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Targeting the Hepcidin-Ferroportin Axis to Develop New Treatment Strategies for Anemia of Chronic Disease and Anemia of Inflammation

Chia Chi Sun¹, Valentina Vaja^{1,2}, Jodie L. Babitt¹, and Herbert Y. Lin^{1,†}

¹Program in Membrane Biology, Division of Nephrology, Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA

²Foundation Istituto Di Ricovero e Cura a Carattere Scientifico Ca' Granda, University of Milan, Milan, Italy

Abstract

Anemia of Chronic Disease (ACD) or Anemia of Inflammation (AI) is prevalent in patients with chronic infection, autoimmune disease, cancer and chronic kidney disease. ACD is associated with poor prognosis and lower quality of life. Management of ACD using intravenous iron and erythropoiesis stimulating agents (ESAs) are ineffective for some patients and are not without adverse effects, driving the need for new alternative therapies. Recent advances in our understanding of the molecular mechanisms of iron regulation reveal that increased hepcidin, the iron regulatory hormone, is a key factor in the development of ACD. In this review, we will summarize the role of hepcidin in iron homeostasis, its contribution to the pathophysiology of ACD, and novel strategies that modulate hepcidin and its target ferroportin for the treatment of ACD.

Keywords

Hemojuvelin; BMP6; Hepcidin; Anemia of Chronic Disease; Anemia of Inflammation

Introduction

Anemia of Chronic Disease (ACD), also known as Anemia of Inflammation (AI), is the most prevalent anemia in hospitalized patients worldwide. It occurs in patients with acute or chronic inflammatory conditions including infections, cancer, rheumatoid arthritis, and chronic kidney disease [1]. ACD is a heterogenous disorder that is typically characterized by a normocytic anemia, changes in erythropoietic responses, low serum iron and low transferrin saturation, but unlike in true dietary iron deficiency, iron is retained in the macrophages and there may be an increase in total body iron [2, 3]. Until recently, the molecular mechanisms and pathogenesis of the iron distribution abnormalities in ACD were unknown. It is now clear that inflammatory cytokines released during acute infection or chronic disease can alter systemic iron metabolism by inducing excess synthesis of hepcidin, the iron regulatory hormone [4-8]. Since hepcidin inhibits iron export from cells by blocking

[†]Corresponding author: Herbert Y. Lin, M.D., Ph.D., FASN, Massachusetts General Hospital, Center for Systems Biology, Program in Membrane Biology/Division of Nephrology, Richard B. Simches Research Center, 185 Cambridge St., CPZN-8216, Boston, MA 02114, Phone: 617-726-5661, Fax: 617-643-3182, Lin.Herbert@mgh.harvard.edu.

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ferroportin activity, excess hepcidin is the root cause of the hypoferremia and iron-restricted erythropoiesis seen in ACD [9, 10]. Treatment of the anemia, when necessary, has included administration of intravenous iron and erythropoiesis stimulating agents without directly addressing the high levels of hepcidin responsible for ACD. Moreover, concerns over adverse effects from these therapies have driven the need for alternative treatments. This review will summarize the current knowledge of iron metabolism and hepcidin regulation, and will examine emerging new treatment strategies for ACD that directly address the pathophysiology of this disease by antagonizing the hepcidin-ferroportin axis.

Systemic Iron Homeostasis

Iron is an essential micronutrient incorporated into proteins responsible for cellular respiration, survival and growth. It is also required for the generation of hemoglobin in red blood cells. Excess iron, however, can be toxic, resulting in free radical production and dysfunctional lipid membranes that ultimately leads to cell death and organ damage [11]. Therefore, systemic iron balance needs to be tightly regulated by the pathways that supply, utilize, recycle, and store iron [12].

Duodenal enterocytes absorb dietary iron and export the iron supply into the circulation to be utilized for cellular processes including erythropoiesis [13]. Reticuloendothelial macrophages, which recycle iron from senescent erythrocytes, supply most of the iron demanded by the bone marrow for erythropoiesis [14]. Intracellular iron is also stored with ferritin in the liver and where it accounts for a third of the body's iron stores. Intracellular iron can be exported from the hepatocytes when needed [15].

Hepcidin- the Central Regulator of Iron Homeostasis

Hepcidin is the key regulatory protein that controls intestinal iron absorption and distribution of iron from body stores including reticuloendothelial macrophages [14]. Hepcidin is a 25 amino acid secreted peptide hormone that is produced in the liver in response to a number of signals including iron levels. Hepcidin functions by binding to and initiating the degradation of ferroportin, the only known iron exporter. Ferroportin is present on the cell surface of duodenal enterocytes, macrophages, and hepatocytes. Thus, downregulating ferroportin will inhibit the transfer of cellular iron into the plasma from these cell types [9, 15, 16].

Regulation of Hepcidin

During normal iron homeostasis, increased circulating iron levels strongly signal the liver to upregulate hepcidin expression. High serum hepcidin levels decrease intestinal iron absorption and block iron export from tissue stores into the bloodstream in order to protect the body against excess total body iron accumulation and excessive distribution of iron into the circulation. Conversely, limited circulating iron levels downregulate hepcidin synthesis, allowing an influx of bioavailable iron from the duodenal enterocytes and tissue iron stores. Erythropoietic stimulators [17, 18] and hypoxia [19, 20] also negatively regulate hepcidin to increase iron availability for erythropoiesis. The molecular mechanisms for these inhibitory pathways of regulating hepcidin expression have been reviewed extensively elsewhere and will not be reviewed here [12, 21, 22]. In addition to increased circulating iron levels, inflammatory cytokines are potent stimulators of hepcidin synthesis, which play a main pathogenic role in the functional iron deficiency seen in ACD.

Pathophysiology of ACD

ACD is a well-known clinical entity commonly observed in patients with various underlying diseases that includes under its umbrella: anemia of infection, anemia of cancer, anemia of

rheumatoid arthritis, and anemia of chronic kidney disease. The etiology of ACD is multifactorial and is characterized by an immune cell activation and inflammatory cytokine response that blunts erythropoietin production, impairs erythropoiesis, decreases red cell life span and dysregulates iron homeostasis [1]. The hematologic features of ACD are distinct from typical iron deficiency anemia (IDA) without inflammation. In IDA, erythropoietic drive is increased in an effort to maintain sufficient oxygenation, resulting in the production of microcytic and hypochromic red blood cells. In contrast, ACD is typically a normochromic and normocytic anemia and microcytosis is not usually observed unless there is concurrent iron deficiency [1]. It is hypothesized that the normocytic anemia is due to inflammation induced blunting of erythropoiesis.

Inflammatory cytokines also induce changes in iron distribution. Patients with ACD have low serum iron concentration, low or normal total iron binding capacity, and low transferrin saturation and low reticulocyte counts [1, 23]. Importantly, a key feature of ACD is an accumulation of iron in the reticuloendothelial macrophages despite reduced circulating iron levels. Thus, little circulating iron is available for hemoglobin synthesis, even in the setting of adequate or high body iron stores. It is possible that humans developed this mechanism to sequester iron as a defense against certain invading pathogens, many of which require iron for growth [24, 25]. However, the diversion of iron from the circulation into macrophages effectively causes functional iron deficiency and iron restricted erythropoiesis, and eventually, if not corrected, results in anemia. It is important to note that functional iron deficiency of ACD is distinct from true iron deficiency in IDA where iron is depleted in both the circulation and macrophages [26].

Research over the last 10 years suggests that the functional iron deficiency in ACD can be attributed to excess levels of the main iron regulatory hormone hepcidin. Overexpression of hepcidin in mice recapitulates classic ACD features of hypoferremia and iron retention in macrophages [8, 27, 28]. Moreover, resection of a large hepatic adenoma secreting high levels of hepcidin in a patient with severe IDA resolved the anemia [29]. These observations collectively implicate excess hepcidin in the pathogenesis of ACD and suggest that hepcidin inhibitors may be a useful new therapeutic strategy for treating ACD. The design of hepcidin inhibitors has been directed by recent work elucidating the molecular mechanisms of hepcidin regulation and action.

BMP6-HJV-SMAD Signaling: The Central Pathway for Hepcidin Expression

Recent advances in understanding the molecular mechanisms of hepcidin regulation came from studying patients with the genetic iron overloaded disorder hereditary hemochromatosis. Nearly all of these patients harbor mutations in genes encoding the hemochromatosis protein (*HFE*) [30], transferrin receptor 2 (*TFR2*) [31], and hemojuvelin (*HJV/HFE2/RGMc*) [32], and have low hepcidin levels relative to iron stores. Furthermore, patients with very rare mutations in hepcidin itself have a severe early-onset form of this disease [33]. These data suggest that hepcidin deficiency has an important role in the pathogenesis of hereditary hemochromatosis, and that *HFE*, *TFR2*, and *HJV* are involved in regulating iron homeostasis upstream of the hepcidin synthesis pathway. *HFE* and *TFR2* are thought to function as part of an “iron sensor complex”. Their precise roles in the molecular regulation of hepcidin are still unclear and have been reviewed elsewhere [34-37].

Shortly after it was linked to juvenile hemochromatosis in 2004, *HJV* was reported to be a bone morphogenetic protein (BMP) co-receptor, and BMP signaling was demonstrated to be required for hepcidin expression and iron metabolism [38-40]. BMPs belong to the transforming growth factor beta (TGF- β) superfamily of ligands and are involved in cellular and systemic functions during embryonic and adult life [41]. BMP ligands bind to BMP type

I and type II serine threonine kinase receptors to activate the canonical SMAD pathway and modulate the transcription of target genes.

HJV is a glycosylphosphatidylinositol (GPI)- linked membrane-associated protein that binds to BMPs and enhances their effectiveness to activate the BMP-SMAD signaling pathway to stimulate hepcidin transcription in hepatocytes [39, 42] (Figure 1, black arrows). Analysis of the hepcidin promoter has identified two distinct SMAD binding elements responsible for upregulating hepcidin transcription by this pathway [43-46]. Although several BMP ligands can bind HJV and induce hepcidin expression [38, 39, 42, 47], BMP6 appears to be the key endogenous regulator of hepcidin expression *in vivo*, at least in mice [38, 40]. The BMP type I receptors ALK2 and ALK3 also appear to be important for HJV-mediated hepcidin regulation in mice [47, 48]. Indeed, deletions in genes encoding the ligand BMP6 [38, 40], the BMP co-receptor HJV [32], the BMP type I receptors ALK2 and ALK3 [48], or the intracellular signaling molecule SMAD4 [46], all independently result in inappropriately suppressed hepcidin expression and tissue iron overload in mice, supporting the central importance of the BMP6-HJV-SMAD signaling pathway in hepcidin regulation and iron homeostasis.

Regulation of Hepcidin By The Inflammatory Pathway

Inflammation due to infection, autoimmune disease or cancers stimulates the synthesis of many pro-inflammatory cytokines such as, interferon- γ (IFN- γ), interleukin 1 (IL-1) and interleukin-6 (IL-6), and pro-inflammatory cytokines have been shown to induce excess hepcidin production [7, 19]. Indeed, patients with inflammation characterized by high C-reactive protein levels (>10mg/dL) and patients with multiple myeloma (known to secrete excess IL-6) all have inappropriately elevated levels of hepcidin [49]. Injection of lipopolysaccharide [50] into healthy humans induces an acute inflammation marked by IL-6 production that is correlated with increased hepcidin production and hypoferrremia [51]. Similar disturbances in iron metabolism and increased hepcidin synthesis were also observed in mice injected with pro-inflammatory cytokines IL-1 and IL-6 [7, 52]. Long-term hepcidin production, due to its ability to inhibit ferroportin function on duodenal enterocytes and macrophages, leads to poor iron absorption from the gut and increased iron retention that is a hallmark of ACD [7, 53].

A well-characterized molecular mechanism by which inflammation regulates hepcidin is the IL-6/ Janus kinase 2 (JAK2)-Signal transducer and activator of transcription 3 (STAT3) pathway. Ligand binding to the IL-6 receptor activates JAK2, which in turn phosphorylates the transcription factor STAT3. Translocation of phosphorylated STAT3 into the nucleus and binding to the canonical STAT3 binding site in the proximal hepcidin promoter results in upregulation of hepcidin gene expression [54-56] (Figure 1, black arrows). These data provide rationale for inhibiting the IL-6-STAT3 pathway to block hepcidin production.

Interestingly, there appears to be crosstalk between this pathway and the BMP6-HJV-SMAD signaling pathway. A functional SMAD-binding site in the hepcidin promoter has been shown to be necessary for IL-6 mediated hepcidin expression [55, 57, 58]. Indeed, abolishment of the BMP pathway as observed in liver specific *Smad4*^{-/-} mice results in blunted responses to IL-6 mediated hepcidin transcription [46]. BMP inhibitors which sequester BMP ligands or block BMP receptor signaling have also been shown to inhibit IL-6 mediated hepcidin transcription *in vitro* [42, 59]. These studies provide the rationale for developing BMP signaling inhibitors as pharmacologic inhibitors of hepcidin for the treatment of ACD as discussed below.

Current Management of ACD

Anemia often complicates the underlying chronic diseases and is consistently a predictor of poor prognosis of the disease, longer hospitalization, cognitive impairment, heart failure and increased morbidity [60-64]. Although survival benefits have not yet been proven in prospective randomized controlled trials, treatment of anemia has been demonstrated to improve the quality of life and energy levels for hemodialysis, cancer and rheumatoid arthritis patients with concurrent ACD [65-67]. The treatment of choice for ACD is to cure the underlying chronic disease; however, this is not possible for many ACD patients. Current therapeutic management of ACD can involve increasing hemoglobin levels by blood transfusions, erythropoiesis stimulating agents (ESAs) and/or iron administration.

The management of one form of ACD, i.e. the anemia of chronic kidney disease (CKD) was changed markedly in the 1980s when the US Food and Drug Administration (FDA) approved the use of the recombinant human erythropoietin epoietin alfa for treatment of anemia of CKD in hemodialysis patients [68]. It is thought that diminished production of erythropoietin is an important aspect of the pathogenesis of anemia of CKD. Replenishing the deficiency with epoietin alfa improved hemoglobin levels, reduced blood transfusions, improved quality of life scores, energy levels and work capacity in patients with anemia of CKD [69]. Although the insufficient production of erythropoietin seen in anemia of CKD is not shared with other types of ACD (anemia of malignancy, chemotherapy, infection or inflammation), administration of epoietin alfa and other similar ESAs was used and shown to benefit these patients [66, 70-75].

Some patients with CKD and other types of ACD are poorly responsive to ESAs, leading to a requirement for higher dosing to achieve target hemoglobin levels. Recent clinical trial results from the Correction of Hemoglobin in Outcomes and Renal Insufficiency (CHOIR) and Trial to Reduce cardiovascular Events with Aranesp Therapy (TREAT) studies revealed that patients with CKD receiving ESA doses to achieve target hemoglobin levels of >13g/dL had a higher incidence of adverse outcomes including cardiovascular events, stroke, progression of cancer and death [76, 77]. Additionally, a number of trials studying the use of ESAs for cancer and myelosuppressive therapy associated anemia have demonstrated an increased incidence of tumor progression and death [78-81]. These findings have prompted the US FDA to require a black box warning on the labels of ESA products, with recommendations for limited use in cancer patients, and a downward adjustment of hemoglobin target levels in CKD patients [70, 82, 83].

A commonly shared phenotype between CKD and the other subtypes of ACD is iron block or functional deficiency, which is associated with increased serum hepcidin levels [49, 84]. Because of the functional iron deficiency in ACD, iron supplementation is frequently administered either alone or in combination with ESA therapy. Oral iron supplements are widely available, inexpensive, and easy to administer. However, they are less effective or ineffective compared to intravenous (IV) iron therapy due to hepcidin-mediated block in intestinal iron absorption [85-87]. The DRIVE (Dialysis Patient's Response to IV iron and with Elevated Ferritin) study revealed that anemic hemodialysis patients receiving IV ferric gluconate and ESAs had a faster and more robust response in hemoglobin levels and reticulocyte hemoglobin levels compared to those receiving ESAs alone [88]. Subsequent studies have corroborated the observation that correction of the functional iron deficiency with IV iron supplementation can significantly improve the effects of ESAs [89] and can reduce the required ESA dosage [90, 91].

Although these data suggest that IV iron can overcome functional iron deficiency in ACD, a recent double-blinded trial found no additional benefit of providing IV iron over oral iron or placebo in patients with chemotherapy-associated anemia receiving concomitant ESA

therapy [92]. The negative result in this study could be due to higher total body iron load (reflected by higher ferritin levels) or to different iron dosing amounts in this patient population compared to the other studies. Interestingly, a reanalysis of the data suggested that treated patients who had lower hepcidin levels responded with significantly higher hemoglobin levels compared to those with higher hepcidin levels [93].

Paradoxically, providing supraphysiologic levels of IV iron would worsen the underlying iron restriction in ACD. IV iron is a potent stimulus for hepcidin production and has been shown to increase hepcidin levels by three-fold in CKD patients on hemodialysis within 24 hours of administration [94]. The rise in hepcidin could be predicted to increase iron sequestration in the macrophages due to further inactivation of ferroportin, rendering the administered iron unavailable for erythropoiesis once the initial iron bolus is incorporated into red cells and body stores.

The long term effects of IV iron therapy and concurrent increase in iron stores in ACD patients have not been investigated, but safety concerns are warranted given the known toxic effects of iron overload [95, 96]. In particular, supraphysiologic iron therapy poses an increased risk of infection in ACD patients [97, 98]. Based on the premise that some pathogens require iron for growth, providing excess iron may be detrimental [97]. The early termination of the Pemba study highlights this concern when children who lived in malaria endemic regions and received iron indiscriminate of iron status had a higher incidence of sudden illness and death from malaria and other infections than those in the placebo group [99]. Although iron therapy is still used to manage anemia in ACD patients at risk of infections, caution is recommended [100].

Thus, treating ACD using ESAs and iron therapy can have limited effectiveness in some patients and are not without potentially serious adverse events. Importantly, these therapies do not address a main root cause of the pathophysiology of ACD, namely, excess hepcidin and reduced ferroportin activity.

New Therapeutic Strategies for ACD that Target the Hepcidin-Ferroportin Axis

Alternative therapies for ACD that target the hepcidin-ferroportin axis are attractive treatment options. Therapeutics that decrease hepcidin production and increase ferroportin activity would improve iron bioavailability from the diet and would mobilize existing body iron stores for erythropoiesis, without the adverse risks from supraphysiologic iron or ESA therapies. A number of strategies that inhibit hepcidin function (Direct Hepcidin Antagonists), prevent the transcription of hepcidin (Hepcidin Production Inhibitors) or promote resistance of ferroportin to hepcidin action (Ferroportin agonists/ stabilizers) are currently under investigation (Figure 2, red lines), and are reviewed below.

Direct Hepcidin Antagonists

Anti-Hepcidin Antibodies—Therapeutic monoclonal antibodies have revolutionized the management of cancers and autoimmune diseases [101]. One strategy under investigation for neutralizing hepcidin activity is with anti-hepcidin antibodies. Studies performed by Amgen demonstrated that a humanized anti-hepcidin monoclonal antibody (mAb2.7) can reverse iron restriction and prevent anemia progression in a mouse model of anemia of inflammation when used in combination therapy with ESA [102]. However, neither anti-hepcidin antibodies nor ESA alone could prevent the anemia progression in their model. Recently, the authors performed a pharmacokinetics study on normal cynomolgus monkeys and determined that a dose of 300mg/kg per week of another monoclonal anti-hepcidin antibody (Ab12B9m) was required to effectively decrease free hepcidin levels below baseline [103]. Even though Ab12B9m has a high affinity ($K_d=1\text{pM}$) for hepcidin and a

long half life estimated at 16.5 days, the high rate of hepcidin production (7.6nmol/kg/h) would require large quantities of the antibody to achieve significant blocking effect [103]. A US patent (#7820163) was recently assigned to Eli Lilly and Company for the use of anti-hepcidin monoclonal antibodies as treatment for anemia [104]. These antibodies bind with high affinity ($K_d=80\text{pM}$) to human and monkey hepcidin and can block its action on ferroportin [104]. In a cynomolgus monkey IL-6-induced hypoferremia model, anti-hepcidin monoclonal antibody therapy prevented the hypoferremia for up to 12 hours [104]. The antibodies are currently in phase 1 clinical trials for the treatment of anemia according to the Lilly company website [105].

Short Interference RNA and Anti-Sense Oligonucleotides Against Hepcidin—

RNA interference (RNAi) and gene silencing anti-sense oligonucleotides that target transcription or translation of hepcidin represent another approach to developing therapeutics for ACD. Amgen recently described a short hairpin RNA (shRNA) strategy against hepcidin that successfully reduced its expression and corrected anemia in a mouse model of anemia of inflammation [102]. Profound suppression of hepcidin alone was sufficient to redistribute stored body iron into the circulation and to correct the anemia in these mice. Moderate hepcidin suppression alone was unable to correct the anemia and required ESA co-administration in order to be effective [102]. Alnylam Pharmaceuticals is currently developing hepcidin RNAi (ALN-HPN) to silence hepcidin gene expression and increase serum iron levels [106]. They expect to file an Investigational New Drug application in 2012 for ALN-HPN for the treatment of erythropoietin resistant ACD and iron refractory anemias [106].

Significant technical challenges will need to be overcome in order to apply RNAi therapeutics for treatment in humans. The challenges include effective design of the RNAi without off-target effects, instability of RNAi *in vivo*, lack of biocompatibility of the delivery system and non-specific targeted delivery to organs/cells [107]. Viral delivery systems, while highly efficient in delivering shRNA or RNAi can carry risks of random genome integration and induction of unfavorable immunological responses [108]. Notably, shRNA against hepcidin delivered in an AAV8 capsid serotype virus system caused some deaths in mice [102].

Anti-sense oligonucleotides that inhibit translation of hepcidin or its regulators such as HJV are currently in discovery stages of development. Xenon Pharmaceuticals and ISIS Pharmaceuticals have been collaborating since 2010 to develop this technology to treat anemia of infection [109]. Systemic delivery of anti-sense molecules result in preferential delivery to the liver, making them appealing agents to target hepcidin [110]. While the approach is theoretically feasible to dampen hepcidin production, it has not been demonstrated that anti-sense hepcidin oligonucleotides are effective in increasing serum iron concentration and ameliorating inflammatory anemia *in vivo*. In addition, anti-sense oligonucleotides share similar technical challenges with RNAi therapies as described above.

Hepcidin Binding Proteins—Lipocalins are secreted proteins whose four-peptide loop cavity forms a binding site with high structural plasticity [111]. Their simple structure and natural ability to recognize and bind various small hydrophobic ligands and specific cell-surface receptors make them suitable for engineering a new class of therapeutic proteins for specific blocking purposes. [112]. These engineered lipocalins are known as anticalins. Scientists at the Technical University Munich and Pieris AG have developed an anticalin PRS-080, which exhibits high affinity binding ($K_d=0.1\text{nM}$) to human hepcidin based on ELISA studies and surface plasmon resonance [113, 114]. They also demonstrated that pretreatment with 95mg/kg of PRS-080 can completely neutralize the short-term hypoferremia induced by injection of synthetic human hepcidin in mice [113]. According to

their company website, Pieris AG recently received support of six million Euro from a EU FP7 Grant to progress PRS-080 from preclinical development through Phase 1b clinical trials [114]. Further characterization of this hepcidin blocking anticalin will be needed to determine its safety, tolerability and efficacy for relieving hepcidin-mediated iron blockade in ACD.

Hepcidin Binding Spiegelmers—Aptamers are synthetic single-stranded oligonucleotides that bind ligands with high affinity, representing a novel class of oligonucleotide structures suitable for blocking purposes [115]. Spiegelmers (Spiegel, which means “mirror” in German) are mirror image aptamers trademarked by NOXXON Pharma. Spiegelmers are attractive therapeutic agents because of their high resistance to nuclease activity, good stability *in vivo*, and low immunogenicity [115, 116]. NOX-H94 is a polyethylene glycol conjugated (PEGylated) Spiegelmer developed by NOXXON Pharma AG that specifically targets and binds to human hepcidin [117]. It was demonstrated to be effective *in vitro* by blocking hepcidin-induced ferroportin degradation in cells [118]. Additionally, it was able to increase serum iron concentration in a IL-6 induced high hepcidin model in cynomolgus monkeys [118]. Preclinical studies suggest spiegelmers are safe, well tolerated and effective in animals [119-123]. The company has initiated a first-in-human clinical trial to evaluate safety, tolerability and efficacy of NOX-H94 [117]. Administration of PEGylated aptamers was recently shown to lead to an accumulation of the oligonucleotides in macrophages throughout the body, it is currently unknown whether chronic systemic administration of PEGylated aptamers will lead to adverse effects [124].

Hepcidin Production Inhibitors

Given the rapid rate of hepcidin production [103], targeting the positive regulators of hepcidin to reduce its expression may be more effective than direct blockade of hepcidin action. Two signaling pathways have been targeted thus far to inhibit hepcidin synthesis: BMP6-HJV-SMAD pathway inhibitors and IL6-STAT3 pathway inhibitors.

BMP6-HJV-SMAD Pathway Inhibitors—The BMP family of ligands represents the largest subgroup of the TGF β superfamily. Twenty BMP family members have been identified with diverse functions including embryogenesis, osteogenesis, neurogenesis, and iron metabolism [125, 126]. Like other members of the family, BMP6 has osteogenic potential [127]. However, BMP6 is crucial in regulating hepcidin and iron metabolism since the main phenotype of the *Bmp6* knockout mice is impaired hepcidin production and severe iron overload [38, 40]. Blocking hepcidin transcription by targeting the hepatic BMP pathway and more specifically BMP6 may therefore be a useful strategy for treating ACD.

Dorsomorphin is a small molecule inhibitor of BMP receptor type I kinases that was identified in a zebrafish embryo screen [59]. It was also shown to inhibit BMP-, HJV-, and IL-6-stimulated hepcidin expression in cultured hepatocytes and block iron induced hepcidin mRNA in zebrafish and in mice [59]. However, dorsomorphin (also known as compound C) is a relatively non-selective kinase inhibitor that also inhibits AMP kinase [59, 128]. A derivative of dorsomorphin, LDN-193189 was shown to have improved potency and selectivity as a BMP inhibitor [129] and was able to inhibit excessive BMP signaling *in vivo* [130]. We have recently succeeded in using LDN-193189 to reverse anemia associated with streptococcal peptidoglycan-polysaccharide (PG-APS)-induced chronic arthritis in rats [131]. Compared to mock treatment, LDN-193189 treatment over a period of 4 weeks reduced hepatic hepcidin mRNA levels, increased serum iron concentration, increased ferroportin expression in splenic macrophages, and importantly, improved hemoglobin levels and hematocrit in anemic rats. Consistent with our data, LDN-193189 treatment prevented an acute inflammatory anemia induced by turpentine injections in mice [132].

However, profiling of LDN-193189 against a panel of 123 human kinases revealed that it not only blocks the BMP pathway, but also potently inhibits VEG-F and components of the MAPK-ERK pathway [133]. Thus, LDN-193189 is not as specific a BMP inhibitor as initially thought and caution must be used when interpreting results from studies using LDN-193189.

We have shown that a soluble form of the human hemojuvelin protein linked to the constant region of IgG1 (HJV.Fc) can decrease BMP-mediated hepcidin expression *in vitro* [42]. Additionally, HJV.Fc administration into healthy rodents blocked SMAD activation, decreased hepcidin expression, mobilized splenic iron stores, and increased serum iron levels [42]. In collaboration with Ferrumax Pharmaceuticals, our recent study in rats with PG-APS induced ACD indicated that pharmacologic inhibition of hepatic BMP signaling with HJV.Fc reduced hepcidin mRNA within 24h [131]. Longer term treatment for 4 weeks showed that HJV.Fc inhibited phosphorylation of hepatic SMAD 1/5/8, increased splenic ferroportin levels and serum iron levels, and rescued the anemia in ACD rats [131]. The safety and efficacy of HJV.Fc in human patients has not yet been determined.

Anti-BMP6 monoclonal antibody therapy is another option to specifically block BMP6 mediated hepcidin regulation. Administration of anti-BMP mAb in healthy mice decreased hepatic hepcidin expression and increased serum iron levels [38]. In the Hfe transgenic mouse model of excess hepcidin and iron deficiency anemia, anti-BMP6 administration improved the anemia by lowering hepcidin levels [134]. Several caveats surround the targeting of BMP6 as a therapeutic strategy. Whether BMP6 is the most important BMP in the physiologic regulation of hepcidin in humans has not yet been shown. Due to high amino acid sequence similarity among the BMP family of ligands [135], one technical challenge is to generate antibodies that can specifically recognize BMP6 without significant cross-reactivity to other BMPs. Finally, the safety of lowering BMP6 in humans is unknown.

Recently, heparin was implicated in sequestering BMP activity and lowering hepcidin expression [136]. Heparin is a highly sulfated glycosaminoglycan that is widely used pharmacologically as an anti-coagulant. Pretreatment of cultured hepatoma cell lines with heparin decreased basal levels of BMP-SMAD signaling and inhibited BMP6-mediated hepcidin transcription. Additionally, injection of heparin (50mg/kg) into healthy mice decreased hepatic SMAD phosphorylation and reduced hepcidin expression, leading to mobilizing of spleen iron stores and increased circulating iron. Moreover, deep vein thrombosis patients treated with heparin have decreased serum hepcidin levels and increased serum iron levels [136]. Whether heparin will become a useful strategy to treat ACD remains to be determined, since heparin is known to have significant adverse side effects including life-threatening bleeding, heparin-induced thrombocytopenia, hyperkalemia, alopecia and osteoporosis [137].

Alcohol loading in mice has been shown to decrease hepcidin mRNA and increase iron absorption through the intestines [138]. The mechanism was recently elucidated to involve inhibition of the BMP-SMAD pathway [139]. Chronic alcohol consumption in humans is associated with excess iron accumulation in the liver and hepcidin may be a contributing factor to the progression of alcoholic liver disease [140]. The therapeutic potential of using alcohol for the treatment of ACD is an interesting strategy that must be tempered by the well-known deleterious side effects of excess alcohol use.

IL-6 Pathway Inhibitors—Inhibitors of the IL-6 pathway have been shown to downregulate hepcidin expression and improve anemia of inflammation in Multicentric Castleman's Disease, a rare lymphoproliferative disorder marked by excessive production of IL-6 in the lymph nodes and associated with hypochromic and microcytic anemia [141].

Serum hepcidin levels were also elevated in these patients, most likely due to IL-6 mediated hepcidin synthesis. Most MCD patients (5 out of 6) when treated with the anti-IL-6 receptor antibody (anti-IL-6R) Tocilizumab for 6-12 months, had lower serum hepcidin levels, normalization of hemoglobin levels, and experienced beneficial effects on disease symptoms such as reduced fatigue, increased weight and alleviation of fever [141]. Another study demonstrated that IV administration of anti-IL-6R antibodies ameliorated anemia in a monkey model of collagen-induced arthritis [142]. In this model, anti-IL-6R antibody therapy effectively lowered hepcidin levels, decreased CRP levels within one week and improved hematological parameters over a treatment period of 4 weeks [142].

Blocking antibodies to the IL-6 ligand also resulted in similar hepcidin lowering effects. The anti-IL-6 chimeric monoclonal antibody, Siltuximab was recently demonstrated to increase hemoglobin levels by 2.1g/dL in an open label, dose finding, phase I study in MCD patients [143]. The major complication of blockade of IL-6 activity appears to be increased risk of infections [144, 145].

In addition to IL-6 ligand/receptor blockade, inhibition of the JAK1/2-STAT3 signaling cascade can also lower hepcidin expression. The JAK2 inhibitor AG490 and synthetic peptide inhibitor of STAT3 (PpYLKTK) have been actively studied to target cancers with elevated JAK/STAT activity [146-148]. AG490 inhibits the phosphorylation of STAT3 by JAK2 while the PpYLKTK peptide disrupts pSTAT3 dimerization, which is required for binding target genes. Faith and colleagues demonstrated that both compounds were able to decrease phosphorylation of STAT3 and downregulate IL-6 mediated hepcidin expression in a mouse liver co-culture system [149]. The use of STAT3 inhibitors to modulate hepcidin production *in vivo* has not yet been reported.

Vitamin D—Vitamin D is a hormone synthesized by the skin upon exposure to UV light and activated in the kidney. Primarily known for regulating calcium and promoting bone health, vitamin D has also been implicated in a wide range of cellular activities including differentiation of hematopoietic cells and down regulation of pro-inflammatory cytokines transcripts [150, 151]. Vitamin D deficiency is associated with a higher prevalence of anemia of inflammation in elderly people [152]. Vitamin D deficiency is also a common occurrence in CKD and hemodialysis patients [153]. In a recent pilot study, vitamin D supplementation had an EPO sparing effect in vitamin D deficient hemodialysis patients [154]. The results were consistent with observations from a retrospective study where vitamin D repletion in anemic CKD patients not on hemodialysis correlated with a lowering of EPO dose requirements [155].

A mechanism for the EPO sparing effects of vitamin D is suggested by recent data demonstrating a hepcidin lowering effect of vitamin D. *In vitro* treatment with vitamin D of monocytes isolated from hemodialysis patients downregulated hepcidin transcription [156]. Furthermore, oral administration of vitamin D in healthy volunteers lowered serum levels of hepcidin by 50% compared to baseline levels within 24 hours and persisted for 72 hours [156]. Supplementation with vitamin D has also been reported to have beneficial effects on increasing erythropoiesis [157] and decreasing inflammation [158]. These initial results are promising and a randomized controlled study is warranted to determine whether correction of vitamin D deficiency can ameliorate anemia of chronic disease.

Ferroportin Agonists/Stabilizers

Hepcidin excess prevents iron absorption from the diet and blocks iron release from body stores by binding to and inducing the degradation of the iron export protein ferroportin. In addition to reducing hepcidin production and blocking its activity, agents that stabilize ferroportin on the cell surface may be useful for correcting the functional iron deficiency in

ACD. In a study characterizing the molecular mechanism of ferroportin disease with parenchymal iron overload and resistance to hepcidin, the thiol form of Cys326 in ferroportin was found to be essential in hepcidin-ferroportin interaction [159]. In a high-throughput screening approach using HEK cells expressing ferroportin-GFP fusion protein, they discovered thiol modifier compounds that prevented ferroportin-hepcidin binding and blocked internalization of ferroportin in the presence of excess hepcidin [160]. This screening approach also identified molecules such as cardiac glycosides, which do not interfere with hepcidin binding but appear to prevent ferroportin internalization [160]. An anti-ferroportin mAb developed by Eli Lilly (Patent Application #20110129480), which binds to the extracellular loop of ferroportin can block the hepcidin-ferroportin interaction while maintaining ferroportin function [161]. These emerging strategies would promote ferroportin activity and allow continuous iron influx, potentially preventing iron-restrictive erythropoiesis due to hepcidin excess. The safety and efficacy of these approaches in humans have not yet been determined.

Conclusion

Recent advances in the iron metabolism field provided valuable insights into the molecular pathophysiology of ACD as well as new potential targets for therapy. Targeting the hepcidin-ferroportin axis with novel therapeutics that inhibit the BMP6-HJV-SMAD and the IL-6-STAT3 pathways for hepcidin production, antagonize hepcidin activity, or promote ferroportin function may lead to better management of the iron maldistribution and its contribution to the consequent anemia of this common but clinically important disorder.

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Figure 1. Hepcidin regulation by BMP6-HJV-SMAD and IL-6-STAT3 signaling pathways

The BMP6-HJV-SMAD and IL-6-STAT3 signaling pathways both activate hepcidin transcription in the liver (black arrows). In response to iron sufficiency, circulating bone morphogenetic protein 6 (BMP6) binds transmembrane BMP receptors type I (BMP-RI) and type II (BMP-RII) and BMP co-receptor hemojuvelin (HJV) to create a multiplex on the hepatocyte membrane to activate the SMAD signaling cascade. Activated intracellular SMAD1/5/8 proteins then complex with the common mediator SMAD4 and translocate to the nucleus to induce hepcidin expression through BMP- responsive elements (BMP-REs) localized on the hepcidin promoter.

In an inflammatory setting, pro-inflammatory cytokines like IL-6 are released. Upon binding to its receptor, IL-6 initiates signaling through activated JAK1/2 proteins to phosphorylate the transcription factor STAT3. Activated STAT3 then binds to a STAT3-responsive element (STAT3-RE) on the proximal hepcidin promoter. Both STAT3-RE and the adjacent BMPR-RE are required for IL-6 mediated hepcidin expression. Hepcidin protein is secreted into the bloodstream to result in ferroportin inhibition, leading to iron retention in the reticuloendothelial macrophages and reduced iron absorption in the intestinal epithelia.

Figure 2. Inhibitors that target the hepcidin-ferroportin axis are potential therapeutic avenues for treating ACD

Since excess hepcidin leads to ACD, blocking the signaling pathways responsible for hepcidin synthesis, neutralizing hepcidin's effect on ferroportin or promoting ferroportin function may ameliorate ACD (red lines).

Hepcidin production can be effectively inhibited by the following BMP6-HJV-SMAD inhibitors: soluble HJV.Fc protein (Ferrumax Pharmaceuticals), monoclonal anti-BMP6 antibodies, or the glycosaminoglycan heparin sequester BMP6, preventing its interaction with BMPR and membrane anchored HJV; the dorsomorphin derivative LDN-193189 inhibits BMP type I receptor activity. Alcohol may also inhibit hepcidin synthesis by dampening the BMP-SMAD pathway.

Anti-inflammatory therapeutics suppress IL-6 mediated hepcidin gene expression by: blocking antibody to IL-6 Siltuximab (Janssen Biotech Inc); neutralizing antibody to IL-6 receptor Tocilizumab (Genentech); or STAT3 pathway inhibitors, which block the phosphorylation of STAT3 (AG490) or its transcription factor binding activity (PpYLKTK). Vitamin D can also downregulate hepcidin transcription but the mechanism is unknown.

Hepcidin protein expression or activity may be inhibited directly by: hepcidin siRNA [106]; hepcidin anti-sense oligonucleotides (Xenon Pharmaceuticals and ISIS Pharmaceuticals); or direct hepcidin antagonists, including anti-hepcidin antibodies (Amgen, Eli Lilly), hepcidin sequestering anticalins (Pieris AG) or hepcidin binding spiegelmers (NOXXON Pharma). Ferroportin agonists/ stabilizers, which modify the ferroportin-hepcidin interaction or increase ferroportin's resistance to hepcidin (Eli-Lilly), may theoretically ameliorate anemia by maintaining ferroportin activity and allowing iron influx.