6. Summary and Conclusions

Anemia is a very prevalent public health issue associated with an increased risk of morbidity and mortality, especially in pregnant women and young children throughout the world. Wide variety of causes are responsible for anemia but more often they coexist such as iron deficiency anemia (IDA), Anemia of inflammation (AI) and anemia related to kidney diseases. Many therapeutic trials are carried out all over the world but none have been effective to eradicate the anemia completely, due to various side effects. 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 with involvement of inflammatory process in the pathogenesis of AI. It has been documented clinically in some cases, that patients with inflammatory disorders often develop an iron-restricted anemia known as ACI or AI. 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/STAT3 or BMP/SMAD pathway. The hepatic peptide hepcidin controls iron efflux to plasma from enterocytes and macrophages through degradation of the iron export channel FPN causing cellular iron retention with increased accumulation of intracellular iron leading to hypoferremia.

In the present study, we identified a natural compound GDP (ZINC Database ID: ZINC08215481), that show sufficiently good binding affinity with hepcidin using molecular modelling techniques further validated by biochemical and biophysical method. Keeping in view the above objective, antagonist potential of GDP was hypothesised on the basis of its ability to block molecular recognition interactions of hepcidin-FPN complexation. 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 and apart from that we also discover that encapsulated (NH+GDP) was found to inhibit NF-κB activation thus, modulating IL-6/JAK2/STAT3-hepcidin axis decreasing Hamp mRNA transcription.
Important finding of present study are as follows:

1. Through virtual screening of 68,752 natural compounds via molecular docking, GDP was identified as a promising hepcidin-binding agent. The molecular dynamics simulations helped to identify the important hepcidin residues involved in stabilization of hepcidin-GDP complex.

2. *In vitro* studies revealed that GDP triggered FPN stabilization (FPN-GFP cell lines) with effective FPN-mediated cellular iron efflux in HepG2 and Caco-2 cell line. Flow cytometer and protein expression showed that GDP prevented hepcidin-mediated FPN internalization with reduced iron storage ferritin level.

3. One dose response study in normal wild type mice showed that GDP along with FeSO$_4$ decreases Hamp mRNA expression with increased FPN expression and reduced iron storage ferritin level in spleen and enterocyte for cellular-mediated iron export.

4. GDP + FeSO$_4$ ameliorates turpentine-induced AI in mice with increased haemoglobin level. Tissue specific iron distribution showed that GDP + FeSO$_4$ decreases iron accumulation in spleen and liver via stabilization of FPN for effective iron-mediated erythropoiesis thus, improving hypoferremia.

5. Coincidently, GDP+FeSO$_4$ also decreases Hamp mRNA level pointing towards involvement of IL-6/STAT3 pathway. Protein expression revealed reduced nuclear translocation of pSTAT3 with decreased Hamp mRNA level in LPS-induced anemic mice.

6. TEM and SEM analysis showed that no significant change in the size and structure of NH+GDP was observed which confirms the integrity of liposome structure. MTT assay showed that after all toxological studies encapsulated (NH+GDP) was most compatible encapsulating delivery vehicle.

7. NH+GDP decreased LPS-induced phosphorylation and degradation of IκB-α with reduced translocation of NF-κB from cytosol into nucleus. A significant decrease in
levels of pro-inflammatory cytokine (IL-6, TNF-α, and IL-1β) was observed indicating suppressed inflammatory response in U937 macrophage cells.

8. A significant decline in hepcidin level and *Hamp* mRNA expression was observed in HepG2 and Caco2 co-culture cells indicating suppressed activation of IL-6/JAK/STAT pathway. Immunoblot analysis showed that NH+GDP treatment down regulates JAK2/STAT3 pathway with decreased translocation of pSTAT3 into the nucleus.

9. A significant decrease in IL-6 level was observed with reduced nuclear translocation of pSTAT3 and pJAK2 in acute mice model. Protein expression showed decrease phosphorylation of JAK2 and STAT3 activation with reduced transcription of *Hamp* mRNA level.

10. A significant increase in serum iron concentration, haemoglobin level and erythrocyte number was observed in chronic AI model. Protein expression analysis showed suppressed JAK2 and STAT3 phosphorylation with decreased hepcidin protein expression. Tissue specific section revealed iron accumulation in anemic group. However, NH+GDP treatment reversed this effect with decreased iron deposit in spleen section.

Current approaches to correct AI involve transfusion or erythropoietin (EPO) administration to treat seriously ill patients. These approaches are accompanied by many complications. The present findings clearly demonstrate that GDP can inferred as a robust candidate for hepcidin binding agent to regulate normal iron homeostasis thus, improving hemoglobin synthesis. Further, these findings pave the way for the design of hepcidin binding agents or a novel therapeutic agent to overcome the limitations associated with current therapies in inflammation-induced anemic conditions and suggest being a potential drug to relief AI with higher preclinical and clinical relevance.

Pharmacodynamics and pharmacokinetics study of GDP and NH+GDP:

In relevance to clinical studies, further we will investigate the role of GDP and NH+GDP in PG-APS female wistar anemic induced rats. For long-term experiment, AI condition was induced in female wistar rats by (IP) administration of PG-APS (15ug rhamnose/g of body
weight), resulting in anemia within 2 week interval (2 injections/week). Meanwhile, during treatment we will analyse CBC blood parameter to check haemoglobin count. The experimental set up include eight group of female wistar rats, control, control+NH, control+GDP, positive control (FeSO₄+absorbic acid), negative control (FeSO₄), anemic, anemic+NH+GDP, anemic+GDP. To evaluate whether GDP or NH+GDP treatment could alleviate anemia in these animals, anemic rats (2 weeks post-PG-APS) were treated with GDP or NH+GDP in dose dependent concentration. Further if significant result were observed we, will check all biochemical assay, haematological parameter, tissue histology, western blotting and pharmacokinetics and pharmacodynamics parameter.

Further the experimental work will be carried out by Poonam Sagar (ICMR-JRF) at NABI Mohali to explore the pre-clinical relevance action of GDP and NH+GDP on female wistar rats.