibitor desidustat can treat EPO resistance via improved iron utilization and decreased inflammation.

1. Introduction

Chronic kidney disease (CKD), caused by obesity, diabetes, hypertension, inflammatory diseases, and cancer, has a high risk of morbidity and mortality (Kim et al., 2019). Anemia, caused by CKD, generally worsens with the progression of kidney disease and contributes to the overall disease burden. The primary cause of CKD-induced anemia is insufficient erythropoietin (EPO) production by the kidneys, though other factors like chronic inflammation, blood loss, vitamin deficiencies, decreased iron absorption, and utilization contribute to the pathogenesis (Sahitt and Lin, 2012). High levels of hepcidin in serum, increased inflammation, and reduced iron absorption and utilization are associated with end-stage renal disease in CKD (Rubah et al., 2015; Małyszko et al., 2012). Erythropoiesis stimulatory agents (ESA), such as recombinant human erythropoietin (rhEPO), substantially benefit patients with CKD (Hayat et al., 2008). However, ESA treatment is associated with adverse cardiovascular events, and many CKD patients exhibit a poor hematologic response to ESA, mainly due to inadequate iron supply to the erythron (Santos et al., 2020). Other causes of ESA resistance include infection, neoplasia, severe hyperparathyroidism, aluminum intoxication, vitamin B12 deficiency, inadequate dialysis, myelosuppressive agents, and antibody-mediated pure red cell aplasia (Santos et al., 2020; Ganz and Nemeth, 2016). There is significant variability and loss of sensitivity in the patient response to rhEPO, and this resistance to rhEPO therapy can increase the mortality and morbidity in CKD patients (Fukuma et al., 2012). In addition, rhEPO can also generate antibodies against rhEPO (Eckardt and Casadevall, 2003). It is also possible that the anemia and hyporesponsiveness to rhEPO in CKD patients could be due to suboptimal response of the body to the hypoxia, even in the presence of adequate EPO release in the body (Hung et al., 2014). Due to persistent inflammation and increased hepcidin levels, iron absorption and utilization is deranged in CKD patients (Nemeth et al., 2004). Hepcidin is a hepatic hormone that decreases iron absorption from the gastrointestinal tract and locks the iron in tissue stores like the liver and macrophages by inhibiting ferroportin, the tissue iron exporter (Nemeth et al., 2004).

Thus, due to hyporesponsiveness to rhEPO, there is a significant

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unmet need to treat CKD-induced anemia. Prolyl hydroxylase (PHD) inhibitors offer a potential treatment alternative in CKD-induced anemia (Haase, 2011). PHD inhibitors stabilize hypoxia-inducible factor (HIF), which improves erythropoiesis by increasing EPO generation and decreasing hepcidin (Joharapurkar et al., 2018). A major advantage of PHD inhibitors is the optimum utilization of body’s hypoxia responsive system that helps improve hematopoiesis using endogenous stimulators. Desidustat is a novel PHD inhibitor to treat anemia of CKD (Parmar et al., 2019). Desidustat has demonstrated potential for combating CKD-associated and chemotherapy-induced anemia in animal models by EPO release and enhancing iron utilization (Jain et al., 2016, 2019; Joharapurkar et al., 2021). Chemotherapy-induced anemia is a significant concern in cancer patients, which affects the quality of life and even survival. ESA-associated cardiovascular toxicities and the possible risk of tumor progression are the major hindrances of using ESAs in treating chemotherapy-induced anemia. PHD inhibitors may improve the erythropoiesis in the cancer patients without the overt risk of tumor progression, and thus, could be a valuable alternative to the ESAs (Beck et al., 2017). This study aimed at assessing the impact of desidustat on hyporesponsiveness to EPO in a rat model of CKD induced by cisplatin and turpentine oil.

2. Results

2.1. Effect of desidustat on development of EPO resistance and bone marrow histology

The rats treated with cisplatin and turpentine oil (vehicle control group) demonstrated significantly reduced hemoglobin in the blood (p < 0.05) when compared to the normal control group. At the same time (i.e., two weeks of cisplatin injection), the serum creatinine and urea in the treated (vehicle control group) rats were found to be 0.60 ± 0.03 mg/dL and 67.2 ± 6.3 mg/dL compared to 0.37 ± 0.01 mg/dL and 32.5 ± 1.8 mg/dL, respectively, in the normal control group. The serum LDH levels of rats treated with cisplatin and turpentine oil was increased to 252.5 IU/L, compared to 110.2 IU/L in the normal control group. Treatment of rhEPO (1 μg/kg, once a week) increased the hemoglobin in anemic rats by 15.5 ± 6.8% at the third week of the treatment and finally reduced it by 17.5 ± 15.5% at the end of the eight-week treatment period (Fig. 1A). Desidustat (15 and 30 mg/kg) treatment increased the hemoglobin up to four weeks of treatment (p < 0.05), compared to the vehicle control group. Treatment of rhEPO alone caused resistance in 55.5% of rats (five out of nine), whereas cotreatment of 15 mg/kg dose of desidustat with rhEPO induced rhEPO resistance in 33.3% of rats (three out of nine). Further, cotreatment of a 30 mg/kg dose of desidustat with rhEPO caused rhEPO resistance in only 22.2% of rats (two out of nine) by the end of the treatment.

In the anemic rats, a significant increase in the iron levels was found serum, liver, and spleen by rhEPO treatment (p < 0.05), as compared to the vehicle control group (Fig. 1B–D). Serum hepcidin and liver hepcidin expression were increased, and ferroportin expression was decreased by rhEPO treatment (p < 0.05), as compared to the vehicle control group (Fig. 1E–G). Ferroportin expression was significantly increased, while hepcidin expression was significantly decreased by both the doses of desidustat (p < 0.05), as compared to vehicle control group. Serum IL-6 and IL-1β were increased (p < 0.05) by rhEPO treatment, as compared to vehicle control group (Fig. 1H–I). Desidustat treatment (15 and 30 mg/kg) reduced serum iron (p < 0.05), as compared with vehicle control group, and also reduced liver and spleen iron. Desidustat at 15 and 30 mg/kg reduced serum hepcidin, IL-6 and IL-1β levels (p < 0.05), as compared to the vehicle control group.

Treatment of rhEPO significantly (p < 0.05) increased myeloid to erythroid ratio and lymphoid to erythroid ratio as compared to vehicle control, which were significantly (p < 0.05) reduced by simultaneous desidustat treatment at 15 mg/kg and 30 mg/kg. Desidustat at 15 mg/kg reduced the myeloid to erythroid ratio by 81% and myeloid to lymphoid ratio by 84%, compared to only rhEPO treatment group (Fig. 2A–B). The dose of 30 mg/kg reduced the myeloid to erythroid ratio by 97% and lymphoid to erythroid ratio by 86% compared to only rhEPO treatment group. Since 15 mg/kg desidustat was significant, the same dose was used in further experiments.

2.2. Effect of desidustat on the progression of EPO resistance

Treatment of rhEPO (1 μg/kg, up to 2 weeks and thereafter 2 μg/kg for the next two weeks) increased hemoglobin levels in anemic rats up to
four weeks of treatment (Fig. 2A). The dose of rhEPO needed was 5 μg/kg to maintain normal hemoglobin levels after five weeks (Fig. 2B). At the end of eight weeks of treatment, rhEPO treated animals had hemoglobin levels similar (p > 0.05) as compared to the vehicle control group. The cumulative rhEPO dose used per animal used throughout the eight week treatment period in the rhEPO treatment group was 64 μg/kg. When desidustat (15 mg/kg) treatment was given with rhEPO, the dose of rhEPO was required was 2 μg/kg at 5 weeks of treatment to maintain

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**Fig. 2.** Effect of desidustat on bone marrow histology on development of EPO-resistance in anemia induced by cisplatin and turpentine oil in rats. A, representative image Myeloid: Erythroid and Lymphoid: Erythroid cells ratio. The data expressed as mean ± SEM, n = 9 per group. B, representative images of the bone marrow cell population at the end of the treatment period. * indicate P < 0.05 compared with vehicle control, $ indicate P < 0.05 compared with rhEPO treatment.

**Fig. 3.** Desidustat protected against EPO refractory state in anemia induced by cisplatin and turpentine oil in rats. A, hemoglobin response over time, B, cumulative rhEPO dose given over time, C, antibody titer, and D, Serum EPO, at the end of 8 weeks of rhEPO and desidustat treatment. The data expressed as mean ± SEM, n = 9 per group. * indicates P < 0.05 compared with vehicle control, $ indicates P < 0.05 compared with rhEPO treatment.
normal hemoglobin level, which amounted to the cumulative dose of 24 μg/kg (Fig. 2B). Thereafter, the rhEPO was stopped, and desidustat treatment was continued for the next three weeks. At the end of treatment, hemoglobin levels in the desidustat treated group were significantly (p < 0.05) higher when compared with vehicle control. Treatment of rhEPO showed induction of significantly higher levels of anti-EPO antibody (p < 0.05) as compared to normal control, vehicle control or desidustat treated group (Fig. 3C). Treatment of rhEPO, alone or with desidustat significantly (p < 0.05) increased the serum EPO levels (Fig. 3D), when compared to the vehicle control group.

2.3. Effect of desidustat on cessation of EPO therapy

The effect of desidustat was evaluated in anemic rats after stopping the rhEPO treatment. rhEPO (5 μg/kg) treatment for two weeks significantly (p < 0.05) increased hemoglobin after two weeks of treatment, as compared to vehicle control (Fig. 4A). Two weeks after discontinuation of the rhEPO treatment, hemoglobin level of this group was not significantly different (p > 0.05) from vehicle control group. However, desidustat treatment, initiated after discontinuation of rhEPO treatment, showed significantly (p < 0.05) improved hemoglobin levels as compared to the vehicle control group. Serum and spleen iron was increased by rhEPO treatment by 127 ± 8.9 and 18.7 ± 11.9%, respectively, while liver iron was decreased by 33.6 ± 11.6%, when compared with vehicle control (Fig. 4B-D). Desidustat treatment reduced serum, liver and spleen iron 39.3 ± 7.0, 45.9 ± 9.4, and 36.7 ± 2.9%, respectively, when compared with rhEPO treatment.

3. Discussion

Chronic kidney disease is caused by multitude of factors including erythropoietin (EPO) deficiency, abnormal iron metabolism, hemolysis, chronic inflammation, reduced erythrocyte survival duration, infection, oxidative stress, and nutritional deficits (Babitt and Lin, 2012). A severe form of CKD decreases the quality of life and increases the risk of cardiovascular diseases and mortality (Santos et al., 2020). Erythropoiesis-stimulating agents (ESA) such as epoetin alfa or beta, biosimilars, and longer-acting agents ESAs are generally used to treat anemia associated with CKD (Hayat et al., 2008). CKD is a chronic inflammatory state where the persistent inflammation may contribute to the variability in Hb levels and hyporesponsiveness to erythropoietin stimulating agents (ESA) that precipitates into severe anemia (Gluba-Brzózka et al., 2020). Hyporesponsiveness to ESA or EPO resistance occurs in a substantial population of CKD patients, when the patient cannot reach normal hemoglobin (HB) levels using a recommended dose of ESA (Sibbel et al., 2015). In EPO-resistant patients, a continuing EPO treatment increases the risk of death due to increased blood pressure, increased blood viscosity, and deranges platelet function (Santos et al., 2020). Thus, improving EPO resistance in patients with CKD is vital for improving anemia and also for reducing morbidity and mortality.

The present work aimed to study the effect of desidustat, a prolyl hydroxylase (PHD) inhibitor in anemia of CKD with erythropoietin hyporesponsiveness, in a rat model of CKD induced by cisplatin and turpentine oil.

Here we have observed that a single dose of cisplatin and once weekly administration of turpentine oil-induced a sustained anemia in rats. Cisplatin causes direct damage to nuclear and mitochondrial DNA, which activates cellular pathways leading to apoptosis, fibrosis and induces a robust kidney damage (Shi et al., 2018). Cisplatin-induced renal tubular damage causes anemia due to a persistent EPO deficiency state (Wood and Hrushesky, 1995). After single dose of cisplatin, periodical administration of turpentine oil-induced chronic inflammation with increased hepcidin. These data are consistent with reports indicating that anemia of chronic kidney disease are associated with increased hepcidin (Nemeth et al., 2004). Combination of cisplatin and turpentine oil treatment is used in this study to get a robust inflammation and kidney damage that mimics the decreased EPO secretion, and increased hepcidin as observed in CKD patients (Nemeth et al., 2004). Cisplatin-turpentine oil treatment induced an overt anemic state with significant reduction in hemoglobin, with apparently no change in the serum, liver, or spleen iron. Though serum hepcidin was not significantly increased, we found enhanced hepcidin expression and reduced ferroportin (FPN1) expression in liver. Increased serum levels of IL-1β and IL-6, and LDH levels indicated that the anemia was associated with a chronic state of inflammation and kidney damage, which was also substantiated with a significant uremia. The population of erythroid precursor cells in the bone marrow was reduced, and the ratio of myeloid and lymphoid to erythroid precursor cells have been significantly increased. Consistent with clinical observations, these data indicate that reduced erythropoiesis in CKD is associated with chronic inflammation, and is characterized by inefficient iron utilization, mild hemolysis, increased urea and creatinine levels, and bone marrow inefficiency, contributing together to the overt anemia (Babitt and Lin, 2012; Ma Izyzko et al., 2012; Gluba-Brzózka et al., 2020; Sibbel et al., 2015).

Despite a significant reduction in liver hepcidin expression and overt reduction in hemoglobin by cisplatin-turpentine oil treatment, serum and tissue iron were not significantly different in anemic rats, compared to the normal rats. In this experiment, iron was measured 72 h after the last.
Four to five weeks of rhEPO treatment induced resistance in the anemic rats. Desidustat, along with rhEPO, increased hemoglobin and decreased the development of EPO resistance at 15 mg/kg and 30 mg/kg dose. The dose of 15 mg/kg was selected from our previous studies (Nemeth et al., 2004; Haase, 2011). In the current study, we have observed that the 15 mg/kg dose of desidustat normalizes the hemoglobin just before the start of EPO refractory phase and the higher dose of desidustat (30 mg/kg) causes accelerated and causes supra-physiological erythropoiesis. Based on this observation, the 15 mg/kg dose of desidustat was used for further experiments.

In patients with CKD, after development of EPO resistance, increasing EPO dose may help in improving hemoglobin levels, but eventually leads to toxicity or worsening of EPO resistance leading to overt anemia (de Oliveira Júnior et al., 2015; Provatopoulou and Ziroyiannis, 2011), and discontinuation of EPO treatment (Alves et al., 2015) leaving blood transfusion as the only treatment option (Koncicki and Fishbane, 2017). Here, in this study we have observed that, desidustat treatment reduced the requirement of EPO in maintaining normal adequate hemoglobin level in the EPO-resistant state, and improved the responsiveness to EPO in the anemic rats. Also, desidustat reduced the requirement of rhEPO to maintain hemoglobin levels, reduced hyporesponsiveness to rhEPO, and attenuated the drop in hemoglobin caused by the cessation of EPO treatment. The generation of anti-EPO antibodies is a major reason for resistance to exogenous EPO and discontinuation of EPO therapy in patients (Verhelst et al., 2004). With immunosuppressive therapy or a blood transfusion can help only half of the patients recover the effectiveness of EPO after immunotherapy or renal transplantation (Bennett et al., 2005). In this study, we observed a significant induction of anti-EPO antibodies in rEPO resistant animals and it was significantly attenuated by desidustat treatment. Recently, another PHD inhibitor roxadustat has also been reported to increase hemoglobin in EPO-resistant patient (Cai et al., 2021). The anemia induced by cisplatin-turpentine oil in rats is associated inefficient EPO secretion, caused due to the fibrotic damage to the EPO secreting cells in the renal tissue. Treatment of either rhEPO or desidustat improved the serum EPO levels to a similar extent. However, the EPO release by rhEPO was not able to maintain normal hemoglobin because of higher anti-EPO antibody titer in these rats. On the other hand, desidustat treatment showed a robust increase in hemoglobin, which might be because of reduced anti-EPO antibody titer. It appears that the endogenous EPO may not be neutralized by exogenous rHuEPO inducing anti-EPO antibody. On the other hand, the decrease in anti-EPO antibodies by desidustat might be due to the reduced requirement (and hence reduced dosing) of exogenous rEPO and a decrease in inflammation. We also observed a decrease in myeloid to erythroid and lymphoid to erythroid ratio, suggesting an increase in erythroid progenitor in desidustat treated rats, which substantiates the increased sensitivity of the anemic rats to endogenous erythropoietin.

A decrease in EPO responsiveness is mainly due to systemic inflammation (Alves et al., 2015). Uremia causes blunting the bone marrow response to EPO (Alves et al., 2015) and increase in urea improves IL-6 and also reduces requirement of erythropoietin (Yuen et al., 2005). Also uremia alters erythrocyte morphology (Kong et al., 2001). Chronic inflammation induced by turpentine oil can induce the expression of hepcidin, the iron regulatory peptide (Langdon et al., 2014) We have previously observed that the kidney damage and systemic inflammation can reduce EPO secretion (Jain et al., 2016, 2019). It is reported that in both healthy, non-diseased (with normal hemoglobin) mice (Gammella et al., 2015) and humans (Robach et al., 2009; Ashby et al., 2010), hepcidin is down-regulated by rhEPO. On the other hand, high dose erythropoietin treatment induced EPO resistance in anemic rats, which is associated with anti-rHuEPO antibodies and higher hepcidin expression (Fernandes et al., 2014). Also, clinically a significant positive relation between hepcidin and erythropoietin resistance is observed in anemic patients (Petuline et al., 2017). Our data is consistent with these reports, since we have found EPO resistance and increased hepcidin with the presence of anti-EPO antibody. Desidustat decreased serum hepcidin and released iron from the liver and spleen in the blood. These effects were due to increased ferroportin expression, which released iron from the liver and spleen after desidustat treatment.

Decreased hepcidin and increased serum iron is the result of EPO action, but in case of EPO resistance, increase in hepcidin locks the iron to deposit in the tissues and the serum iron is still increased due to lack of optimum utilization in the bone marrow (Fernandes et al., 2014). There is an increased absorption of iron to match the erythropoietic demand in an EPO resistance state (Fernandes et al., 2014). Here, we have also observed an increase in serum iron in EPO resistance animals. Consistent with this report, we have observed increase in the serum and tissue (liver and spleen) iron in erythropoietin-resistant state indicating an inefficient erythropoiesis despite high erythropoietin and iron in serum. Also, the inefficient hematopoiesis that results in premature destruction of RBCs may also lead to increase in serum iron (Hasegawa et al., 2021). However, when the iron was measured within 24 h of the last dose of EPO and turpentine oil, an acute effect of the inflammatory stimuli on hepatic iron and iron levels was evident in anemic rats, and it was significantly attenuated by desidustat treatment. The decrease in serum iron levels by desidustat treatment also indicate an optimum iron utilization in maintaining hemoglobin levels. Desidustat could impact iron homeostasis by indirectly decreasing hepcidin production in the liver and increasing transcription of genes that promote the dietary uptake and transport of iron, although this hypothesis warrants further investigation.

Desidustat is a prolyl hydroxylase inhibitor, which stabilizes HIF. Apart from enhancement of erythropoietin secretion and decrease in hepcidin, HIF can induce significant antiinflammatory effect through mediators like adenosine (Bartels et al., 2013; Bowser et al., 1985), and regression of inflammation by HIF could be the important mechanism for reduction in the anti-EPO antibody titer and improved erythropoiesis sensitivity in the current study. The antiinflammatory effect of HIF is demonstrated abundantly in renal tissue as well as in circulation, and inactivation of HIF seems to promote multiple inflammatory pathways (Kobayashi et al., 2012) in most of the issues, including intestine. In addition, we have observed that the kidney injury in the current study by cisplatin-turpentine oil was associated with increased levels of inflammatory cytokines IL-1β and IL-6 and treatment with desidustat reduced these levels. The antiinflammatory and antioxidant effects of desidustat are reported (Joharapurkar et al., 2021) which indicates that stabilization of HIF by desidustat can reduce the production of inflammatory cytokines. Increase in inflammatory interleukin cytokines is reported in EPO resistance (MacDougal and Cooper, 2002; Pesce et al., 2014) and decrease in these markers is also correlated with reduction in the hepcidin levels (Suega and Widi, 2019). However, further probe into the antiinflammatory mechanism of action of desidustat is warranted. Cisplatin, being used as a chemotherapeutic drug, is also widely used for inducing a model for chemotherapy-induced anemia in rats. Thus, our data indicates a potential benefit of desidustat in chemotherapy induced anemia, though a controlled clinical trial is required to ascertain this benefit.

In conclusion, we present here that prolyl hydroxylase inhibitor desidustat can correct EPO resistance in anemia by promoting erythropoietin synthesis and decreasing hepcidin. This effect seems to be through the stabilization of HIF which induces an antiinflammatory effect resulting in reduction of anti-EPO antibodies. As a result, desidustat may decrease the development of EPO resistance, reduce the requirement of EPO to maintain hemoglobin, and decrease the drop in hemoglobin after cessation of EPO therapy.
4. Material and methods

4.1. Animals

Institutional Animal Ethics Committee approved animal procedures conducted in this work at Zydus Research Centre, a facility accredited by AAALAC International. Male Sprague Dawley rats (6–8 weeks old) were used in all the experiments. Male Sprague Dawley rats housed in groups at controlled temperature (25 ± 3 °C) and humidity conditions (50–70%), with a 12-h light/dark cycle. The animals had access to a standard chow diet and water ad libitum.

4.2. Chemicals and analysis

Desidustat was synthesized at Zydus Research Centre (ZRC), Ahmedabad, India. All other chemicals were purchased from Sigma Aldrich. Blood hemoglobin content was measured by tail bleeding using the QuantiChrom hemoglobin assay kit (Bioassay). Serum hepcidin levels were measured using an ELISA kit (CUSABIO Technology LLC). Serum IL-1p levels (Biolegends), and IL-6 levels (Sigma Aldrich) were measured using ELISA kit. Serum and tissue iron was measured using a total iron estimation kit (Pointe Scientific Inc.). Erythropoietin was measured using an ELISA kit (R&D Systems). Tissue was processed using a reported method for iron estimation using a kit (Pointe Scientific Inc.) (Rebouche et al., 2004).

Desidustat was administered by oral gavage in 0.5% aqueous carboxymethylcellulose (CMC), and Recombinant Human Erythropoietin (rhEPO, ZYROP 10000 injection) was diluted in normal saline.

4.3. Induction of anemia

Male Sprague Dawley rats were given a single intraperitoneal (IP) injection of cisplatin (5 μg/kg). After a week of cisplatin treatment, all the animals were treated once a week with turpentine oil (5 mL/kg) by subcutaneous (SC) route for the next ten weeks. The induction of anemia in terms of a decrease in hemoglobin was confirmed after two weeks of cisplatin injection.

4.4. Effect of desidustat on development of EPO resistance in anemia

The anemic rats were randomized into four groups based on their hemoglobin levels (thirty six anemia animals, each group containing nine anemic rats). These groups were assigned the following treatments, namely vehicle, human recombinant erythropoietin (rhEPO, 1 μg/kg, SC, three times a week), rhEPO and desidustat (15 μg/kg, PO, alternate day), and rhEPO and desidustat (30 μg/kg, PO, alternate day). A separate group of rats was sham-treated as a normal control group (n = 9). All the treatments were given for eight weeks, during which time blood hemoglobin was estimated every week. At the end of the treatment period (24 h after the last dose of turpentine oil), animals were bled retro-orbitally under isoflurane anesthesia and euthanized after collecting liver and spleen. Tissue samples were collected in liquid nitrogen and kept at −70 °C until further analysis.

4.5. Effect of desidustat on the requirement of EPO for hemoglobin maintenance in anemia

Twenty-seven anemic rats were randomized into three groups based on their hemoglobin levels (n = 9/group). These groups were assigned the following treatments: vehicle control, human recombinant erythropoietin (rhEPO, 1–5 μg/kg, SC, three times a week), rhEPO, and desidustat (15 μg/kg, PO, alternate day), and rhEPO. All the treatment was continued for eight weeks. A separate group of rats was sham-treated as a normal control group (n = 9). The dose of rhEPO was adjusted at weekly intervals to maintain the hemoglobin levels at 16.0 ± 0.5 g/dL. Hemoglobin was estimated every week for four weeks. After four weeks, hemoglobin was measured twice weekly. The dose of rhEPO was increased gradually from 1 μg/kg to 5 μg/kg, as and when required to maintain the hemoglobin levels of each group. The rhEPO treatment was stopped when the animals attained normal hemoglobin levels of 16.0 ± 0.5 g/dL. The desidustat treatment was continued for eight weeks of treatment. At the end of the treatment period, animals were bled retro-orbitally under isoflurane anesthesia. Anti-EPO antibodies were measured in serum.

4.6. Effect of desidustat on hemoglobin after discontinuation of rhEPO therapy

Eighteen anemic rats were treated with human recombinant erythropoietin (rhEPO, 5 μg/kg, SC, three times a week, n = 18) for two weeks, and a vehicle-treated control group (n = 9) was also maintained in them. After two weeks, the rhEPO treatment was stopped, and all the rhEPO treated rats were randomized into two treatment groups, namely, vehicle and desidustat (15 mg/kg, PO, alternate day), for the next two weeks (n = 9). A separate group of sham-treated rats was the normal control group (n = 9). At the end of the treatment period (24 h after the last dose of turpentine oil), animals were bled retro-orbitally under isoflurane anesthesia and euthanized after collecting liver and spleen. Tissue samples were collected in liquid nitrogen and kept at −70 °C until further analysis. Total iron content was measured in the spleen and liver.

4.7. Measurement of anti-rhEPO antibody titer

Serum samples were diluted 1:6000 before the estimation of antibody levels. The detection of anti-EPO antibodies was carried out by ELISA, using rHuEPO as antigen, rabbit anti-human EPO antibody (Sigma Aldrich; E2531, 500 ng/mL, 1:50,000 dilution), and donkey anti-rabbit IgG conjugated (Jackson Immuno Research Inc., 711-035-152, 1:10,000 dilution) with horseradish peroxidase. The substrate tetramethylbenzidine (Sigma Aldrich) was added, and the reaction was stopped by adding sulphuric acid 1.25 mol/L. The optical density was read at 450 nm.

4.8. Bone marrow histology

The bone marrow from the tibia was collected in bovine serum albumin and flushed through a syringe with bovine albumin to obtain a fine suspension. The suspension was centrifuged at 10,000 rpm at 4 °C. The supernatant and pellet were resuspended in bovine serum albumin. A drop of the suspension was placed on a slide, and a smear was prepared. Slides were cooled and air-dried. The dried smear was fixed with methanol. It was then stained with May-Grumwald stain and Giemsa stain. The stained slides were washed with distilled water and then air-dried. One hundred cells were screened per animal and were quantified for myeloid, erythroid, and lymphoid cells. The ratios of myeloid to erythroid and lymphoid to erythroid were calculated for each animal.

4.9. Liver hepcidin and ferroportin mRNA isolation and gene expression

Expressions of ferroportin (FPN, FPN.5-AAGATCGCAGAACCTTCCGCACA-3 and R:5-TCGGTGCTGAGCTGCAAGACC-3), hepcidin (F5-GAAGCCGACGTGCAATAGAACA-3 and R:5-TTCGATGTCTGGCCGGAAGATAG-30) and β-actin messenger ribonucleic acid (mRNA) in liver tissue were assessed by quantitative PCR (qPCR) (Patel et al., 2021). Total RNA was extracted with TRI Reagent according to the manufacturer’s instructions (cat. No. T9424, Sigma Aldrich, USA). Thereafter, first strand cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystem, USA). The resulting cDNAs were used for qPCR using the QIAGEN Quanti Fast SYBR Green kit (Cat. No. 204052, Qiagen, USA). The qPCR was run in an ABI-7300 (Applied Biosystem, USA).
Biosystems, Foster City, CA, USA). Relative gene expressions were calculated by 2−ΔΔCT method and normalized using β-actin as a housekeeping gene (Patel et al., 2022).

4.10. Data analysis

All analyses were performed using GraphPad Prism version 9.2 (GraphPad Software, San Diego, CA). Data is represented as mean ± SEM with number of animals indicated in individual experiments and figures. The data represented in bar graphs were analyzed by one-way ANOVA followed by Fisher’s LSD multiple comparison test for comparisons within the groups. For data represented in the line graphs, statistical analysis was performed by two-way ANOVA followed by Fisher’s LSD multiple comparison test. P < 0.05 was considered a statistically significant difference (*P < 0.05 against vehicle Control, $P < 0.05 against rHepO treatment).

CRediT authorship contribution statement

Amit A. Joharapurkar: were involved in the concept and design of this work, Formal analysis, drafting of the manuscript, were involved in the design of the experiments, acquisition, Formal analysis, initial drafting of the manuscript. Vishal J. Patel: were involved in the design of the experiments, acquisition, Formal analysis, initial drafting of the manuscript. Samadhani G. Kshirsagar: were involved in the design of the experiments, acquisition, Formal analysis, initial drafting of the manuscript. Maulik S. Patel: were involved in the acquisition. Haridikumar H. Sivashan: were involved in the acquisition. Ghestan Kavadar: were involved in the acquisition. Darshan Valan: were involved in the acquisition. Mukul R. Jain: were involved in the concept and design of this work, Formal analysis, drafting of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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