Introduction

Iron is an essential nutrient for nearly all organisms. In mammals, iron is used as a component of heme and non heme, including hemoglobin and myoglobin, and in iron-containing centers in redox enzymes. For normal iron homeostasis organisms have developed complex cellular mechanism to maintain appropriate iron balance. Iron play a significant role in certain cell activities, including cell proliferation, oxygen transport, energy metabolism and DNA repair (Zhang et al. 2014).

Iron is one of the most abundant transition metals present on earth, many organisms have developed intricate mechanisms to obtain and allocate iron in multicellular organisms (Andrew et al. 2007). Iron in the body is mainly absorbed from the diet and is present in two fundamental forms, *viz* heme and non-heme iron. Iron is absorbed in the proximal small intestine, where iron moves across the apical membranes of the enterocytes for access in the circulation (Anderson et al. 2005). Heme carrier protein and non-heme iron is transported in form of heme iron and is reduced by ferric reductates to Fe$^{2+}$ before being transported across the apical cellular membrane by divalent metal transporter 1 (DMT1) (Shayeghi et al. 2005). Iron is then transported across the basolateral membrane *via* ferroportin (FPN), the only known iron transporting channel or iron exporter. To export iron it’s require ceruloplasmin and hephaestin which entail ferroxidase activity (Vulpe et al. 1999). TfR1-iron mediated process involve absorbed iron which is bound to transferrin (Tf), which binds all plasma iron in circulation (Gkouvatsos et al. 2012). Iron is generally deposit in form of ferritin (a protein cage of stored iron), and is transported back into endosomes, where it is separated from the Tf before being transported into the cytoplasm (Theil et al. 2003). Ferritin level mainly regulates iron uptake and storage in the cells. In condition of low intracellular iron, iron regulatory elements binds to iron regulatory proteins mRNA to absorb or release more iron (Hentze et al. 2004).

Total body iron content about 3-4 g in an adult human (Ganz et al. 2011). Iron looses mainly in form of daily epithelial sloughing and bleeding approximately around 1-2 mg/day. Heme red blood cells exist in two-thirds of iron in the body and through RBC senescence or death of erythrocytes approxiamately 20 mg of iron looses each day. To compensate daily iron losses sufficient amount of iron was absorbed from dietary source (1-2 mg) and stored in form of ferritin (Draft et al. 2009). Phagocytosis of cells primarily occurs through
macrophages, heme which is unbound from hemoglobin is degraded by heme oxygenase to release iron for recycling. Thus, proper iron homeostasis is maintained by adequate iron intake and absorption, minimal blood loss, and the reuse of iron from dying erythrocytes (Lawen et al. 2013).

Anemia is a serious public health problem globally to affect million people worldwide with a range of social and economic consequences (Zimmermann and Hurrell et al. 2007). In iron deficiency, many enzyme systems like cytochrome oxidase, xanthine oxidase plays a central part in the transport of oxygen in the body, which is essential for growth and development of the body (Veena et al. 2013). In infants and small children iron deficiency affects neurotransmitter systems in brain causing changes in behavior such as attention, memory and learning (Tolentino and Friedman et al. 2007). The normal defense system against infection is also adversely affected by iron deficiency (Eldibany and Tonochi et al. 1999). In pregnant women, iron deficiency contributes maternal morbidity, mortality & increases risk of fetal morbidity, mortality & low birth weight (Detels et al. 1999).

Across worldwide, approximately 50% of anemia cases are associated with iron deficiency, although the proportion varies across populations (Hidalgo et al. 2013). Iron deficiency (anemia) is a global health problem, and the World Health Organization (WHO) has reported that iron deficiency manifests as anemia in up to 2 billion people worldwide. According to the National Family Health Survey (NFHS)-(III), more than half of women in India (55%) have anemia, including 39 % with mild anemia, 15 % with moderate anemia and 2% with severe anemia with inflammation and infection (Santosh et al. 2014). Prevalence of iron-deficiency anemia is higher in rheumatoid arthritis in Indian patients with severe infection or inflammation (Aggrawal et al. 2006). Inflammation, hypoxia, infection and iron stores within macrophages lead to hepcidin production in liver, a key causative agent that results in iron sequestration with reduced iron absorption (Nemeth et al. 2014).

Hepcidin, a 25 amino acid cysteine-rich cationic peptide hormone, secreted from liver, and hepcidin synthesis and subsequent secretion is regulated by inflammation, hypoxia, erythropoiesis and iron stores within macrophages (Andrews et al. 2004; Ganz et al. 2003). The inflammation causes iron sequestration in macrophages, resulting into an excessive hepcidin production (Maliken et al. 2011). FPN, only cellular iron transporter, is mainly expressed at the surface of hepatocytes, macrophages and enterocytes. Hepcidin binding to FPN triggers ubiquitination, endocytosis and degradation of FPN, which subsequently leads
to reduced iron absorption (Zhao et al. 2013; Nemeth et al. 2004). The hepatic peptide hepcidin controls iron efflux to plasma from enterocytes and macrophages through degradation of the iron export channel FPN (Figure 1). Elevated hepcidin decreases FPN (cellular iron export) expression with increased iron accumulation leading to hypoferremia with reduced hemoglobin synthesis or ineffective erythropoiesis (Ganz et al. 2011; Park et al. 2001).

Figure 1: Hepcidin and FPN interaction regulates iron homoeostasis: Increased inflammation elevates hepcidin synthesis leading to FPN degradation affecting iron mobilization for normal erythropoiesis. Concentration of hepcidin is in turn regulated by iron, erythropoietic activity, and inflammation (Nemeth et al. 2009).

Hepcidin is synthesized mainly in the liver and triggered by inflammatory stimulators such as IL-6/STAT3 or BMP/SMAD pathway (Wrighting et al. 2006; Parrow et al. 2014). Specifically, BMP6 is an iron-specific ligand that induces hepcidin production (Figure 2). In case of severe iron overload disruption in BMP6 activity interrupted hepcidin production with increase iron deposit in tissues (Meynard et al. 2009; Ramey et al. 2009). Inflammation modulates hepcidin production in liver, inflammatory stimuli including freund’s adjuvant, turpentine, LPS, and heat-killed *brucella abortus* (Frazer et al. 2004; Nemeth et al. 2004;
Deschemin et al. (2013; Sasu et al. 2010) have been shown to increase hepcidin mRNA expression in mice. Inflammatory cytokine IL-6 is the key regulator for the inflammation-mediated hepcidin production (Nemeth et al. 2004; Falzacappa et al. 2007). Recently many strategies are employed to decrease expression of hepcidin via IL-6/JAK2/STAT3 pathway and BMP/SMAD pathway. Earlier studies reported that dorsomorphin inhibit BMP signal inactivating AMP-activated protein kinase activity (Yu et al. 2008). Hydrogen sulfide (H$_2$S) inhibited inflammatory hepcidin with reduced IL-6 levels with SIRT1-mediated STAT3 deacetylation (Xin et al. 2016). Recently small molecule inhibitors of STAT3 (curcumin, PpYLKTK and AG490) decreases expression of hepcidin by inhibiting the IL-6/STAT3 signalling pathway (Fatih et al. 2010; Zhang et al. 2011). S-propargyl-cysteine (SPRC) more stable than H$_2$S, suppresses hepatic hepcidin and corrected hypoferremia induced by lipopolysaccharide (Wang et al. 2016).

**Figure 2.** Hepcidin expression is transcriptionally up regulated via BMP/SMAD and IL6 JAK-STAT3 pathway.

Single infusion of IL-6 increased hepcidin production with decreased serum iron in humans (Nemeth et al. 2004). Inflammation leads to the activation of inflammatory cytokines such as IL-6, which then binds to the IL-6 receptor and subsequently activate the JAK/STAT3 pathway. Phosphorylated STAT3 dimer then binds to the hepcidin promoter and activates its transcription (Falzacappa et al. 2007). Hepcidin production can be stimulated by other cytokines like IL-1, in mouse hepatocytes and play a role in activation of hepcidin.
production (Lee et al. 2005). However, these interpretations have not been endured in human studies (Nemeth et al. 2003).

Anemia of inflammation (AI) is one of the most common manifestations of iron deficiency in the patients with inflammatory conditions (Theurl et al. 2009; Adamson et al. 2008). AI is the most prevalent form of anaemia in hospitalized patients and it is indicated by low serum iron, ferritin levels in spite of normal or increased iron stores suggest involvement of inflammatory process in the pathogenesis of AI. AI is responsible for hypoferremia, with consequent iron-restricted erythropoiesis (Nemeth et al. 2014). AI has been witnessed for many years; it is only recently that the discovery of the peptide hormone hepcidin has assisted to elucidate the mechanism of AI development. Various studies have reported that hepcidin, a 25-amino acid cysteine-rich cationic peptide hormone, secreted from liver, is the key causative factor in AI (Deicher et al. 2004; Ganz et al. 2009). Recently, research on the pathogenesis of AI has focused on the role of hepcidin, a 25-amino acid cysteine peptide synthesized mainly in the liver and triggered by inflammatory stimulators such as IL-6/JAK/STAT3 or BMP/SMAD pathway (Ruchala et al. 2014). IL-6, secreted from macrophages, is responsible for inflammation-mediated hepcidin induction through activation of JAK2/STAT3 pathway, with release of other inflammatory molecules (IL-1β or TNF-α), which actively not interfere in pathway regulation of hepcidin induction. (Niemand et al. 2003). LPS-induced inflammation increases IL-6 induction with increase hepcidin mRNA transcription in in vitro and in vivo demonstrating that human hepcidin is a type II acute-phase reactant and the association of hepcidin to LPS-mediated inflammation proved a key role mediator of AI. Many different techniques validated hepcidin as a marker for iron deficiency as indicated by hepcidin concentration which was further influenced by iron supplementation in hemodialyzed HD patients. Oral iron or intravenous iron supplementation will be of great advantage in identifying patients with unresponsive to iron replacement treatment. In haemodialysis treatment iron replacement is an important aspect, which is common in iron deficient patients (Pagani et al. 2011).

Increased production of hepcidin is observed in AI, which causes FPN degradation, thereby retaining iron in spleen and liver with reduced serum iron and increase iron deposit in tissues (Figure 3). Hence, targeting hepcidin could be a novel approach to treat AI. Therefore, inhibition of hepcidin could serve as a potential strategy as a remedy for AI (Ganz et al.2003; Ganz et al.2009).
**Figure 3:** Hepcidin expression is regulated by intracellular iron, erythropoiesis, and inflammation. Elevated hepcidin degrade FPN channel on enterocytes, macrophages, and hepatocytes via causing iron accumulation leading to hypoferrimia (Ganz et al. 2012).

Role of hepcidin agonists and antagonists in treatment of AI has been already established (Sun et al 2012). The presently employed approaches to prevent hepcidin-mediated FPN degradation include inhibiting hepcidin expression (Babitt et al. 2007; Liu et al. 2012; Poli et al. 2014), using anti-hepcidin agents (Leung et al.2013; Poli et al. 2011) and FPN binding agents (Fung et al. 2013). The explicitly employed therapeutic agents include anti-hepcidin antibodies (Sasu et al. 2010), BMP inhibitors (e.g. dorsomorphin; Paul et al. 2008), antagonists of BMP signaling (e.g. soluble hemojuvelin; Babitt et al. 2007) and inhibition of SMAD signaling (e.g. glycol-split non-anticoagulant heparins; Poli et al. 2011). Anti-hepcidin Spiegelmer NOX-H94 a biostatic aptamer was reported to prevent hepcidin-induced FPN degradation (Schwoebel et al. 2013). LY2928057, a humanized IgG4 monoclonal antibody is a high affinity FPN binding agent that inhibits the hepcidin-FPN binding (Leung et al. 2013). *Angelica sinensis* polysaccharide (ASP) was reported to suppress the expression of hepcidin in rats with AI (Liu et al. 2012). Tocilizumab a monoclonal antibody was identified as an effective inhibitor of hepcidin production (Song et al. 2013). Fursultiamine is identified as a hepcidin antagonist that blocks the interaction of hepcidin-FPN disulfide bond (Fung et al. 2013). Recently, anti-hemojuvelin antibodies are reported to ameliorate anemia due to their hepcidin suppressive potential (Kovac et al.2016). Many
approaches are used to ameliorate AI that includes transfusion or erythropoietin (EPO) administration for treatment of seriously ill patients (Tonelli et al. 2009). Patients with milder conditions can take iron supplemental therapy, which is safe and economical, but it often has low efficacy. However, these therapies are accompanied by many complications (Nielsen et al. 2005; Teehan et al. 2004). Therefore, finding a new treatment paradigm to treat AI are imperative.

Therefore in present study we, target to treat inflammation-induced anemia (anemia of chronic disease) termed as AI that’s restrict iron absorption due to elevated hepcidin level (Weiss et al. 2005; Nemeth et al. 2003). A systemic approach using virtual screening, molecular docking and molecular dynamics studies, a natural compound GDP (ZINC Database ID: ZINC08215481) was identified to show sufficiently good binding affinity with hepcidin. Further in vitro and in vivo studies confirmed the role of GDP in preventing hepcidin-mediated FPN degradation, reversing iron restrictive effect of inflammation with increase in haemoglobin level. GDP has been established as a promising candidate for inhibiting hepcidin-FPN interaction, thus promoting an effective iron-mediated erythropoiesis. Earlier studies reported that GDP was also used as a drug in conjugation with a key monosaccharide for the glycosylation of protein and lipid. GDP-man as nucleotidyl transferase family contains enzyme members with activated part of mannosylation of mannose and the biomolecule containing GDP-man. *Leishmania* mannose biosynthetic pathway provides a novel therapeutics route that inhibits the enzymes used in gene deletion mutants. *Leishmania* parasite lacks the enzyme activity which was inhibited by GDP-man hexamer, which can further target as a drug for anti-*Leishmania*. At low ionic strength and increase pH, GDP-man hexamer as a new drug candidate can be designed to inhibit the hexameric form of enzymes (Davis et al. 2004).

To overcome concerns of stability, toxicity and to enhance the bio-availability suitable delivery mechanisms are required. Liposomes are artificial bilayer membranes with non-toxic and non-immunogenic properties and can be used as a carrier for hepcidin blocker (GDP). Liposomes can be easily absorbed and degraded in vivo due to as they have structure similar to cellular membranes. GDP is a natural hepcidin antagonist and apart from directly inhibiting hepcidin action, GDP also suppresses IL-6 levels in U937 macrophages cells thus, modulating NF-κB activation in IL-6 JAK/STAT3-hepcidin axis. An effective approach would be to develop liposome encapsulate GDP (NH+) to ameliorate AI which may be
mediated by suppression of *Hamp* mRNA transcription. Furthermore, encapsulation of liposomes as a carrier can reduce drug consumption; improve absorption efficiency and lower toxicity. To enhance the efficacy and stability of GDP on iron availability, GDP was encapsulated within the lipid vesicle having different surface potential. Encapsulated NH+GDP with single positive charge (NH+) was found to be most compatible encapsulating delivery vehicle after all toxological studies. Next, we aimed to evaluate the the underlying mechanism of NH+GDP on inflammation mediated NF-κB activation through IL-6/STAT3 hepcidin axis in *in vitro* and *in vivo* and assessed its therapeutic potential against AI

Considering, the above observation, following objectives were undertaken.

To increase iron bioavailability we selected a Novel compound against hepcidin action through natural compound libraries that might provide a new alternative approach to increase iron absorption for prevention of hepcidin mediated FPN internalization with different *insilico*, *invitro* and *invivo* studies:

i) **In-silico studies**: Screening of the Natural compound from chemical libraries (~70,000) to find potential hepcidin blockers/inhibitor and molecular interaction of selected compound with hepcidin.

ii) **In-vitro studies**: Investigation of GDP effect on iron bioavailability and ferroportin mediated cellular iron content on HepG2, Caco-2, GFP-FPN cell line.

iii) **In-vivo/Ex-vivo studies**: Chronic effect of GDP treatment on iron homeostasis in mice.

iv) To find a better delivery target system (Encapsulated liposome GDP): GDP will be encapsulated in form of liposome to increase iron Absorption on HepG2 and Caco-2 cell line and effect of encapsulated GDP on LPS induced anemic mice for enhancement of iron bioavailability.