Title:

Heparin: a potent inhibitor of hepcidin expression in vitro and in vivo

Short title: Heparin inhibits hepcidin

Authors

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Key words

Hepcidin, iron homeostasis, anemia, heparin, inflammation
Abstract.

Hepcidin is a major regulator of iron homeostasis, and its expression in liver is regulated by iron, inflammation and erythropoietic activity with mechanisms that involve bone morphogenetic proteins (BMPs) binding their receptors and co-receptors. Here we show that exogenous heparin strongly inhibited hepcidin expression in hepatic HepG2 cells at pharmacological concentrations, with a mechanism that probably involves BMP6 sequestering and the blocking of SMAD signaling. Treatment of mice with pharmacological doses of heparin inhibited liver hepcidin mRNA expression and SMAD phosphorylation, reduced spleen iron concentration while increasing serum iron. Moreover, we observed a strong reduction of serum hepcidin in five patients treated with heparin to prevent deep vein thrombosis and this was accompanied by an increase of serum iron and a reduction of C-reactive protein levels. The data show an unrecognised role of heparin in regulating iron homeostasis and indicate novel approaches to the treatment of iron restricted iron deficiency anemia.
Introduction
Iron maldistribution is implicated in various diseases, including hemochromatosis, iron loading anemias, anemia of inflammation and neurodegenerative disorders. Body iron absorption and distribution are controlled by hepcidin, a small peptide secreted by hepatocytes which is expressed as a prepropeptide of 84 amino acids and is processed to the mature hormone of 25 residues by furin. Hepcidin binds and induces the degradation of ferroportin, the only known cellular iron exporter, and thus it regulates iron absorption from duodenum and iron release from reticuloendothelial macrophages. Inappropriately low levels of hepcidin are associated with the various forms of genetic hemochromatosis, while high levels of hepcidin are found in inflammatory conditions, where they contribute to anemia. Liver hepcidin expression is regulated by iron, erythropoietic activity and inflammation, with mechanisms that strongly rely on bone morphogenetic protein (BMP) signaling pathways. Hemojuvelin, which is a main hepcidin regulator, acts as a BMP coreceptor, and the modulation of its level by proteases tightly regulates hepcidin expression. Hepcidin expression in hepatic cells is strongly stimulated by BMP2, BMP4, BMP6 and BMP9, and is reduced by inhibitors of the BMP/SMAD signaling pathway, such as noggin or dorsomorphin. BMP6 is considered the physiological BMP regulator of hepcidin, since its expression is iron-dependent, and BMP6 knockout mice show massive liver iron overload. Hepcidin is stimulated also by the inflammatory cytokine IL-6, in a pathway that involves STAT3, but is dependent on the presence of SMAD4.

Methods to control hepcidin expression can be based on the modulation of BMPs activity. These proteins belong to the transforming growth factor-β (TGF-β) family and exhibit a wide range of biological effects on various cell types. The recombinant BMP2, BMP4 and BMP7 induce osteoblastic differentiation of myoblasts or osteoblastic cells in vitro assays with mechanisms that are modulated by various factors, including the antagonists noggin and chordin and the heparan sulfate proteoglycans (HSPGs) present on the surface and in the extracellular matrix of various cell types, including hepatocytes. The consequences of the binding of BMPs to HSPGs vary, depending if HSPGs are cell-associated or in a free form. Moreover, HSPGs can bind also BMP antagonists and receptors. Accordingly, removal of cell-associated HSPGs by treatments with chlorate or heparinase reduced or increased BMP signaling. Heparin is a glycosaminoglycan analog to HSPGs and it is known to bind BMPs, which were originally purified from heparin columns. In cellular systems, the addition of heparin inhibited the signaling by BMP7 and by BMP6, while potentiated the biological activity of BMP2 and BMP4, or inhibited BMP2 binding to BMP receptors. Altogether it is proven that heparin has strong and
variable effects on BMP osteogenic activity. Heparin and its derivatives were shown to inhibit also a wide range of cytokines and growth factors, implying their potential employment in the treatment of various important pathologies, including tumor and viral diseases.\textsuperscript{29,35,36} Here, we analyzed the effects of exogenous heparin on BMP signaling and hepcidin expression in hepatic cell lines. Moreover we analyzed hepcidin expression in mice treated with pharmacological doses of heparin and in patients who had to be treated to prevent deep vein thrombosis.

**Materials and methods**

*Cell culture.* HepG2 cells were cultured in Minimal Essential Medium (PAA Laboratories GmbH, Headquarters-Austria) with 10% fetal bovine serum (PAA), 40 \( \mu \)g/ml gentamicin and 1 mM L-glutamine (PAA). The cells were maintained at 37°C under 5% \( \text{CO}_2 \). For studies of SMAD1/5/8 and STAT3 phosphorylation or activation, 10\(^5\) cells/well were seeded in 12-well plates, grown for 24 h and for other 16 h in 0.5% FBS. Then they were incubated with recombinant BMP2, BMP6, IL-6 (R&D Systems, USA), (R&D), heparin (UFH) (Calciparina, Italfarmaco), or low molecular weight heparin (LMWH) (Enoxaparin Sodium, Clexane, Sanofi Aventis) or pentasaccharide Fondaparinux sodium (Arixtra, GlaxoSmithKline).

*Immunoblot analysis.* Cell extracts were lysed in lysis buffer (200mM Tris-HCl pH8, 100mM NaCl, 1mM EDTA, 0.5% NP-40 and 10% glycerol) containing a mixture of protease inhibitors (Sigma). For assays examining phosphorylated SMAD and STAT3 expression, 1mM sodium orthovanadate (Sigma) and 1mM sodium fluoride (Sigma) were added to the lysis buffer as phosphatase inhibitors.

Cells lysates were analyzed on 10% SDS-PAGE and after transfer, the nitrocellulose filters were incubated for 16 h with specific antibodies, washed, and further incubated for 1 h with secondary peroxidase-labeled antibodies (Anti-mouse IgG Dako, Glostrup, Denmark or Anti-rabbit IgG Pierce). The primary antibodies used were: rabbit anti-PhosphoSMAD1/5/8 antibody (1:1000; Cell Signaling technology), rabbit anti-SMAD5 antibody (1:1000; Cell Signaling technology), anti-PhosphoSTAT3 antibody (1:1000; Cell Signaling technology) and rabbit anti-β–actin antibody (1:1000; Sigma). Bound activity was revealed by advance enhanced chemiluminescence (ECL) kit (Amersham, Uppsala, Sweden) and detected using KODAK Image Station 440CF (Kodak, Rochester, NY).

*RNA extraction and Real-Time reverse-transcriptase–polymerase chain reaction (RT-PCR).* RNA was purified from cells or mouse livers using the guanidinium thiocyanate–phenol–chloroform method (Trizol) according to the manufacturer’s instructions (Ambion, Austin, TX). Total RNA (1 \( \mu \)g) was used to synthesize the first strand of cDNA with the ImProm-II Reverse Transcription System (Promega), using oligodT. For real-time PCR analysis, specific human or murine assays-on-Demand products (20x) and TaqMan Master Mix (2x) from Applied Biosystems (Foster City, CA) were used, according to the
manufacturer’s instructions, and the reactions were run on ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in a final volume of 20 µL for 40 cycles. The expression levels of hepcidin and Id1 were normalized with levels of HPRT1 mRNA in each sample.

**Mice.** Seven-week-old female mice (Harlan Laboratories, Italy) were cared for in accordance with the European Convention for the Protection of Laboratory Animals and the study was approved by the Institutional Animal Care and Use Committee of the University of Brescia. The mice were daily injected subcutaneously with saline or heparin. After 3-15 days they were sacrificed, and the liver homogenized and analyzed for hepcidin mRNA transcript (by RT-PCR and by real-time RT-PCR) or for phosphoSMAD1/5/8, total SMAD5 and β-actin as a control. For RT-PCR analysis of hepcidin-1 and HPRT1 we used the following primers: hepcidin-1: forward TTGCGATACCAATGCAGAAGAG, reverse AATTGTTACAGCATTATAGCAGAAGA and HPRT1: forward GCTTGCTGGTGAAGACCTCTCGAAGGAGAAG, reverse GCCTGACTGACTTAGGTGGAAGGCGCAT. The PCR were run for 25 cycles. Liver and spleen iron concentration were determined spectrophotometrically by using bathophenantroline, following the procedure described in ref. 37, except that the heating at 110°C was omitted. Blood was collected from the tail and serum iron determined spectrophotometrically with a commercial kit (Randox laboratories, Ltd).

**Patients.** To preliminarily explore the effects of heparins on hepcidin in humans, we evaluated a time course of serum hepcidin concentration and of indices of iron status in five patients (median age 87 years, range 67 to 93) admitted to the Section of Internal Medicine at University Hospital of Verona, because of an intercurrent illness (pneumonia, n=3; decompensated heart failure, n=1; decompensated heart failure and urinary tract infection, n=1). In all patients, heparin thromboprophylaxis was indicated according to current guidelines 38, with no contraindication. All patients gave informed consent for the collection of a small adjunctive aliquot of blood (1 ml) for hepcidin measurement during scheduled blood drawn in the morning for monitoring of their disease conditions. Serum hepcidin was measured by SELDI-TOF-MS as previously described 39. The protocol was approved by the local ethical committee.

**Statistical analysis.** Comparison of values between untreated and treated cells or mice was performed by Student t test for unpaired data. Differences were defined as significant for P values less than 0.05.

**Results.**

*Unfractionated heparin strongly inhibits hepcidin expression in HepG2 cells.*
Exogenous heparin was previously shown to affect the osteogenic activity of different BMPs in various cell lines\textsuperscript{26,31,33}, thus it was of interest to evaluate how it modifies the activity of BMPs and hepcidin expression in hepatic HepG2 cells. We initiated by using unfractionated heparin (UFH). Treatments for 16 h with different concentrations of UFH (from 0.4 to 400 µg/ml) caused a strong dose-dependent inhibition of hepcidin mRNA, represented in a logarithmic scale in figure 1A and in linear scale in the inset. Heparin at concentrations > 4 µg/ml reduced hepcidin level below 4% of the basal (Fig. 1A) and inhibited also the phosphorylation of SMAD1/5/8 induced by the BMPs (Fig. 1B). Then we tested other commercial and clinically used heparin preparations. The low molecular weight (LMWH) Enoxaparin was also inhibitory of hepcidin mRNA (Fig. 1A) and of pSMAD1/5/8 (not shown), but it was less potent than UFH and at the lowest concentrations tested (0.4-4.0 µg/ml) it was even stimulatory. The pentasaccharide Fondaparinux slightly repressed hepcidin expression and SMAD1/5/8 phosphorylation only at very high concentrations (>200 µg/ml) (Fig. 1A and B). Thus, all the heparins tested inhibited hepcidin expression and SMAD phosphorylation with a potency that increased with the length of the saccharidic chain. In time course experiments we found that a decrease of hepcidin mRNA was evident 30 min after addition of 200 µg/ml LMWH and continued linearly for 4 h (Fig. 1C). At 4 h we obtained maximum repression also with 4 and 40 µg/ml LMWH and with 0.4 and 4 µg/ml UFH (not shown). In other experiments we maintained the cells in 4 µg/ml LMWH for up to 7 days, hepcidin mRNA remained below 10% of the basal for the whole period (Supplemental S1), and this had no evident effect on cell proliferation and viability (not shown). These data showed that the inhibitory effect of heparin on hepcidin is strong, fast and may last for a long time.

\textit{Heparin strongly inhibits BMP6-mediated hepcidin induction.}

Heparin binds the BMPs, thus we hypothesised that hepcidin inhibition is caused by heparin sequestering the BMPs. To verify this, we analyzed how the heparins modified BMP6-induced hepcidin expression. BMP6 alone (16 h, 10 ng/ml) increased hepcidin mRNA level of 5-7 folds. The presence of UFH not only suppressed hepcidin induction by BMP6, but at high concentrations reduced hepcidin level to values below 5% of the basal, as in the absence of exogenous BMP6 (Fig. 2A). LMWH was also inhibitory, but was less potent than UFH and Fondaparinux was only marginally inhibitory (Fig. 2A). The strong similarity of the inhibition plots of the three heparins in the presence and in the absence of added BMP6 supports the hypothesis that they act by suppressing BMPs activity. This is further strengthened by the evidence that UFH inhibited BMP6-induced SMAD1/5/8 phosphorylation in a dose dependent way, parallel to hepcidin inhibition, while Fondaparinux had little or no effect on pSMAD1/5/8 and hepcidin (Fig. 2B).
Heparin inhibits IL-6-mediated hepcidin induction.

The inflammatory cytokine IL-6 is an activator of hepcidin expression. HepG2 cells treatment with IL-6 (4 h, 50 ng/ml) induced hepcidin mRNA of 2.5 fold, increased the phosphorylation of STAT3 but not that of SMAD1/5/8 (Fig. 3A). A parallel treatment with BMP6 (10 ng/ml) induced hepcidin (5-7-fold) and pSMAD1/5/8, but not pSTAT3, as expected (Fig. 3A). To study the effect of heparin, the cells were first incubated with LMWH (16 h, 200 μg/ml) to suppress hepcidin, then BMP6 or IL-6 were added and the cells were grown for other 6 h in the presence of heparin and of the stimuli. The presence of heparin blunted both the BMP6 and the IL-6-mediated induction of hepcidin mRNA (Fig. 3B). It suppressed pSMAD1/5/8 induction by BMP6, while did not affect the induction of pSTAT3 by IL-6 (Fig. 3A and B). Similar effects were obtained with lower concentrations LMWH (4-40 μg /ml, not shown) and with 4 μg /ml of UFH (Supplemental Fig. S2). Moreover, we also analyzed Id1 expression by real time RT-PCR, a gene that is tightly regulated by the BMP/SMAD pathway. It was induced 5 fold by BMP6 and strongly inhibited by UFH even in the presence of BMP6 (Fig S2). Interestingly, it was induced when BMP6 was added after washing UFH away. Thus, Id1 was modulated by heparin and BMP6 in parallel with hepcidin, as expected. Altogether the data confirm that heparin inhibits hepcidin expression by reducing the basal and BMP6-induced pSMAD1/5/8 signaling.

To verify if heparin affected soluble or membrane-bound molecules, the cells were first incubated with heparin for 16 h to suppress hepcidin and pSMAD1/5/8, then washed, added of BMP6 or IL-6 and incubated for 6 h in heparin-free medium. In this period hepcidin mRNA increased to about the 40% of the basal in the control cells without stimuli, while the addition of BMP6 produced an induction of pSMAD1/5/8 and of hepcidin mRNA comparable to that of the cells that were not treated with heparin (Fig. 3A and C). More interestingly, the response to IL-6 remained blunted, with a level of hepcidin mRNA non significantly different from the one of the cells not exposed to IL-6. In these cells the level of pSMAD1/5/8 did not increase, although STAT3 phosphorylation was stimulated as in the presence of heparin (Fig. 3B and C). These results support the hypothesis that the suppression of BMP signaling caused by heparin is due to the sequestering of BMPs, (mainly BMP6) since the response was fully activated by exogenous BMP6. The lack of BMP6 may explain why hepcidin and pSMAD remained repressed and hepcidin was only poorly induced by IL-6. In other experiments the cells were first induced with BMP6 (6 h, 10 ng/ml), and then added of UFH 4 μg/ml and incubated for other 16 h in the presence of BMP6. We observed an inhibition of hepcidin mRNA and of pSMAD1/5/8 similar to that in the absence of previous BMP6 stimulation (Fig. 3D).
**Heparin has a stronger inhibitory activity on BMP6 than on BMP2 signaling**

Heparin effect on BMP osteogenic activity varied with the experimental conditions and BMP type 31-33. Thus we studied the heparin effect on BMP2 and BMP6, which belong to different BMP subgroups 19 and strongly induce SMAD signaling and hepcidin in HepG2 cells. In these experiments BMP2 was more potent than BMP6 (Fig. 4), but we observed a remarkable batch to batch variability of the potency for either BMPs (not shown). The presence of 4 μg/ml UFH fully blocked the dose-dependent induction of hepcidin by BMP6, as expected. More interestingly, it reduced only to 30-50% the induction by BMP2 (Fig. 4). In other experiments we found that hepcidin stimulation by BMP2 (10 ng/ml) was only marginally inhibited by increasing concentrations of LMWH, and was reduced only ~10-fold by incubation with UFH (Supplemental S3). We concluded that heparin has a much more potent inhibitory effect on BMP6 than BMP2 in HepG2 cells.

**Heparin inhibits hepatic hepcidin expression in vivo.**

Next we asked if heparin has the capacity to modulate the expression of hepcidin also in vivo. In initial experiments the mice were subcutaneously injected daily with 50 mg/kg/d with UFH for 7 days and the liver analyzed. RT-PCR analysis showed a decrease of hepcidin1 mRNA, and also of Id1 mRNA, and blotting showed a reduction of pSMAD1/5/8 in liver homogenates (Fig. 5A). Thus heparin reduced liver hepcidin expression and SMAD signaling in vivo at concentrations higher than the pharmacological ones, without causing evident local hemorrhages. Similar results were obtained by treating the mice for 5 days with pharmacological concentration of UFH 5 mg/kg/d (not shown). In other experiments the mice were treated for 15 days with lower UFH doses (2 mg/kg/d), and then liver mRNA quantified by real time RT-PCR calibrated on the housekeeping HPRT1 mRNA. The level of hepcidin transcript was rather variable in the three control saline-treated animals, but it strongly decreased in the heparin treated mice (Fig. 5B). Heparin treatment caused also an increase in serum iron concentration and a large reduction of spleen iron concentration (Fig. 5B).

**Heparin reduces circulating hepcidin levels in treated patients.**

After the evidence that pharmacological concentrations of heparin reduced hepcidin level in mice, we analyzed hematological parameters and serum hepcidin level of five hospitalized patients who had to be treated with heparin to prevent deep vein thrombosis. All the patients had normal hematocrit values, signs of inflammation with high levels of C reactive protein and of serum
hepcidin, and low serum iron and transferrin saturation. They were treated for 4-5 days with usual pharmacological doses of LMWH. We observed a strong decrease of serum hepcidin, to 15-20% of the initial level, in all five patients after 2-5 days of treatment (Fig. 6A). Serum iron (Fig. 6B) and transferrin saturation (not shown) increased in all the patients analyzed. Also C reactive protein decreased drastically in all the subjects (Fig. 6C). The data suggest an increase in systemic iron availability during the treatment in all the patients analyzed.

Discussion.
We demonstrated that heparin inhibits hepcidin expression in hepatoma HepG2 cells in a dose-dependent manner. The effect is evident after 30 min, is complete after 4 h (Fig. 1C), and hepcidin mRNA can be maintained below 10% of basal level for a week or longer in the presence of heparin (Supplemental Fig. S1). We used three different commercial heparin types of clinical use: the unfractionated heparin (UFH, of 12-15 kDa) had an effect approximately 10 fold more potent than LMWH (of 4.5 kDa), and the smaller pentasaccharide fondaparinux (1.7 kDa) had little inhibitory activity (Fig 1, 2A and B). This size-dependent potency of heparin is not surprising and it has been already observed on the biological activity of growth factors, including fibroblast growth factors (FGFs) 41, vascular endothelial growth factors (VEGFs) and HIV-Tat 42. Noticeably, all these growth factors are inhibited by their direct interaction with heparin and its sequestration in the extracellular environment. By analogy we postulate that heparin binds BMPs to generate complexes that are unable to stimulate SMAD signaling and hepcidin expression. This is consistent with all our data, including the evidence that BMP signaling and hepcidin expression were fully restored by the addiction of BMP6 to cells pretreated with heparin (Fig. 3C). This demonstrates that heparin does not desensitize cells and does not act on membrane-associated BMP receptors, as indicated to occur in murine osteoblastic NC3T3 cells 34. Also the kinetics of inhibition (Fig. 1C) and of recovery of hepcidin expression (few hours) are compatible with heparin sequestering the endogenous BMPs, known to be expressed by HepG2 cells 7 and to have autocrine activity on hepcidin expression in hepatic cells 43. Interestingly, heparin did not affect the STAT3 signaling induced by IL-6, but still suppressed its effect on hepcidin regulation (Fig. 3). This is in full agreement with previous observations that the STAT3 signaling induced by IL-6 stimulates hepcidin expression only in the presence of a functional SMAD pathway15,18.

Heparin is a strongly acidic molecule that binds any positively charged molecule, but it exerts a biological activity only on molecules with high affinity binding, as it occurs with antithrombin. Thus, it was of interest to find that heparin had no effect on IL-6 signaling, and that it had a different effect on the two BMPs we tested. Heparin inhibition was more potent on the signaling
and hepcidin induction by BMP6 than that by BMP2 (Fig. 4), and this goes along with previous findings that BMP6 has a higher affinity binding to heparin (Kd of 6.3 nM) \(^{44}\) than BMP2 (Kd= 15-20 nM) \(^{45}\). The binding to BMP2 may be responsible of osteoporosis, a known side effect of prolonged heparin treatments described 40 years ago \(^{46}\), while the binding to BMP6 seems to affect systemic iron homeostasis. We found that low concentrations of LMWH stimulated rather than inhibit hepcidin expression in HepG2 cells (Fig. 1 and 2). These variable effects were already observed for the BMP osteogenic activity \(^{19,32,28,25,47}\) and are probably due to competition with endogenous heparan sulfates and/or binding to BMP antagonists. Further work will clarify the role of cellular heparan sulfates proteoglycans in recruiting BMPs to hepatic cells and the possible competition with exogenous heparin. Heparins and glycosaminoglycans bind a variety of factors in a specific manner that depends on fragment size and sequence. Thus it would be of interest to define the size and structure of the heparin fragments that have the highest inhibitory activity. We expect that such activity is linked to affinity binding to BMP6, the putative physiological regulator of hepcidin. It was shown that such affinity was reduced in the heparins that have been desulfated in position 2O and 6O, indicating that both sulfate types are involved in the binding \(^{44}\). Moreover, BMP6 did not bind octasaccharides obtained by fragmentation of heparin, indicating that it has affinity for longer fragments \(^{44}\) in agreement with the higher potency of UFH.

We show that daily heparin treatments cause a significant downregulation of liver hepcidin in mice, evident after 5 and 15 days treatment (Fig. 5). This response occurred with high (50 mg/kg/d) and with low pharmacological doses of UFH (2 mg/kg/d). For example the strong reduction of liver hepcidin mRNA shown in Figure 5B was obtained with treatments of UFH that correspond to 400 U/kg/d, which are in the range of ones used in clinical settings. These doses were sufficient to increase serum iron and to mobilize iron from splenic macrophages. Thus, heparin-induced reduction of hepcidin levels has the expected effects on systemic iron status. The inhibition of hepcidin is not attributable to heparin anticoagulant activity, however, we found a strong decrease of liver C reactive protein mRNA after the treatments with 5 mg/kg/d (about 50%, not shown), consistent with its known anti-inflammatory activity \(^{48}\), which might indirectly affect hepcidin expression. These initial findings stimulated the analysis of serum hepcidin levels in a small group of hospitalized patients who had to be treated with low doses of heparin to prevent deep vein thrombosis. We noted effects in the same direction of those observed in cellular and animal models, with a major decrease of serum hepcidin evident as soon as at day 1 or 2 and continuing for the short period of observation of 2-5 days (Fig. 6A). Serum iron (Fig. 6B) and transferrin saturation increased (not shown). The change in serum iron were so evident that we were surprised that it had not been reported before. Indeed, we found that Braunsteiner et al, in 1959 already
described the doubling of serum iron levels after short treatments with heparin. We observed also a reduction of C reactive protein level (Fig. 6C), as already reported to occur in patients. Serum ferritin marginally increased in 3 of the 4 subjects (not shown) and it did not seem to respond to the inflammatory status but rather to the iron status of the subjects. This initial study has inherent limitations, but it indicates unrecognized effects of heparin on iron homeostasis that may be beneficial. The inhibition of liver hepcidin and the increases in serum iron may be attributed both to the anti-inflammatory activity of heparin, which would reduce IL-6 expression, and to the anti-hepcidin activity we describe in the cells, which would reduce BMP6. They act in the same direction, but in different ways: the anti-inflammatory activity is attributed to heparin interfering with leukocytes extravasation, while the anti-hepcidin activity seems to occur at a molecular level by heparin sequestering the BMPs. Thus, the fast decrease in hepcidin levels and the trend of iron status improvement may be due to the specific anti-hepcidin activity, to a reduction of inflammation (but we did not measure IL-6), or to a combination of the two. Whatever the mechanism, this seems to be the first evidence that heparin has a direct effect on iron homeostasis, indicating novel therapeutic approaches for anemia.

The importance of hepcidin-targeted therapeutics has been recently reviewed and thought to have applications for various diseases, including chronic kidney diseases. The approaches under study include hepcidin agonists based on short peptides of the hepcidin N-terminal region, neutralizing anti-hepcidin antibodies, inhibitors of BMP type I receptors such as dorsomorphin, soluble hemojuvelin, which acts as an antagonist of BMP signaling, and erythropoiesis stimulating agents such as erythropoietin. Heparin adds to this list, and it has the unique advantage of being a drug that has been used in clinical setting for more than 70 years. Its toxicity (mainly thrombocytopenia) and its costs are extremely low compared to other drugs used for the treatment of inflammatory anemia, such as erythropoietin. Moreover, heparin has can be modified experimentally to improve its anti-hepcidin activity.

In conclusion, more accurate studies on a larger number of animals and for longer time are necessary to verify if and how heparin treats the anemia due to hepcidin overexpression, although some relevant data can be obtained from patients who have to be treated with heparin. Moreover, the evidence that heparin has a strong anti-hepcidin activity in vitro and in vivo stimulates the study and development of modified sulfated polysaccharides with low anti-coagulant and high anti-hepcidin activity. This should be feasible, since heparins with low anticoagulant activity were shown to have strong anti-inflammatory activity and novel biotechnological K5 polysaccharide derivatives are promising molecules. In addition, the effect of heparin suggests that heparan sulfated proteoglycans are deeply involved in the BMP signaling pathways that regulate hepcidin...
expression in hepatic cells. The role of these endogenous molecules on hepcidin regulation is still unexplored, and so is their activity on the various heparin binding molecules involved in the liver BMP signaling.

Acknowledgments
The work was partially supported by Euroiron1 grant 200-037296 and by Murst-Cofin-2006 to PA. We are particularly grateful to Prof. Marco Rusnati for reading the manuscript and helpful discussions and to Dr Laura Silvestri for helpful suggestions.

Authorship
M. Poli designed the research and analyzed data. P.A. and D.G wrote the paper. N.C and A. N. contributed vital new analysis. D.F, S. L. and F. M. performed research and analyzed data.

Conflict of Interest Disclosure
A patent application regarding the use of heparin for hepcidin inhibition has been submitted by the University of Brescia.

References


Legend to the figures

**Figure 1. Effect of heparin on hepcidin expression and BMP signaling in HepG2 cells.**

A: The cells were grown for 16 h in the absence of exogenous BMPs with the indicated concentrations of heparin: UFH, LMWH and Fondaparinux pentasaccharide, and the hepcidin mRNA levels quantified by real-time RT-PCR in relationship to HPRT1 mRNA. The data are expressed in a logarithmic scale, the inset shows the inhibition plot of UFH in a linear scale. Data are means and SD of three independent experiments in triplicate. B: western blotting of phosphorylated SMAD1/5/8, total SMAD5 and of β–actin as calibrator of the cell extracts after the incubation with UFH and with Fondaparinux. Data from two independent experiments in triplicate. C: cells were incubated for the indicated time with 200 μg/ml LMWH, and the level of hepcidin mRNA quantified by real-time RT-PCR. Representative of 4 independent experiments.

**Figure 2. Effects of heparin on BMP6-induced expression of hepcidin and signaling.**

A: The HepG2 cells were grown for 16 h in the presence of 10 ng/ml BMP6 and of the indicated heparin types and concentrations; the level of hepcidin was analyzed by real-time RT-PCR in relationship to HPRT1 mRNA and expressed in a logarithmic scale. Data are means and SD of three independent experiments in triplicate. B: western blotting of phosphorylated SMAD1/5/8, total SMAD5 and of β–actin as calibrator of the cells extracts before and after the incubation with UFH and with Fondaparinux. Representative of three independent experiments.

**Figure 3. Effect of heparin on hepcidin stimulation by BMP6 and IL-6.** Panel A: HepG2 cells were incubated for 6 h with 50 ng/ml IL-6 or 10 ng/ml BMP6, and then hepcidin mRNA level evaluated by qRT-PCR in relationship to HPRT1 mRNA. Panel B: as in panel A except that the cell were first incubated for 16 h with 200 μg/ml LMWH, and then added of the stimuli and grown in the presence of heparin for 6 h. Panel C: as in panel B, except that the cells were washed before addiction of the stimulating factors, and grown for 6 h in the absence of heparin. Panel D: The cells were grown for 6 h in the presence or absence of 10 ng/ml BMP6, then added UFH 4 μg/ml and grown for other 16 h. The schemes of the experiments are represented above the graphs, where stimuli is the incubation with IL-6 or BMP6, and heparin treatment is with 200 μg/ml LMWH (A-C) or with 4 μg/ml UFH (D). Bottom: western blotting of phosphorylated STAT3 (pSTAT3), of phosphorylated SMAD1/5/8 (pSMAD1/5/8), total SMAD5 and of β–actin as calibrator representative of each panel A, B, C and D respectively. The real time RT-PCR data are means and SD of two independent experiments in triplicate. The asterisks indicate significant difference.
(p<0.05) from the non-stimulated control. W.B. are representative of at least two independent experiments. The vertical solid lines separate lanes of the same electrophoresis experiment that were spliced out in the figure preparation to eliminate irrelevant or uninformative lanes.

**Figure 4: Heparin and BMP2 or BMP6.** HepG2 cells were grown for 16 h in the indicated concentrations of BMP2 or BMP6 in the presence or the absence of UFH 4 μg/ml, and then hepcidin mRNA was evaluated.

**Figure 5: In vivo effect of heparin in mice.** Panel A: mice were daily treated subcutaneously for 7 days with 50 mg/kg/d UF heparin and the level of hepatic hepcidin mRNA evaluated by RT-PCR, HRPT1 was used for calibration, the corresponding real time RT-PCR values were 2 and 2.7% of the control, for the mice treated with 50 mg/kg/d UFH. Lower: western blot of the liver homogenates for evaluation of phosphorylated SMAD1/5/8 and of total SMAD5. β-actin was used as load control. The real-time RT-PCR evaluation of Id1 mRNA in the treated mice is shown on the right. Panel B: Mice were treated subcutaneously for 15 days with the pharmacological concentration of 2 mg/kg/d UF heparin or with saline, and liver hepcidin mRNA: was evaluated by real-time RT-PCR of in relationship to HPRT1 mRNA. The animals were analysed for spleen iron and for serum iron concentration. The horizontal bars represent the mean values.

**Figure 6. Serum hepcidin and iron in patients under heparin treatment.** Panel A: time-course of serum hepcidin levels in 5 subjects during heparin therapy. Panel B: time-course of serum iron concentration in four subjects. Panel C: time-course of circulating C reactive protein concentration in four subjects. The data on CRP and serum iron were not available for one subject.
Figure 1

A. Hepcidin mRNA, fold change over basal.

B. Western blot analysis:
- pSMAD1/5/8
- SMAD5
- Actin

C. Hepcidin mRNA, % of basal.
Figure 2

Panel A: Graph showing the effect of heparin concentration on Hepcidin mRNA fold change over basal. The x-axis represents Heparin concentration (μg/ml) ranging from 0 to 400, and the y-axis represents Hepcidin mRNA fold change. Three different heparins are compared: Fondaparinux, LMWH, and UFH.

Panel B: Western blot analysis showing the expression levels of pSMAD1/5/8, SMAD5, and Actin with BMP6 10 ng/ml treatment. The left panel shows results with UFH, and the right panel shows results with Fondaparinux. The molecular weight markers are indicated:
- 60kDa
- 40kDa
Figure 3
Figure 4

Hepcidin mRNA, fold change over basal

BMP (ng/ml)

BMP2+UFH
BMP2
BMP6+UFH
BMP6

For personal use only.
Figure 5

A

RT-PCR

Hepcidin

HPRT1

-82 bp

-117 bp

W. Blotting

pSMAD1/5/8

SMAD5

Actin

Saline 50 50 mg/kg/d

UFH

Id1 mRNA / HPRT1

mRNA Id1 / HPRT1

Saline

UFH

B

Liver hepcidin

Spleen iron

Serum iron

mRNA hepcidin / HPRT1

Fe μg/mg wet weight

Serum Iron μg/dl

Saline

UFH

Saline

UFH

Saline

UFH

Figure 5
Figure 6
Heparin: a potent inhibitor of hepcidin expression in vitro and in vivo

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