Hepcidin is a key regulator of body iron concentration and distribution and also its involvement in AI suggests it to be appropriate target to maintain iron homeostasis (Camaschella et al. 2013). Lipopolysaccharide (LPS) produced in gram-negative bacteria cell wall, constitutes major component of bacterial endotoxin. LPS is recognized by Toll-like receptor (TLR4) on host cell surface and initiates an inflammatory signalling cascade. Inflammatory pathways up-regulate the expression of TLR4-activated macrophages signal via release of various inflammatory factors. This occurs primarily via translocation of nuclear factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Kinjyo et al. 2002). NF-κB signalling pathways have been considered the classical pathways to modulate the inflammatory response. Once activated, nuclear factor κB translocates into the nucleus to release inflammatory factors. Activation of these pathways regulates oxidative stress response and accelerates inflammatory response (Song et al. 2007; Xagorari et al. 2000). However, previous studies reported that inflammatory disorders result in the secretion of pro-inflammatory cytokines such as IL-6, which then binds to the IL-6 receptor on the membrane of hepatocytes to activate the JAK2/STAT3 pathway via phosphorylation. The phosphorylated STAT3 dimer then translocates to the nucleus, binds to the hepcidin promoter and activates its transcription (Wrighting et al. 2006). Elevated hepcidin eventually binds to the FPN leading to its lysosomal degradation thus, resulting in intracellular iron accumulation, blockage of iron egress from the cells, impairing iron absorption from duodenal intestine and macrophage (Nemeth et al. 2014). Therefore, it causes iron retention within the cells leading to hypoferremia, resulting in ineffective iron-mediated erythropoiesis (Nemeth et al.2004). Hence, transcriptional re-programming of hepcidin could be a novel approach in treating AI symptoms. The genetic programming of hepcidin regulation constitutes two major pathway; IL-6/STAT3 pathway (Nemeth et al. 2004; Verga et al. 2006) and BMP/SMAD4 pathway (Parras et al. 2014; Zhao et al. 2013). During infectious and inflammation stimuli, macrophages release pro-inflammatory cytokines which stimulates the immune response indicating the symptoms of inflammation.

Earlier studies reported that Decursin, (coumarin component in A. gigas) inhibit expression of matrix metalloproteinase-9 and cytokines via the NF-κB pathway and its derivatives has inhibitory effects on lung inflammation (Kim et al. 2006; Yang et al. 2009).
Nodakenin, has been reported to suppress LPS-induced inflammatory response in macrophage cell by inhibiting TNF-α and NF-κB pathway (Rim et al. 2012). Moreover, different strategies were employed to decrease expression of hepcidin via IL-6/JAK/STAT3 pathway. 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 H₂S, suppresses hepatic hepcidin and corrected hypoferremia induced by LPS (Wang et al. 2016). AMP-activated protein kinase (Wang et al. 2016) as a novel therapeutic target ameliorate AI by promoting suppressor of cytokine signalling 1(SOSCO1) mediated JAK2 degradation. However, these approaches are limited with poor pharmacokinetics profile (AG490 and PpYLKTK), lack of specificity, stability (STAT3 inhibitors), and competing iron chelating properties (curcumin) with decreased metabolic profile.

Previously in chapter 5, we found that GDP has a dual role that may act at transcriptional as well as post-translational level. We had demonstrated the binding of GDP to hepcidin and disruption of hepcidin-FPN complex. GDP+FeSO₄ also decreases the Hamp mRNA level, pointing towards involvement of IL-6/JAK/STAT3 pathway (Angmo et al. 2017). 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). Furthermore, encapsulation of liposomes as a carrier can reduce drug consumption; improve absorption efficiency and lower toxicity. In the present study, we demonstrated that GDP apart from directly binding and inhibiting hepcidin action was also found to inhibits NF-κB activation thus, modulating IL-6/JAK/STAT3-hepcidin axis. 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. Further, we aimed to investigate the underlying mechanism of NH+GDP on inflammation mediated NF-κB activation modulating IL-6/STAT3 hepcidin pathway in vitro and in vivo and assessed its therapeutic potential against AI.
5. Characterization and encapsulation of GDP into liposome vesicle

5.1. Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

Liposomal formulations were prepared by the thin film hydration method with some modifications. Briefly, (1,2-dioleoyl-3-trimethylammonium–propane(chloride salt; DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine–N-Lactosyl (LacPE), 1,2-Dioleoyl-sn-glycero-3- phophoethanolamine (DOPE). Later, the film was hydrated with 2 ml of PBS at 10 μM GDP (Table 16). To characterize the physiochemical structure and unilamellar size, SEM and TEM images of NH+ and NH+GDP were obtained. SEM confirms that there were no significant changes in the structure of the NH+GDP as compared to control (NH+) indicating that encapsulation of GDP did not cause any distortion in the structure of the NH+ (Figure 28A). TEM imaging reconfirms size range of 100 nm for both NH+ and NH+GDP with equal unilamellar size which confirm the integrity of liposome structure after encapsulation (Figure 28B).

Table 16: Characterisation of Liposomes with GDP

<table>
<thead>
<tr>
<th>NAME</th>
<th>LIPOSOme COMPOSITION</th>
<th>CHARGE</th>
<th>POTENTIA l (mV)</th>
<th>SIZE (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH+GDP</td>
<td>DOPE 60, DOTAP 30, lacPE10 (GDP 10μM)</td>
<td>+</td>
<td>24 - 27</td>
<td>138-152</td>
</tr>
<tr>
<td>Control NH+</td>
<td>DOPE 60, DOTAP 30, lacPE10</td>
<td>+</td>
<td>25-28</td>
<td>132-154</td>
</tr>
<tr>
<td>NH++GDP</td>
<td>DOPE 60, DOTAP 30, lacPE10(GDP 10 μM)</td>
<td>++</td>
<td>45-50</td>
<td>125-126</td>
</tr>
<tr>
<td>Control NH++</td>
<td>DOPE 60, DOTAP 30, lacPE10</td>
<td>++</td>
<td>36-44.2</td>
<td>116-117</td>
</tr>
<tr>
<td>NH</td>
<td>DOPC45, DOPE45, Cholesterol10 (GDP 10μM)</td>
<td>Neutral</td>
<td>-5 to -6</td>
<td>135-130</td>
</tr>
<tr>
<td>Control NH</td>
<td>DOPC45, DOPE45, Cholesterol 10.</td>
<td>Neutral</td>
<td>-4 to -5</td>
<td>135.3-136.7</td>
</tr>
</tbody>
</table>
5.2. Evaluation of cellular toxicity of NH+ and NH++ with or without GDP formulation on HepG2 and Caco2 cells

5.2.1 Cell viability

MTT cell viability assay was performed and cytotoxicity of the liposomal formulations was investigated with both types of liposomes (NH+ and NH++) at different concentration (100, 1000μg) on HepG2 and Caco2 cell lines using MTT assay. We treated the cell line with both liposomal formulation and found that NH++ relatively showed higher cytotoxicity than NH+ at indicated concentrations may be due to cellular membrane damage by NH++ vesicles to a greater extent. Furthermore, the percentage of viable cells were more when treated with NH+ as compared to NH++ liposomes which prove that NH++ liposomes are more toxic to the cells (Figure 29A-B). We investigated the cytotoxicity of the liposomal formulations (NH+ and NH++) at indicated concentration and found that comparative to NH+ formulation, NH++ is more toxic to the cell lines, as most of the cells surface is negatively charged thus, it has higher permeability for cationic charged particles, therefore double positive charge particles (NH++) penetrate the cell membrane more abruptly as compared to the particles with less positive or negative and neutral charge thus, resulting in cellular damage.
Results

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Figure 29: Evaluation of cytotoxicity of cationic liposomes (NH+ and NH++) without encapsulation on HepG2 and Caco2 cells. A-B) Viability of liposome formulation (NH+ and NH++) was determined using MTT assay at different concentration indicating NH++ more toxic than NH+ on both HepG2 and Caco2 cells. Data represent means ± SD of three independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey’s post test. (***) $p < 0.05$; (****) $p < 0.001$.

5.2.3 Dose response study of NH+GDP with and without encapsulation of GDP on indicated LPS concentration

We treated the cells with encapsulated (NH+GDP and NH++GDP) and control (NH+ and NH++) at indicated LPS concentration. Additionally, a dose response experiment was conducted with NH+ and NH++ formulation with and without GDP at indicated concentration of LPS on HepG2 cells. The white bar is control NH+ and NH++ liposome and the green bar is non encapsulated GDP and encapsulated NH+, NH++GDP at 10 μM. The indicated red and black bar represent LPS of different concentration (20, 30, 40 and 50 μg) with or without encapsulated NH+GDP and NH++GDP. Cellular toxicity revealed that NH++ was more toxic at different LPS concentration in a dose dependent manner comparatively to NH+. Non-treated cells were used as 100% viability control (dotted line). In agreement, we choose the less toxic NH+ formulation (DOPE60, DOTAP30, lacPE10) as ideal lipid composition to encapsulate the GDP(10μM) for further studies (Fig 30 A-B).
Results

Figure 30: Cytotoxicity of encapsulated NH+ and NH++ GDP formulation with different LPS concentration on HepG2 cells: A-B) Viability of HepG2 cells were determined using MTT assay. Cells were treated with encapsulated (NH+GDP and NH++GDP) and control (NH+ and NH++) at indicated LPS concentration and among all the concentration NH+GDP was found to be less toxic comparatively to NH++GDP.

5.3. In vitro MANT-GDP Internalization of NH+GDP in HepG2 and Caco2 cells with encapsulated drug release of NH+GDP

To confirm the internalization of NH+GDP inside the cells, MANT encapsulated NH+GDP was validated at different concentration (10μM, 50μM) in both HepG2 and Caco2 cells. The blue fluorescence inside the cells clearly indicated the internalization of NH+GDP (Figure 31A). Evaluation of in vitro drug release from encapsulated NH+GDP was done by dialysis method. The dialysis method provided a correlation with the in vivo release profile. The in vitro release behaviour of the NH+GDP is summarized as the cumulative release percentage. The release rate of the NH+GDP was measured at 37°C in PBS at pH-6.8 for over a period of twelve hours and the measurement was taken at 253 nm. The value of concentration corresponding to the absorbance was calculated from the GDP standard curve. Release data expressed as percent GDP released over 12 h determined for the liposomal formulation. The release profile of GDP from NH+ showed that 78.98% of the drug was released in 6 h, followed by a steady release rate. The release behaviour of GDP from the NH+liposomal formulation exhibited a biphasic pattern characterized by a steady and continuous release for a period of 12 h (Figure 31B).
Results

Figure 31: Internalization of MANT-NH+GDP inside HepG2 and Caco2 cells and cumulative release profile of encapsulated GDP (NH+GDP): A) Internalization of encapsulated MANT-NH+GDP at different concentration in HepG2 and Caco2 cell line. B) Release profile of encapsulated NH+GDP at different time interval at pH 6.8.

5.4. Dose dependent concentration of LPS at different time interval on HepG2 cells

Earlier studies reported that LPS induced inflammation increases *Hamp* mRNA expression in HepG2 and macrophages cells (Matak et al. 2009). We, treated the HepG2 cells with LPS at indicated concentration at different time intervals and among all the concentration the time course assessment suggested 6h-1μg as the peak point of hepcidin mRNA (*Hamp*) induction in HepG2 cells (Figure 32).

Figure 32: LPS-induced inflammation increases hepcidin level in HepG2 cells: Time course evaluation suggested that increase in *Hamp* mRNA level (12 fold) was observed at LPS (1μg-6h) in HepG2 cells. Data represent means ± SD of three independent experiments. Differences were analyzed using One-way ANOVA followed by Tukey’s post test. (***), p<0.001.
5.5. *Invitro* studies

5.5.1. Study design:

To evaluate the effects of NH+GDP *in vitro*, we used a Conditioned Medium (CM) model consisting of U937 macrophage cells and co-culture model consisting of HepG2 and Caco2 cells to mimic the pathophysiological conditions *in vivo*. U937 macrophage cells were stimulated with LPS for 6h and treated with NH+GDP till 1h (termed as pre-treatment) followed by exposure of U937-CM to the HepG2/Caco2 co-culture model, which was termed as “post treatment” (Figure 33).

![Flowchart representation of CM model of U937 cells and further exposure of U937-CM to HepG2 and Caco2 co-culture cells.](image)

**Figure 33.** Flowchart representation of CM model of U937 cells and further exposure of U937-CM to HepG2 and Caco2 co-culture cells.

5.5.2 NH+GDP inhibit LPS-induced nuclear translocation of NF-κB suppressing pro-inflammatory cytokine expression in U937 macrophage cell line

We treated U937 macrophages cells with LPS(10nM) to induce inflammation activating NF-κB signalling pathway with transcription of pro-inflammatory cytokine release. Our target was to investigate mechanistic action of NH+GDP that might interfere in NF-κB signalling pathway attenuating pro-inflammatory cytokine production. LPS-induced transcriptional regulation of inflammation is required for activation of NF-κB pathway in macrophages cells (Sakurai et al. 2002). To explore the mechanistic action of NH+GDP, U937 macrophage cell lines were stimulated with LPS to activate the NF-κB signalling pathway. Inactive state of NF-κB binds to its inhibitor protein IκB-α in the cytoplasm, but after cellular stimulation IκB-α is phosphorylated at specific serine residues and undergoes polyubiquitination, which free NF-κB, allowing it to be translocated to the nucleus (Xagorari et al. 2000). Interestingly, we found that NH+GDP attenuates phosphorylation and degradation of IκBα that prevents nuclear translocation of p65 subunit of NF-κB, from cytosol into the nucleus thus, decreasing induction of pro-inflammatory cytokines levels in U937 macrophages cells (Figure 34A). This decrease was paralleled with reduced transcription of pro-inflammatory cytokines (IL-6, TNF-α and IL-1β) mRNA and protein expression along with decreased nitric oxide (NO)
production in U937 cells in a dose dependent manner (Figure 34B-F). These results indicate that NH+GDP inhibit LPS-induced phosphorylation and degradation of IκB-α thus, decreasing transcription of pro-inflammatory cytokine (IL-6, TNF-α, and IL-1β) expression in U937 macrophages cells.

Figure 34: NH+GDP suppresses NF-κB pathway reducing pro-inflammatory cytokines release: A-B) NH+GDP decreases phosphorylation of IκB-α that prevents the nuclear translocation of the p65 subunit of NF-κB from cytosol into the nucleus thus decreasing the induction of inflammatory cytokines (IL-6). C) Reduced NO level was observed with NH+GDP treatment in dose dependent manner. p values were calculated using one-way ANOVA. **: p ≤0.05. D-E) NH+GDP significantly decreases the mRNA and protein expression of TNF-α and IL-1β pro-inflammatory cytokine level. F) Treatment with NH+GDP significantly decreases protein expression in IL-6, TNF-α and IL-1β. p values were calculated using one-way ANOVA. **: p ≤0.05, ***: p ≤0.001. Differences were analyzed using One-way ANOVA followed by Tukey’s post test. (*, #) p < 0.01; (**, ##) p < 0.001, p < 0.05.
5.5.3 To investigate the effect of encapsulated (NH+GDP) or non-encapsulated (GDP) on expression of IL-6 in U937 macrophages cells

We investigated the effect of GDP and NH+GDP on IL-6 level in U937 macrophages cell. LPS-induces inflammation increases IL-6 secretion through TLR-4 mediated NF-κB signalling pathway (Kinjyo et al.2002). Additionally, in comparison to non-encapsulated (GDP), encapsulated (NH+GDP) was more effective decreasing IL-6 in dose dependent concentration (Figure 35). This assay marked compelling evidence that encapsulated NH+GDP was more effective in reducing inflammation-mediated IL-6 secretion as compared to non-encapsulated GDP.

![IL-6 concentration graph](image)

Figure 35: Effect of NH+GDP and GDP on IL-6 level: LPS-induced inflammation elevates IL-6 level in dose dependent concentration. In comparison to non-encapsulated (GDP), NH+GDP was more effective in suppressing IL-6 level in U937 macrophages cell. *p* values were calculated using one-way ANOVA. **: *p* ≤0.05.

5.6 NH+GDP suppresses hepcidin expression by down regulating IL-6/JAK/STAT3 pathway

5.6.1. Target specific NH+GDP inhibit Hamp mRNA expression by decreasing IL-6 secretion in HepG2 and Caco2 co-culture model

In agreement with the decreased IL-6 level in U937-CM, subsequently there was reduced binding of IL-6 to its receptor to activate IL-6/JAK2/STAT3 pathway for Hamp mRNA transcription in HepG2 cells. Consistent results were observed with decreased translocation of phosphorylated pJAK2/pSTAT3 activation (Figure 36A) into the nucleus thus, reducing Hamp mRNA and hepcidin level in HepG2 and Caco2 co-culture model (Figure 36 B-C).
Figure 36: Target specific NH+GDP inhibit Hamp mRNA expression by decreasing IL-6 secretion in HepG2 and Caco2 co-culture model: A) NH+GDP inhibited LPS-CM-induced JAK2/STAT3 activation; tubulin was used as an internal control. B-C) Decrease in Hamp mRNA expression and hepcidin level were observed in HepG2 and Caco2 co-culture cells. D) Immunofluorescence images clearly indicate that NH+GDP suppressed pSTAT3 nuclear translocation induced by IL-6. E) Increase FPN protein expression revealed that NH+GDP prevents hepcidin-induced internalization of FPN in HepG2 and Caco2 co-culture cells. F) LPS-mediated inflammation decreases iron uptake from DMT1 transporter whereas, NH+GDP treatment reverses this effect with increase cellular iron uptake and reduced iron storage ferritin in Caco2 cells. Data were normalized to mRNA expression of a housekeeping gene, GAPDH. p values were calculated using one-way ANOVA. ‘*’ with p≤ 0.01 control vs LPS and ‘**’ with p≤ 0.05 LPS+NH+GDP vs LPS vs. ‘#’ with p≤ 0.01 control vs LPS and ‘##’ with p≤0.05 LPS+NH+GDP vs LPS.
Results

Immunoblot analysis indicated that LPS increases the translocation of phosphorylated STAT3 (pSTAT3) into the nucleus due to the activation of JAK/STAT pathway, whereas NH+GDP treatment down regulates JAK2/STAT3 pathway and hence decreases translocation of pSTAT3 into the nucleus (Figure 36D). Concomitantly, FPN protein expression decreases due to hepcidin-mediated internalization of FPN. Whereas, NH+GDP reverses this effect in HepG2 and Caco2 co-culture cells (Figure 36E). As expected, LPS-induced inflammation decreases iron transport across the apical membrane due to hepcidin-mediated FPN degradation with increases iron accumulation leading to hypoferremia. Whereas, NH+GDP treatment facilitates more iron to transport across the apical membrane (DMT1) with reduced iron storage ferritin level in Caco2 cells corresponding to effective iron-mediated cellular efflux (Figure 36F). These results suggested that NH+GDP attenuates IL-6 secretion in U937-CM model by suppressing activation of JAK2/STAT3 pathway in HepG2 cells with increase iron absorption in Caco2 cells.

5.7. NH+GDP targeting IL-6/JAK/STAT3 pathway in acute mice model

The in vitro studies provided a clear evidence that NH+GDP attenuates NF-κB pathway activation thus, reducing pro-inflammatory cytokine (IL-6, TNF-α and IL-1β) levels. In correspondence, subsequently there was reduced binding of IL-6 to its receptor to activate IL-6/JAK2/STAT3 pathway for Hamp mRNA transcription. IL-6 is a major regulator of inflammatory stimulus in hepatocytes. The transcriptional regulation of hepcidin is regulated by IL-6 (Nemeth et al.2004) through JAK2/ STAT-3 signalling pathway (Pietrangelo et al. 2007; Verga et al. 2007; Wrighting et al. 2006). Infections and systemic inflammatory diseases increases hepcidin production with rise in hepcidin concentrations in blood. Inflammation-induced hepcidin increases pro-inflammatory cytokines production (IL-6) with increase transcription of Hamp mRNA level leading to hypoferremia (Niemand et al. 2003). To elucidate the IL-6/STAT3 pathway involved in hepcidin expression BALB/c mice were challenged with LPS (IP) for 6h and further treated with NH+GDP (IP) for 30 minute. In association, we found significant decrease in serum IL-6 (Figure 37A) with decreased phosphorylation of JAK2 and STAT3 activation (Figure 37B) thus, reducing transcription of Hamp mRNA level (Figure 37C). These results indicated that NH+GDP down regulated the inflammation-mediated activation of Hamp mRNA expression via suppressing IL-6/JAK/STAT3 pathway in LPS-induced acute model.
Results

Figure 37: NH+GDP suppresses LPS–induced Hamp expression in acute model: A-B) NH+GDP significantly reduced serum IL-6 level suppressing the phosphorylation of JAK2/STAT3 pathway. Tubulin was used as an internal control. C) Consistently NH+GDP decreases LPS-mediated Hamp mRNA expression relieving LPS-induced inflammation in acute model. Results are normalized to GAPDH and expressed as mean ± SD for n animals (n=5/group). p values were calculated using one-way ANOVA. **: p ≤0.01 LPS+NH+GDP vs LPS;**: p ≤0.05 LPS+NH+GDP vs LPS and *: p ≤0.01 Control vs LPS

5.8 NH+GDP ameliorates AI in chronic model thus, maintaining normal iron homeostasis

5.8.1 Study Design:

Figure 38: Diagrammatic representation of dose interval and time progression towards anemia.
To induce the chronic AI model, BALB/c mice were challenged with LPS (IP) on the first day followed by Zymosan (IP) for one week to develop AI. Next AI mice were treated with NH+GDP (IP) every 24h for 2 weeks as depicted in (Figure 38).

5.8.2 NH+GDP reduces *Hamp* mRNA expression by suppressing IL-6/STAT3 pathway in chronic AI model

Treatment with NH+GDP significantly increases serum iron concentration (Figure 39A). with rise in haemoglobin level and erythrocyte number thus, correcting inflammation-induced AI state (Table 17). As expected, NH+GDP markedly reduced serum IL-6 levels more than 30% (Figure 39B) with suppressed JAK2 and STAT3 phosphorylation (Figure 39C). Consistent results were observed with decreased hepcidin protein expression (Figure 39D), thereby, down regulating IL-6/STAT3 pathway. Moreover, reduced *Hamp* expression indicates the decrease in serum ferritin level for effective iron-mediated erythropoiesis thus, improving hypoferraemia (Figure 39E). The spleen plays a significant role in chronic inflammation and immune response. Dysregulation of splenic iron is another hallmark of AI with reduced circulating iron level. The tissue iron deposit and splenic iron content was reversed by NH+GDP reducing iron restrictive effect of inflammation with effective iron-mediated efflux (Figure 39F-G). These data indicate that NH+GDP successfully ameliorates inflammatory hepcidin and improves AI symptoms in chronic model thus, maintaining the normal iron homeostasis.

**Table 17: Complete blood count (CBC) indices of mice injected (IP) with normal saline or LPS+ Zymogen and treated with NH+GDP (i.p. i.e. 30 mg/kg body) for 2 week.**

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Control</th>
<th>Control+ NH+GDP</th>
<th>Anemic</th>
<th>Anemic+ NH+GDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (mg/dl)</td>
<td>16.7 ± 0.34</td>
<td>15.8 ± 1.02</td>
<td>11.2 ± 0.11 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.4 ±1.44 &lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>49.35 ± 0.45</td>
<td>47.2 ± 0.55</td>
<td>42.60 ±1.433 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.6 ± 0.36 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>17.00 ± 1.55</td>
<td>15.00 ± 0.22</td>
<td>14.9 ±0.54 &lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.9 ± 1.55 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RBCs (10⁹ /L)</td>
<td>09.18 ± 1.33</td>
<td>09.00 ±0.33</td>
<td>7.625 ±1.0 &lt;sup&gt;d&lt;/sup&gt;</td>
<td>09.03 ± 0.28 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Platelets (10⁹ /L)</td>
<td>1203 ± 0.551</td>
<td>1098 ±1.466</td>
<td>1954 ±2.56</td>
<td>2728 ± 0.71</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; *n* = 5

<sup>a, b, c, d</sup> Anemic + NH+GDP is highly significantly from Anemic. (*p* < 0.05)
Figure 39: NH+GDP reduces inflammatory hepcidin expression by suppressing IL-6/STAT3 Pathway in Chronic AI Model: A) Elevated serum iron concentration was observed with NH+GDP treated group. B) NH+GDP significantly attenuates serum IL-6 level evoked by LPS+Zymosan-induced inflammation. C-D) NH+GDP suppressed phosphorylation of JAK2/STAT3 thus, decreasing hepcidin protein expression, tubulin was used as an internal control. E) In parallel, significant decrease in serum ferritin level was observed in NH+GDP treated group indicating effective iron egress for erythropoiesis. F) Splenic iron level indicate decrease in iron content level after treatment with NH+GDP. G) Increased iron deposit was observed in anemic state, whereas NH+GDP reversed this effect with decrease iron accumulation in spleen. (Results are normalized to GAPDH and expressed relative to controls. n = 8/group. p values were calculated using One-way ANOVA. '*' with $p \leq 0.01$ control vs anemic, '**' $p \leq 0.05$ NH+GDP vs anemic, '***': $p \leq 0.001$ NH+GDP vs anemic.)
Discussion

AI is one of the most multifaceted illness with worst prognosis and prevalent in patients with inflammatory disorders and malignancies. It is the second most prevalent anemia caused due to prolonged infection or inflammation (Ganz et al. 2009; Aggarwal et al. 2009). Hepcidin, a hepatic origin peptide hormone, plays a crucial role in iron homeostasis (Nemeth et al. 2004). Two major pathways regulating the activity of hepcidin are BMP/SMAD pathway particularly associated with body iron status and IL-6/STAT3 pathway regulating the inflammation associated hepcidin levels (Wrighting et al. 2006; Parrow et al. 2014; Babitt et al. 2007). Many conventional remedies were developed for AI that include synthetic form of erythropoietin (EPO), blood transfusion and iron supplementation. However, synthetic erythropoietin is associated with increased mortality and poor survival rates, such as heart diseases, high blood pressure and porphyria (cause by enzyme deficiency) and allergic reaction to medicines (Tonelli et al. 2009). Moreover, blood transfusion is associated with increased risk of disease transmission and immunomodulation, such as hepatitis, allergic and acute haemolytic reactions (Vincent et al. 2002). Additionally, oral iron supplementations are efficacious but poorly tolerated due to non-absorbed iron mediated gastrointestinal tract diseases (Teehan et al. 2004). These observations highlight the immediate requirement for alternative therapies and illustrate the significant implications of our findings in discovering a natural screened compound (GDP) with less toxicity and increase iron bioavailability in clinical management of AI.

Earlier studies have demonstrated the anti-inflammatory agents like hydrogen sulphide (Xin et al. 2016), and AG490 (Zhang et al. 2011) which attenuated hepcidin expression in vitro and in vivo experiments. However, their basic limitation was their stability, complex delivery mechanism, non-specific delivery and unclear metabolic profile. Despite the vast contribution of hepcidin for AI, it should be taken into account that other molecules also released during inflammatory response, including IL-1β, TNF-α and NO (Niemand et al. 2003). As macrophage activation is a hallmark of inflammation which trigger signal transduction pathway. Although previous studies demonstrate the effect of anti-inflammatory compound on NF-κB pathway in vitro and in vivo (Rim et al. 2012; Pruett et al. 2009). The mechanistic action beside the NH+GDP attenuating IL-6 and other pro-inflammatory cytokine is suppressing NF-κB activation with reduced nuclear translocation of p65 NF-κB from cytosol into the nucleus thus, down-regulating transcription of pro-inflammatory cytokines release. Decreased IL-6 production reduced binding of IL-6 to its
Results

receptor to activate IL-6/JAK2/STAT3 pathway for Hamp mRNA transcription. Though uncovering all the molecular networks related to signalling pathway is critical, we have only focused only on LPS-induced NF-κB activation along with IL-6 mediated JAK2/STAT3 pathway which is one major contributor to the dysregulation of hepcidin levels. TLR4, a type I trans membrane receptor, is activated by LPS and play a significant role in innate immune system. LPS activate the TLR4-mediated signalling pathway that’s leads to the activation of NF-κB (Kinjyo et al. 2002). As reported earlier, 3,4-dihydroxytoluene (Su K-Y et al. 2014), Resokaempferol (Yu Q et al. 2016) and chloroquonine (Li DY et al. 2017; Jang CH et al. 2006) inhibits NF-κB activation under inflammatory conditions, which is the major inducer of pro-inflammatory cytokines. Earlier many derived natural and synthetic anti-inflammatory compound down regulate NF-κB activation. Earlier many derived natural and synthetic anti-inflammatory compound down regulate NF-κB activation. Herein, we identified the mechanism by which NH+GDP mediates the pro-inflammatory response in macrophages cell line but, we are not validating NH+GDP as an anti-inflammatory compound as it needs more experimental evidence. Indeed, for the first time, we established a link between NH+GDP inactivating NF-κB and IL-6/STAT3 pathway indicating an alternative approach for hepcidin modulation. Earlier, there was no such evidence in previous reports demonstrating that NH+GDP suppress NF-κB signalling attenuating IL-6/STAT3 pathway.

In current years, a remarkable attention on the ability to produce nanocarriers of uniform size, shape, and composition have revolutionized the new era of science and technology (El-sunousi et al. 2013; Sana et al. 2008). Liposomes for drug delivery system offer higher biocompatibility, versatility and lower toxicity as well as increases bioavailability and pharmacokinetics (Laouini et al. 2012). We investigated the cytotoxicity of the liposomal formulations (NH+ and NH++) at indicated concentration and found that comparative to NH+ formulation, NH++ is more toxic to the cell lines, as most of the cells surface is negatively charged thus, it has higher permeability for cationic charged particles, therefore double positive charge particles (NH++) penetrate the cell membrane more abruptly as compared to the particles with less positive or negative and neutral charge thus, resulting in cellular damage. In relevance to previous study (Angmo et al. 2017) in vivo results indicate that encapsulated (NH+GDP) was more efficient and biocompatible than non-encapsulated (GDP) as relative serum iron concentration was increased by 52% due to its uniformity, stability and sustained drug release without any cellular toxicity. Next, we examined the effect of NH+GDP and GDP on IL-6 expression in U937 macrophages cells and relatively to GDP, encapsulated (NH+GDP) was more effective suppressing IL-6 level in
dose dependent concentration. This assay marked compelling evidence that NH+GDP was more effective in reducing inflammation-mediated IL-6 secretion as compared to non-encapsulated GDP.

In the present study, we demonstrated the mechanism of NH+GDP attenuating NF-κB activation suppressing IL-6 secretion which in turn decreases inflammation-mediated IL-6/JAK/STAT3. Through this current study, we have shown its effect on the mechanistic NF-κB targeting IL-6/JAK/STAT3 pathway (Figure 40). Additionally, we encapsulated the GDP compound in the lipid vesicle (NH+) to increase its stability, bioavailability and efficacy by using drug delivery system (Kulkarni et al.2011; Guo et al.1990). NF-κB signalling starts from activation of IκB-α, Once its activated its bind and phosphorylated releasing IκB-α p65 subunit of NF-κB, which is phosphorylated and translocated into the nucleus to regulate target genes. The NF-κB, is one of the most critical transcription factor that regulate the inflammation mediated gene expression in inflammation (LPS) induced macrophages cells. We investigated whether IL-6, secreted from macrophages, is responsible for inflammation-mediated hepcidin induction through activation of JAK/STAT3 pathway. The CM model is a well-established model used in relevant studies as opposed to exposing hepatocytes to LPS induced inflammation (Xin H et al. 2016; Sakamori et al. 2010). STAT3 is a member of STAT family with its roles in cellular transformation, proliferation and metastasis of cancer (Hodge et al. 2005). Interestingly, we found that NH+GDP attenuates NF-κB activation inhibiting expression of IκB-α, with reduced nuclear translocation of p65 NF-κB from cytosol into the nucleus thus decreasing IL-6 secretion in U937 macrophages cell. Reduced IL-6 secretion was observed in LPS-induced CM along with reducing other pro-inflammatory cytokines such as TNF-α, IL-1β and inflammatory marker (NO) release from U937 macrophages cells. IL-6 activated inflammatory pathway (JAK/STAT3) was focussed to examine the hepatic hepcidin expression which is linked to iron homeostasis. In the HepG2 and Caco2 co-culture experiment using LPS-CM, we found that decreased IL-6 secretion in U937 cells reduced the binding of IL-6 to its receptor, further inactivating the phosphorylation of JAK/STAT3 pathway resulting in decreased Hamp transcription in HepG2 cells. However, reduced Hamp transcription is reported with more effective iron egress (ferritin, DMT-1 expression) from Caco2 cells (Gulec et al. 2014). Analogous results were obtained in vivo. Additionally, the effect of NH+GDP has been studied that has evolved as a novel therapeutic target and potential therapy against AI (Figure 40).
Results

Figure 40: Graphical representation of the site of action of the liposomal (NH+GDP) on IL-6/STAT3-hepcidin axis.

Concomitantly, NH+GDP in acute model provide a compelling evidence suppressing IL-6 mediated STAT3 pathway. Next, our target was to evaluate the effect of NH+GDP on LPS+Zymosan induced chronic model in overcoming AI and to maintain normal iron homeostasis. AI is responsible for decreased iron binding ferritin levels (hypoferremia), with consequent iron restricted erythropoiesis; therefore a number of infectious and inflammatory mouse and rats models have been established to date (Langdon et al. 2014; Rivera et al. 2009; Theurl et al. 2012). To induce chronic AI, many well-known models have been established to study the effect of inflammation on iron regulation and erythropoiesis in mice and humans such as turpentine (Wang et al. 2017; Nicolas et al. 2002), LPS followed by zymosan (Lasocki et al. 2008), and complete Freund’s adjuvant (CFA), (Frazer et al. 2004). In the present study, we used LPS+Zymosan induced chronic AI model to test the hypothesis that NH+GDP suppresses the IL-6/JAK2/STAT3 pathway thus, ameliorating iron restrictive AI with increase in haemoglobin level. Our results demonstrate that NH+GDP not only reverses acute inflammatory hepcidin and hypoferremia, but also improved AI symptoms in a
chronic model with effective iron mediated erythropoiesis thus, maintaining normal iron homeostasis.

In conclusion, we demonstrated for the first time, that NH+GDP inhibit the release of pro-inflammatory factors by suppressing activation of NF-κB and JAK2/STAT3 signalling pathways in LPS stimulated U937 macrophages cell thus, decreasing Hamp mRNA transcription. Further, these findings reveal that NH+GDP could be 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.