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RAP-011, an activin receptor ligand trap, increases hemoglobin concentration in Hepcidin transgenic mice

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Abstract

Over expression of hepcidin antimicrobial peptide is a common feature of iron-restricted anemia in humans. We investigated the erythroid response to either erythropoietin or RAP-011, a "murinized" ortholog of sotatercept, in C57BL/6 mice and in hepcidin antimicrobial peptide over expressing mice. Sotatercept, a soluble, activin receptor type IIA ligand trap, is currently being evaluated for the treatment of anemias associated with chronic renal disease, myelodysplastic syndrome, β-thalassemia, and Diamond Blackfan anemia and acts by inhibiting signaling downstream of activin and other Transforming Growth Factor-β superfamily members. We found that erythropoietin and RAP-011 increased hemoglobin concentration in C57BL/6 mice and in hepcidin antimicrobial peptide over expressing mice. While erythropoietin treatment depleted splenic iron stores in C57BL/6 mice, RAP-011 treatment did not deplete splenic iron stores in mice of either genotype. Bone marrow erythroid progenitors from erythropoietin-treated mice exhibited iron-restricted erythropoiesis, as indicated by increased median fluorescence intensity of transferrin receptor immunostaining by flow cytometry. In contrast, RAP-011-treated mice did not exhibit the same degree of iron-restricted erythropoiesis. In conclusion, we have demonstrated that RAP-011 can improve hemoglobin concentration in hepcidin antimicrobial peptide transgenic mice. Our data support the hypothesis that RAP-011 has unique biologic effects which prevent or circumvent depletion of mouse splenic iron stores. RAP-011 may, therefore, be an appropriate therapeutic for trials in human anemias characterized by increased expression of hepcidin antimicrobial peptide and iron-restricted erythropoiesis.

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Authorship and Disclosures

JML executed the experiments, analyzed the data, and wrote the manuscript; SB, AE, and CC analyzed the qPCR data and wrote the manuscript; QLX analyzed the data and wrote the manuscript; VS designed the experiments and wrote the manuscript; CNR designed and executed the experiments, analyzed the data and wrote the manuscript.

Keywords

anemia; iron; erythropoietin; hepcidin antimicrobial peptide; activin receptor type IIA

Introduction

Erythropoietin (Epo) is required for survival of erythroid progenitors [1]. However, hemoglobin production occurs largely independent of Epo [2] and is highly dependent on iron for heme biosynthesis [3]. As committed erythroid precursors begin hemoglobin synthesis, they express very high levels of transferrin receptor (TfR) to facilitate iron acquisition from serum transferrin, the primary source of iron for erythropoiesis. After hemoglobin production is complete, the mature erythroid precursor enucleates, and the resulting reticulocyte is released to circulation [4]. Recent clinical trials have demonstrated that intravenous iron enhances the erythroid stimulating activity of erythropoietin (Epo) in hemodialysis patients with chronic kidney disease (CKD), suggesting that administration of Epo, alone, results in erythropoiesis that is limited by iron availability in these patients [5].

Hepcidin antimicrobial peptide (Hamp) is a potent regulator of iron available to the erythron [6]. It negatively regulates [7] Ferroportin (Fpn) [8, 9], the only transporter known to facilitate elemental iron egress from cells. In adults, the iron that supports erythropoiesis is primarily provided by splenic macrophages which clear aged and damaged erythrocytes from circulation and return the iron recycled from heme to the serum via Fpn. Mice engineered to over express *Hamp1* from a transgene (Tg-*Hamp*) demonstrate iron-restricted erythropoiesis [10, 11]. Over expression of *Hamp* is a central finding in other iron-restricted anemias as well [12–14].

The phenotype of Tg-*Hamp* mice models iron restriction, a feature of anemia of inflammation or anemia of chronic disease [15]. Hamp is known to be elevated in the serum and plasma of patients with adult and pediatric CKD, inflammation, and multiple myeloma [16, 17]. Its central role in driving the hypoferremia associated with anemia of inflammation [18] has made it a popular target for the development of drugs that might enhance erythropoiesis in various disease states [19–24].

Sotatercept is a human fusion protein comprised of the activin receptor type IIA and the Fc domain of IgG_1. Activin receptors bind various ligands of the Transforming Growth Factor β (TGFβ) family of proteins which impact the development of many tissue types [25], including the erythroid compartment [26]. Sotatercept acts by trapping activins A and B as well as several growth and differentiation factors (GDFs) and bone morphogenic proteins (BMPs). Sotatercept prevents receptor binding and subsequent downstream signaling [27]. Although sotatercept was initially developed for its bone building activity [26, 28], it also rapidly and dose-dependently stimulated hemoglobin and other red blood cell (RBC) parameters in healthy female volunteers [29, 30]. Subsequently, these erythropoietic effects have been confirmed and studied in the non-clinical setting [27, 31, 32]. Furthermore, sotatercept is being evaluated for treatment of anemia related to end-stage renal disease [33], myelodysplastic syndrome [34], beta thalassemia [35], and Diamond Blackfan anemia [36].

In the current study, we investigated iron handling during the erythroid response to RAP-011, a murine ortholog of sotatercept, in wild type C57BL/6 mice and Tg-*Hamp* mice. Epo treatment served as a positive control. We assessed hemoglobin response as well as systemic and erythroid-specific markers of iron utilization. Our data demonstrate that RAP-011 acts within 2 days to increase hemoglobin concentration in *Tg-Hamp* mice. Furthermore, while erythropoiesis in response to Epo is iron-restricted, RAP-011 allows for sufficient iron acquisition by erythroblasts.

Methods

Animal Care

All procedures involving mice were approved by The Johns Hopkins University Animal Care and Use Committee. All mice described in this manuscript were 4–5 week old females at day 0 of treatment. Mice were housed in ventilated racks (Allentown Caging Equipment) with a 14 hour light cycle at the Johns Hopkins University barrier facility with access to food and water, ad libitum. Mice were maintained on the 2018SX Teklad Global 18% Protein Extruded Rodent Diet (Harlan Teklad, Madison, WI) which contains 225 parts per million (ppm or mg/kg) iron. An estimated 35 mg/kg iron in the chow is sufficient to meet the mouse daily iron requirement [37]. Because of their rapid growth from 4–8 weeks of age, a greater iron demand is likely during this time of development. However, we expect that wild type mice do not have limitations in iron absorption on this diet which includes over 6 times the mouse daily iron requirement. Sixteen hours before sacrifice the mice were transferred to a clean cage and fasted overnight with only water available ad libitum.

Tg-*Hamp* mice

Mice over expressing the *Hamp1* transgene under the control of the tetracycline regulatory element (TRE) were previously described on a mixed genetic background [10] and then backcrossed10 generations onto the C57BL/6 background [38]. We chose the 5 week time point for our experiments because it provided a strong differential in hemoglobin between C57BL/6 (~13 g/dL) and Tg-*Hamp* (~12 g/dL) female mice. Our previous assessment of hemoglobin in this Tg-*Hamp* line at 8–10 weeks of age was not as low as 4–5 week Tg-*Hamp* mice [38]. We suspect the rapid growth and expanding blood volume of the mice around 5 weeks of age maximizes dietary iron uptake mechanisms, suppresses endogenous *Hamp*, and makes the mice more sensitive to *Hamp* over expression.

Compound

The extracellular domain of ActRIIA is completely conserved among numerous species including mouse, rat, cynomologus monkey and humans. In order to reduce the potential immunogenicity of sotatercept and to maximize the opportunity to maintain exposures in chronic rodent models, a murine ortholog was constructed by exchanging the human IgG1 Fc sequence portion of sotatercept with its murine IgG2a ortholog. The resultant construct, referred to as RAP-011 (ActRIIA-mIgG2aFc) has similar in vitro binding characteristics as sotatercept and has been routinely employed in both cellular and pharmacology studies [27, 32].

Stimulation of Erythropoiesis

The mice were injected with 9 International Units (IU, a dose approximating 600 IU/kg Epo) of Procrit Epoetin Alfa (Amgen Pharmaceuticals, Thousand Oaks, CA) or 450 μg RAP-011 (a dose approximating 30 mg/kg). Because we were interested in assessing the ability of erythroid precursors to utilize iron in response to Epo or RAP-011, a dose finding pilot was performed to identify the doses of each agent that would result in similar hemoglobin response. Epo doses of 600 IU/kg and 1000 IU/mouse were tested before selecting 600 IU/kg. RAP-011 doses of 10 mg/kg and 30 mg/kg were tested before selecting 30 mg/kg. Female C57BL/6 mice or Tg-Hamp mice were treated with intraperitoneal injection of Epo or RAP-011 starting at 4 to 5 weeks of age on experimental days 0, 2, 5, and 8.

Weight and Complete Blood Count

Female mice were anesthetized with intraperitoneal (IP) injection of Avertin (125–240 mg/kg) and then weighed. We collected blood from the retro-orbital sinus into EDTA-treated microtainer tubes (Becton Dickinson, Franklin Lakes, NJ) for complete blood count (CBC), alternating eyes on each experimental day. 50 microliters of blood was collected for CBC and reticulocyte count on day 0 before the first intraperitoneal injection of Epo or RAP-011. 50 microliters of blood was collected for CBC and reticulocyte count again on day 2 before the injection of the test agent. Whole blood samples were analyzed for CBC by the Hemavet 950 (Drew Scientific, Waterbury, CT). Reticulocytes were analyzed using ReticCount (Becton Dickinson, San Jose, CA) according to the manufacturer's instructions. The mice were euthanized on day 10 of the experiment, following retro-orbital blood collection.

ELISA

Whole blood was collected from the retro-orbital sinus into microtainer serum separator tubes (Becton Dickinson) from mice anesthetized with Avertin. Serum was separated and immediately stored at −80°C in single use aliquots. Samples were thawed once, and then analyzed for serum iron or erythropoietin. Mouse Erythropoietin ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Wilcoxon rank-sum test was used to assess the significance of the difference in Epo concentration between treatment groups. Remaining serum was analyzed for hepcidin using the Hepcidin-Murine Compete™ ELISA (Intrinsic Life Sciences, La Jolla, CA).

Analysis of iron stores

Serum iron analysis was performed with the Ferrozine-based Iron/TIBC Reagent Set (Pointe Scientific Inc., Canton, MI). The manufacturer's protocol was amended to use 1ml of Iron Buffer reagent and 50μl of serum. Non-heme tissue iron was analyzed using bathophenanthroline, a colorimetric reagent, as previously described [39].

Flow cytometry

Erythroid maturation was determined essentially as described elsewhere [38, 40, 41]. Briefly, mouse bone marrow was flushed from the femur using RPMI with 2.5% serum and the clumps were dispersed with an 18 gauge needle. Mouse spleens were minced and

digested in 0.05% collagenase and 0.002% DNAse at 37°C for 40 minutes. Marrow and splenocytes were stained with antibodies to Ter119, CD45, CD71, and/or CD44 (BD Pharmingen, Franklin Lakes, NJ). The cell staining was quantified with the FACS Calibur (Beckton Dickenson) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

Statistical Analysis

All analyses were performed in Stata Version 12.1 (StatCorp., College Station, Texas, USA). Paired t-tests were used to assess within-subject change in complete blood count parameters between baseline and Day 10 of the treatment cycle by genotype and treatment condition. Two-sample t-tests were used to assess between-genotype or between- treatment group differences in erythroid response and erythroid progenitors, as well as differences in serum iron concentration and tissue iron stores at Day 10. Because these analyses required necropsy, within-subject change and values at Day 0 and Day 2 could not be determined. Bartlett's test was used for assessing homogeneity of variances across comparison groups. Satterthwaite's degrees of freedom were used in the two sample t-test in the presence of unequal variances. The Wilcoxon rank-sum test was used for Epo concentration because of its non-normal distribution. All p-values were based on tests with 0.05 Type I error rate. More details concerning specific statistical tests are provided in the footnotes for data tables.

Results

RAP-011 increases hemoglobin in Hepcidin antimicrobial peptide transgenic mice

Female Tg-*Hamp* mice and C57BL/6 controls were treated with recombinant human erythropoietin (Epo) or RAP-011, over a 10 day time course starting at 4 to 5 weeks of age. The erythroid response in these groups was compared to mice of the same age, sex, and genotype treated with phosphate buffered saline (PBS) as a vehicle control. At the beginning of the time course, Tg-*Hamp* mice had significantly lower hemoglobin concentration than C57BL/6 controls (Figure S1, $p<0.001$; please see figure legends or table footnotes for specific statistical tests).

As expected, the change in hemoglobin was significantly greater in C57BL/6 mice treated with Epo than in PBS-treated C57BL/6 mice (Table I, $p<0.001$, see also Table S1 and Figure S1A). Tg-*Hamp* mice also showed a significantly greater change in hemoglobin after the 10 day Epo treatment cycle, than PBS-treated Tg-*Hamp* mice (Table I, $p=0.01$, see also Table S1 and Figure S1A) demonstrating that overexpression of hepcidin did not preclude the mice from responding to Epo. Both Tg-*Hamp* and C57BL/6 mice responded to RAP-011 treatment with a significant increase in hemoglobin concentration averaging 2 g/dL or more (Table I, $p<0.001$, see also Table S1 and Figure S1B). Increases in erythrocyte number in both genotypes in response to Epo or RAP-011 were also significantly greater than mice treated with PBS (Table I, $p\leq0.03$ for both).

The change in circulating reticulocytes was greater in both genotypes of RAP-011-treated mice at Day 10 than their corresponding PBS-treated genotype controls, which declined (Table I, $p<0.001$, see also Table S1 and Figure S2B). The percentage of circulating reticulocytes was relatively high in the young mice of both genotypes at baseline (Table S1

and Figure S2) compared to values we have previously reported for these genotypes in older mice at 8–10 weeks of age [38]. After 10 days with PBS treatment, reticulocytes were significantly lower than the values at baseline (Table S1 and Figure S2, $p<0.01$, for C57BL/6 and Tg-*Hamp* mice). The extent by which RAP-011 increased reticulocytes was similar to the effect seen following Epo treatment; however, the timeline for induction was very different. Reticulocytes were significantly higher than baseline at Day 2 for both genotypes of Epo-treated mice (Table S1 and Figure S2A, $p<0.05$), but were lower than baseline by Day 10, similar to PBS-treated mice (Figure S2A and Table S1, $p<0.01$). Thus, while we observed Epo effects on reticulocytes as early as 2 days post-treatment, we did not observe RAP-011 effects on reticulocytes until 10 days post-treatment.

Additional erythrocyte parameters over the 10 Day treatment time course are provided in Table S1.

Erythroid progenitor/precursors in Epo and RAP-011 treated mice

To assess whether the response to RAP-011 involved erythropoietin, we measured serum Epo concentrations in RAP-011-treated mice at Day 10. We found that Epo concentrations were significantly higher in both genotypes treated with RAP-011 when compared to PBS-treated genotype controls (Table II, $p=0.002$). This correlates with increased reticulocytes in RAP-011-treated mice (described above) and suggests that, in addition to the rapid effects of RAP-011, there may also be a secondary mechanism by which RAP-011 could sustain increased red cell parameters through induction of Epo [27].

A single injection of RAP-011 induces expansion of late stage erythroid progenitors in the marrow within two days and returns to equilibrium by 4 days [27]. To assess the response of the erythron to Epo and RAP-011 at the conclusion of our 10 day treatment protocol, we analyzed erythroid progenitors and precursors in the bone marrow and spleens of C57BL/6 and Tg-*Hamp* mice by immunostaining with Ter119 (Ter+), the definitive marker of committed mouse erythroid progenitors. Ten days after the start of treatment, we did not observe a higher percentage of Ter+ cells in the bone marrow of mice of either genotype treated with either Epo or RAP-011 when compared to PBS-treated genotype controls (Table II). However, we did observe a relative expansion of marrow erythroblasts defined by their expression of Ter119 and CD44 (Figure S3). Both C57BL/6 and Tg-*Hamp* mice demonstrated a shift in the distribution toward earlier (that is, $CD44^{high}$) erythroblasts (gates II or III Figure S3) when treated with Epo (Figure S3, panels B and E) or RAP-011 (Figure S3, panels C and F). Because the percentage of Ter+ erythroid progenitors did not change in the marrow (Table II), we hypothesize these observations resulted from two mechanisms. First, increased survival of Epo-sensitive progenitors resulted in increased percentages of erythroblasts in gates II–IV. Second, release to the circulation resulted in relatively decreased percentages of mature erythrocytes in gate V. We expect the expansion of early stage, Epo-sensitive, erythroblasts in the marrow of RAP-011 mice at Day 10 is related to the increase in circulating Epo concentration that we observed in RAP-011-treated mice (Table II).

Spleen weights were greater for Epo-treated C57BL/6 mice than for PBS-treated C57BL/6 mice (Table II, $p<0.001$) after the 10 day treatment course. The percentage of Ter+

splenocytes was not significantly higher in the Epo-treated mice, yet flow cytometry staining with Ter119 and CD44 indicated an increased proportion of CD44-positive erythroblasts (populations II and III) in the spleens of Epo treated C57BL/6 mice (Table S2, $p < 0.005$). In contrast, RAP-011-treated C57BL/6 mice had greater spleen weights (Table II, $p < 0.001$) and higher percentage of Ter+ splenocytes (Table II, $p<0.001$) when compared to PBS-treated C57BL/6 mice. Flow cytometry staining with Ter119 and CD44 indicated an increased proportion of CD44-positive erythroblasts (populations II–IV) in the spleens of RAP-011 treated C57BL/6 mice (Table S2, $p < 0.001$).

Spleen weights were greater in PBS-treated Tg-*Hamp* mice than in PBS-treated C57BL/6 mice (Table II, $p<0.02$), but we observed no difference in the percentage of Ter+ splenocytes, similar to the results for Epo-treated C57BL/6 mice. We did not observe a significant difference in spleen weight or the percentage of Ter+ splenocytes in Epo-treated Tg-*Hamp* mice compared to PBS-treated Tg-*Hamp* mice. However, flow cytometry staining with Ter119 and CD44 indicated a significantly increased proportion of CD44-positive erythroblasts (populations I–III) in the spleens of Epo-treated Tg-Hamp mice compared to PBS-treated Tg-Hamp mice (Table S2, $p<0.05$). In contrast, we observed significantly greater spleen weights and higher percentage of Ter+ splenocytes in Tg-*Hamp* mice treated with RAP-011 when compared to PBS-treated Tg-*Hamp* mice (Table II, $P<0.001$). Flow cytometry staining with Ter119 and CD44 indicated a greater proportion of CD44-positive erythroblasts (populations II–IV) in the spleens of RAP-011 treated Tg-Hamp mice (Table S2, $p<0.001$). In light of our observation that reticulocytes remained elevated in the peripheral blood at Day 10 of the RAP-011 treatment protocol, the percentage of Ter+ splenocytes and the uniquely higher percentage of splenic erythroblasts in population IV (Table S2) seemed to correlate better with sustained reticulocytosis at 10 days than an increase in spleen weight. A single injection of RAP-011 has been shown to have a rapid effect on the expansion of late stage bone marrow erythroid progenitors in mice [27]. Our data demonstrate that repeated injections and a longer treatment time course result in the same unique effect of RAP-011 on late stages (population IV) of splenic erythropoiesis, coupled with modest Epo-induced effects on earlier erythroid progenitors in both the marrow and the spleen. This “preference” for longer term RAP-011 effects in the mouse spleen, as opposed to the mouse marrow, corroborates and extends the recent observation of the splenic effects of RAP-011 in C57BL/6 mice [32] (see supplementary online material for reference 32).

Distribution of serum iron and tissue iron stores after Epo and RAP-011 treatment

To determine how iron was used to support the expansion of erythropoiesis in response to Epo and RAP-011, we measured serum and tissue iron at Day 10 of the treatment time course. Serum iron concentration was significantly lower in mice of either genotype treated with Epo, when compared to PBS-treated genotype controls (Table III, $p=0.01$ for both C57BL/6 and Tg-*Hamp*). However, we observed no such difference in mice treated with RAP-011 when compared to PBS-treated genotype controls ($p=0.63$, 0.39 for C57BL/6, Tg-*Hamp*, respectively). We considered the hypothesis that low serum iron in Epo-treated mice may be related to the increased demand of the expanding erythroid compartment and more effective clearance of iron from the serum. Because RAP-011 treatment resulted in virtually

the same increase in hemoglobin concentration without a change in serum iron, our results suggested a potential difference in tissue iron handling or a difference in the stage of erythroid development which supported expanded erythropoiesis with RAP-011 treatment.

To investigate tissue iron handling, we employed the bathophenanthroline method to quantify tissue iron because it measures only non-heme iron. This allowed our analysis to avoid any confounding by heme iron in splenic erythroblasts. Additionally, we measured the total iron per spleen to adjust for the increase in spleen mass that occurs in Tg-*Hamp* mice and in response to Epo and RAP-011 treatment. This prevented a “dilution” effect of spleen non-heme iron concentration by developing splenic erythroid progenitors. Total splenic non-heme iron stores were lower in C57BL/6 mice treated with Epo when compared to PBS-treated C57BL/6 mice (Table III, p=0.02), suggesting depletion of this active iron source for enhanced erythropoiesis. In contrast, splenic non-heme iron stores were not lower in Tg-*Hamp* mice treated with Epo, compared to PBS-treated Tg-*Hamp* mice, suggesting this group did not successfully utilize splenic non-heme iron stores when treated with Epo (Table III, p=0.80). Consistent with this observation, Epo-treated Tg-*Hamp* mice had the lowest serum iron concentration of all the groups. These results are expected, as Hamp prevents iron egress from macrophages to the serum. Thus, the clearance of serum iron by the Epo-induced expansion of erythroblasts was not balanced by splenic macrophage iron egress in Tg-*Hamp* mice. We did not observe any changes in liver iron stores in response to Epo treatment.

RAP-011 treatment did not result in a decline of serum iron concentration or splenic non-heme iron stores in mice of either genotype (Table III). These results imply that RAP-011 treatment allows for enhanced erythropoiesis without depletion of splenic non-heme iron stores. Liver non-heme iron stores were also unchanged in all groups of mice treated with RAP-011.

Serum iron concentration is negatively regulated by Hamp [42]. Conditions of erythropoietic stress can down regulate *Hamp* expression to increase iron available to the erythron [43–45]. We investigated serum Hamp concentration after 10 days of treatment with Epo or RAP-011 (Table III). We found serum Hamp concentrations were significantly lower in C57BL/6 mice treated with Epo than C57BL/6 treated with PBS at this time point (Table III, p=0.019). Reduced serum Hamp in Epo-treated C57BL/6 mice reflects the expected physiologic response to erythropoiesis [43–45]. Serum Hamp did not decline significantly in Tg-*Hamp* mice treated with Epo because of constitutive expression of Hamp from the transgene. Serum Hamp concentrations were not significantly reduced in response to RAP-011 in mice of either genotype. Liver *Hamp* mRNA expression was highly variable, but followed the same trends (Table S3). From these data we conclude that full suppression of *Hamp* must not be required for the response to Epo or RAP-011, since our Tg-*Hamp* mice (which constitutively express *Hamp* under the control of the transgenic promoter) are able to induce erythropoiesis in response to both agents. However, serum iron is most severely depleted in Tg-Hamp mice treated with Epo because constitutive Hamp expression prevents the replenishment of serum iron with splenic iron stores.

In summary, we observed clearance of serum iron and depletion of splenic iron stores in response to Epo-induced erythropoiesis. In contrast, we found no evidence for depletion of these iron sources with RAP-011-induced erythropoiesis, and no indication that endogenous Hamp was down regulated to off-set iron utilization in RAP-011-treated mice. Combined with our data indicating an expansion of later erythroid developmental stages (IV) in RAP-011-treated mice compared to Epo-treated mice, these data lead us to hypothesize that RAP-011 permits survival or expansion of erythroblasts at late stages of erythroid development that are no longer dependent on iron acquisition. We conclude that RAP-011-induced erythropoiesis allows for conservation of iron when compared to Epo-induced erythropoiesis. This ultimately results in a significantly lower iron demand with RAP-011 treatment than that of Epo-induced erythropoiesis.

Iron-restricted erythropoiesis in response to Epo, but not RAP-011

To determine whether erythroid precursors were sensing the observed differences in serum iron concentration between Epo and RAP-011 treatment groups, we determined the median cell surface expression of the transferrin receptor (TfR/CD71) at each developmental stage of erythropoiesis in the bone marrow (Table IV). We expected this marker to be similar to serum TfR, increasing in response to iron-restricted erythropoiesis [46]. In support of this expectation, we observed higher TfR/CD71 median fluorescence intensity (MFI) in stages II, III and IV of PBS-treated Tg-*Hamp* mice compared to PBS-treated C57BL/6 mice (Table IV, $p<0.01$ for each). This observation is consistent with lower serum iron and iron-restricted erythropoiesis mediated by Hamp at these developmental stages characterized by hemoglobin production. We also observed higher TfR/CD71 MFI in erythroid stages I, II, III and IV for all Epo-treated mice, regardless of genotype, when compared to their PBS-treated control group (Table IV, $p<0.01$ for all groups). In combination with low serum iron concentration, this suggested to us that all Epo-treated mice were experiencing iron-restricted erythropoiesis. In contrast, there was only a marginal increase in TfR/CD71 MFI in RAP-011-treated C57BL/6 mice compared to PBS-treated C57BL/6 mice (Table IV). TfR/CD71 MFI was significantly higher only in gate II ($p<0.05$) for RAP-011-treated Tg-*Hamp* mice compared to PBS-treated Tg-*Hamp* mice (Table IV, $p=0.02$). Within the Tg-*Hamp* mice, we observed a significant step-wise trend in TfR/CD71 MFI across treatment groups, such that RAP-011 treatment resulted in higher median TfR/CD71 MFI than PBS-treatment and Epo-treatment resulted in higher median TfR/CD71 MFI than RAP-011 treatment (PBS<RAP<Epo for erythroblasts I-IV, $p<0.05$ using the nonparametric Cuzick test across treatment groups). We did not observe any consistent changes in mean cell volume (MCV) or mean cellular hemoglobin concentration (MCHC) over the time course. In combination with serum iron concentrations in these mice, the expression of TfR/CD71 suggested to us that marrow erythroblasts are more likely to be iron-restricted with Epo treatment than with RAP-011 treatment. However, increased TfR expression in erythroblasts was sufficient to establish roughly the same MCV and MCHC by Day 10 despite lower serum iron in the Epo-treated groups.

Discussion

RAP-011 is a novel erythroid stimulating agent that successfully induced erythropoiesis in Tg-*Hamp* mice, a mouse model of iron-restricted anemia with some overlapping features of anemia associated with inflammation or chronic disease. In Tg-*Hamp* mice, RAP-011 induced a significant increase in hemoglobin concentration within two days and a lasting reticulocyte response. Hemoglobin concentration in C57BL/6 and Tg-*Hamp* mice also increased in response to Epo over the 10 day time course but with different kinetics than we observed post-RAP-011 treatment.

We observed distinct differences in utilization of iron stores when we compared C57BL/6 mice treated with Epo to C57BL/6 mice treated with RAP-011. Splenic non-heme iron stores were depleted in C57BL/6 mice treated with Epo, but despite utilization of this critical iron source, we found evidence for iron-restricted erythropoiesis (low serum iron and increased median TfR expression on Ter+ erythroblasts) in Epo-treated C57BL/6 mice. In contrast, splenic non-heme iron stores were not depleted in C57BL/6 mice treated with RAP-011. Furthermore, we did not observe extensive iron-restricted erythropoiesis in C57BL/6 mice treated with RAP-011. We considered whether RAP-011 promotes more efficient absorption of dietary iron, or promotes increased iron recycling for erythropoiesis. We expected Hamp to be decreased to support enhanced dietary iron absorption and recycling, but we found no evidence for decreased Hamp in RAP-011-treated mice. Thus, we expect any mechanism for enhanced iron absorption and recycling in RAP-011-treated mice to be hepcidin independent and to involve direct regulation of iron or heme transporters in the duodenum, macrophages, or erythroblasts. A limitation of our study is that we were not able to test this direct regulation of iron and heme transporters. The depletion of splenic non-heme iron stores that we observed in Epo-treated C57BL/6 mice may indicate that the iron has been redistributed, possibly to heme in splenic or marrow erythroid progenitors that do not adequately mature.

Over expression of *Hamp* prevented utilization of splenic non-heme iron stores and resulted in a phenotype of iron-restricted erythropoiesis in Tg-*Hamp* mice. Iron-restricted erythropoiesis was even more severe in Epo-treated Tg-*Hamp* mice than in PBS-treated Tg-*Hamp* mice. RAP-011 successfully induced hemoglobin concentration in Tg-*Hamp* mice, and it did not result in iron-restricted erythropoiesis that was as severe as Epo-treated Tg-*Hamp* mice (as measured by serum iron concentration and median TfR expression in erythroblasts).

We propose that increased TfR expression in marrow erythroblasts reflects iron-restricted erythropoiesis. However, an alternative explanation for increased TfR expression in erythroblasts is that TfR may be transcriptionally up-regulated by Epo through Stat5 signaling [47]. Even if increased erythroblast TfR expression reflects the Epo-induced transcriptional response, decreased serum iron concentration and depleted splenic iron stores in Epo-treated C57BL/6 mice indicate a difference in systemic iron handling in response to Epo and RAP-011 treatments.

The erythroid iron demand seems to be much higher with Epo treatment than with RAP-011 treatment. This may be related to the efficiency with which newly produced erythroid progenitors pass late developmental checkpoints. We found expansion of stage IV splenic erythroblasts to be unique to RAP-011 treated mice. A significant proportion of Epo-induced erythroblasts may not pass the same checkpoint late in development. These data are consistent with recent observations that RAP-011 can rapidly increase the frequency of very late stage erythroid progenitors in the bone marrow. RAP-011 may remove an inhibitor of late stage erythroblasts, such as Activin A or Growth differentiation factor 11 to which iron-restricted erythroid progenitors may be especially sensitive [27].

Hamp is elevated in chronic disease states such as CKD or multiple myeloma [16, 17]. Tg-*Hamp* mice model iron-restricted erythropoiesis associated with chronic disease [10] because they over express *Hamp*. Pharmaceuticals which target the Epo pathway are the primary means to correct anemia in patients with CKD and cancer. However, Epo action is predominantly accomplished by promoting survival of committed erythroid progenitors. Hemoglobin synthesis occurs during a second, largely Epo-independent, stage of erythroid development and requires robust iron acquisition [2]. In keeping with this segregation of molecular mechanisms promoting erythroid progenitor survival versus mechanisms promoting hemoglobin synthesis, clinical trials have demonstrated that hemodialysis patients supplemented with intravenous iron responded more efficiently to epoietin [5]. Additionally, patients with very high expression of hepcidin due to mutations in *Transmembrane protease, serine 6* develop iron refractory iron deficiency anemia (IRIDA) which does not respond to erythropoietin [48]. In our study, RAP-011 was able to correct the anemia of Tg-*Hamp* mice and did not deplete iron stores as we observed for Epo action. Our observations suggest ACE-011, or sotatercept, may be a useful therapeutic intervention in anemias characterized by elevated Hamp and iron-restricted erythropoiesis that do not respond effectively to erythropoietin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table I

Change in complete blood count response to erythropoietin and RAP-011 after 10 day treatment cycle.

Genotype	Treatment	10d ΔHgb, g/dL	10d Δ RBC, M/mcL	10d Δ Retics, %
C57BL/6	PBS (N=13)	0.45 ± 0.92	0.46 ± 0.64	−3.64 ± 1.23
C57BL/6	Epo (N≥13)	1.75 ± 0.89	0.92 ± 0.55	−3.49 ± 3.13
C57BL/6	RAP-011 (N≥12)	2.11 ± 0.90	1.41 ± 0.64	0.96 ± 2.07
TTEST C57BL/6 (PBS vs Epo)		<0.001[&]	0.03[&]	0.56[&]
TTEST C57BL/6 (PBS vs RAP-011)		<0.001[&]	<0.001[&]	<0.001[&]
Tg-*Hamp*	PBS (N≥10)	0.74 ± 0.72	0.50 ± 0.46	−3.66 ± 1.61
Tg-*Hamp*	Epo (N=12)	1.49 ± 0.80	0.89 ± 0.49	−2.60 ± 1.81
Tg-*Hamp*	RAP-011 (N=18)	2.67 ± 0.89	1.73 ± 0.71	1.28 ± 2.46
TTEST Tg-*Hamp* (PBS vs Epo)		0.01[&]	0.03[&]	0.92[&]
TTEST Tg-*Hamp* (PBS vs RAP-011)		<0.001[&]	<0.001[&]	<0.001[&]
TTEST C57BL/6 PBS vs Tg-*Hamp* PBS		0.41[@]	0.86[@]	0.97[@]

Summary values are reported as mean ± standard deviation

[&]based on one-sided t-test for the null hypothesis that the change in Hgb, RBC, or reticulocytes in mice treated with Epo or RAP-011 will be less than or equal to that of PBS-treated mice

[@]based on two-sided t-test

Table II

Erythroid response to erythropoietin and RAP-011 after 10 day treatment cycle.

Genotype	Treatment	Epo, pg/mL* (N≥12)	Ter+, % Marrow# (N≥9)	Ter+, % Spleen# (N≥12)	Spleen, mg# (N≥12)	Spleen (% body weight)
C57BL/6	PBS	0 (0,71)	55 ± 5	46 ± 7	48.9 ± 9.4	0.34 ± 0.05
C57BL/6	Epo	ND	56 ± 5	48 ± 4	65.1 ± 13.2	0.44 ± 0.07
C57BL/6	RAP-011	97 (0,243)	57 ± 3	67 ± 5	77.6 ± 9.9	0.49 ± 0.05
TTEST C57BL/6 (PBS vs Epo)		ND	0.27&	0.13&	<0.001&	<0.001&
TTEST C57BL/6 (PBS vs RAP-011)		0.002^	0.12&	<0.001&	<0.001&	<0.001&
Tg-*Hamp*	PBS	0 (0,137)	59 ± 7	48 ± 6	60.5 ± 11.9	0.41 ± 0.07
Tg-*Hamp*	Epo	ND	52 ± 5	50 ± 8	67.4 ± 14.9	0.45 ± 0.07
Tg-*Hamp*	RAP-011	115 (0, 665)	58 ± 5	69 ± 5	88.4 ± 14.5	0.58 ± 0.13
TTEST Tg-*Hamp*(PBS vs Epo)		ND	0.98&	0.21&	0.12&	0.07&
TTEST Tg-*Hamp*(PBS vs RAP-011)		0.002^	0.61&	<0.001&	<0.001&	<0.001&
TTEST (C57BL/6 PBS vs Tg-*Hamp* PBS)		0.07@	0.21@	0.47@	0.02@	0.02@

*median and range

#mean ± standard deviation

^based on the Wilcoxon rank-sum test

&based on one-sided t-test for the null hypothesis that Ter+ erythroblasts or spleen weight in mice treated with Epo or RAP-011 will be less than or equal to that of PBS-treated mice

@based on two-sided t-test

Table III

Redistribution of serum and tissue iron in response to Epo and RAP-011 after 10 day treatment cycle.

Genotype	Treatment	Serum Fe, mcg/dL (N≥10)	Spleen Fe, mcg (N≥6)	Liver Fe, mcg/g (N≥12)	Hamp (ng/mL) (N≥5)
C57BL/6	PBS	88.0 ± 37.2^	8.20 ± 2.53	94 ± 53	257 ± 117
C57BL/6	Epo	61.0 ± 13.8	5.41 ± 0.74	110 ± 35	122 ± 55
C57BL/6	RAP-011	92.2 ± 21.5	9.65 ± 3.35	101 ± 33	244 ± 66
TTEST C57BL/6 PBS vs Epo		0.01&	0.02&	0.370@	0.019&
TTEST C57BL/6 PBS vs RAP-011		0.63&	0.84&	0.689@	0.407&
Tg-*Hamp*	PBS	65.0 ± 24.5	6.98 ± 2.28	71 ± 19	222 ± 60
Tg-*Hamp*	Epo	38.6 ± 14.5	9.13 ± 6.56	81 ± 72	193 ± 63
Tg-*Hamp*	RAP-011	62.4 ± 20.4	10.03 ± 4.85	86 ± 31	217 ± 67
TTEST Tg-*Hamp* PBS vs Epo		0.01&	0.80&	0.649@	0.227&
TTEST Tg-*Hamp* PBS vs RAP-011		0.39&	0.98&	0.117@	0.447&
TTEST Tg-*Hamp* PBS vs C57BL/6 PBS		0.088@	0.366@	0.166@	0.537@

^Values reported in table are mean ± standard deviation

&based on one-sided t-test for the null hypothesis that serum, spleen iron, or Hamp levels in mice treated with Epo or RAP-011 will be greater than or equal to that of PBS-treated mice

@based on two-sided t-test

Table IV

Median TfR/CD71 expression in marrow erythroid precursors after 10 day treatment cycle.

Genotype	Treatment	I	II	III	IV	V
C57BL/6	PBS (N=13)	322 ± 155	457 ± 120	192 ± 59	93 ± 44	4 ± 2
C57BL/6	Epo (N=14)	692 ± 303	942 ± 260	520 ± 168	245 ± 92	3 ± 1
C57BL/6	RAP-011 (N=14)	335 ± 112	535 ± 136	236 ± 76	88 ± 47	4 ± 2
TTEST[&]C57BL/6 PBS vs Epo		<0.001	<0.001	<0.001	<0.001	0.997
TTEST[&]C57BL/6 PBS vs RAP-011		0.40	0.06	0.05	0.60	0.14
Tg-*Hamp*	PBS (N=11)	363 ± 135	665 ± 175	288 ± 85	147 ± 46	4 ± 1
Tg-*Hamp*	Epo (N=12)	811 ± 184	1094 ± 182	563 ± 157	237 ± 106	3 ± 1
Tg-*Hamp*	RAP-011 (N=18)	477 ± 219	878 ± 344	382 ± 205	150 ± 86	4 ± 2
TTEST[&]Tg-*Hamp* PBS vs Epo		<0.001	<0.001	<0.001	0.009	0.982
TTEST[&]Tg-*Hamp* PBS vs RAP-011		0.05	0.02	0.05	0.44	0.47
TTEST[@] C57BL/6 PBS vs Tg-*Hamp* PBS		0.499	0.004	0.006	0.009	0.509

Summary values are reported as mean ± standard deviation

[&]based on one-sided t-test for the null hypothesis that TfR/CD71 MFI in mice treated with Epo or RAP-011 will be less than or equal to that of PBS-treated mice

[@]based on two-sided t-test