# Structural Requirements of Heparin and Related Molecules to Exert a Multitude of Anti-Inflammatory Activities

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**Abstract:** Chronic inflammatory diseases are common and still remain a therapeutic challenge for both efficacy and safety reasons. Hence, novel therapeutics addressing these issues would for example improve treatment of severe diseases such as psoriasis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis. Inappropriate leukocyte homing to the affected compartments is a common feature of these diseases. Heparin and related polysaccharides have been shown to interfere with leukocyte homing through a variety of effects distinct from their anticoagulant properties. In this review, data on heparin as an anti-inflammatory agent are presented. In addition, structure-activity requirements for the anti-inflammatory properties of heparin are discussed, which should aid the drug development based on structurally modified heparin or other sulfated carbohydrates for treatment of inflammatory diseases.

**Key Words:** Inflammation, Anti-Inflammatory Agents, Heparin, Glycosaminoglycans, Receptors, Lymphocyte Homing, Chemokines, Angiogenesis, Complement.

## INTRODUCTION

While chronic inflammatory conditions - such as rheumatoid arthritis, inflammatory bowel disease or psoriasis - cause substantial physical and mental disabilities in many patients, treatment of these diseases remains a therapeutic challenge for efficacy as well as safety reasons [1-3]. Thus novel treatment options addressing these issues are required for improvement of therapy. One hallmark of inflammation - in general and in these diseases- is the infiltration of leukocytes into the affected tissue or organ. In order to gain access to the tissues, leukocytes have to leave the bloodstream, a process named leukocytes homing or extravasation [4,5]. On one hand, leukocyte extravasation is required for normal immune surveillance. On the other hand, inappropriate leukocyte homing to the affected compartments is a common feature of chronic inflammatory diseases, which therefore is targeted by most of the currently available therapeutic agents.

Heparin, the anticoagulant of choice for more than 60 years, exhibits a multitude of effects distinct from anticoagulation. It's influence on inflammatory responses has long been recognized [6,7]. On the molecular level, heparin's anti-inflammatory effects have been attributed to inhibition of leukocyte extravasation, inhibition of complement activation and reducing the activity of growth- and angiogenic factors [8,9]. The effects of heparin on inflammatory diseases evaluated in disease models, as well as in clinical trials are summarized in Table **1** (*in vivo* disease models) and Table **2** (clinical trials).

From this perspective heparin may be viewed as a multivalent, anti-inflammatory immuno-modulator. However, the application of heparin as an anti-inflammatory drug is limited by its strong anticoagulant activity and the resulting risk to induce bleeding. Another disadvantage of heparin is its animal origin, which is associated with the potential risk of contamination with pathogens. Finally, heparin consists of a complex and variable mixture of glycosaminoglycan (GAG) molecules leading to high batch to batch variability [10].

A suitable basis for the development of anti-inflammatory compounds similar to heparin, but without its disadvantages, is the knowledge of the structural requirements for the anti-inflammatory heparin activities. Hence, in this review we will present the findings on structural requirements for the different anti-inflammatory effects of heparin and related compounds. From the multitude of heparin's anti-inflammatory activities we will here focus on the effects of leukocyte extravasation, growth- and angiogenic factors, as well as inhibition of complement activation.

## LEUKOCYTE EXTRAVASATION

As stated above, unrestrained leukocyte recruitment from the blood stream into tissues is a main pathophysiologic feature of chronic inflammatory diseases. Leukocyte homing is initiated by an interaction of selectin adhesion molecules with appropriate carbohydrate moieties (e.g. sialyl Lewis X), leading to tethering and rolling of the leukocytes along postcapillary venules (Fig. **1A**). Tethering and rolling allows a close interaction of leukocytes and endothelial cells, both expressing/secreting cytokines. Once cytokines bind to their appropriate ligands, the targeted cell is activated, leading to a higher state of leukocyte integrin avidity, allowing the leukocytes to firmly adhere. The final step in leukocyte extravasation is migration into the tissue through the borders of endothelial cells. This process of transmigration is mediated by adhesion molecules expressed at the borders of endothelial cells, namely PECAM, VE-Cadherin, CD99 and the junctional adhesion molecules-A, -B and -C [4,11,12]. Heparin has been demonstrated to interfere with all steps of this

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**Table 1. Effect of Heparin in Animal Models of Inflammation. As Outlined in the Table, Treatment with Either UFH or Different LMWH Preparations have a Pronounced Effect in Several Animal Models of Inflammation. This Effect is Best Documented for Inhibition of Cutaneous Inflammation. However, as Indicated by Sakiniene and Tarkowski [110], the Immunosuppressive Effect of Heparin may Lead to Impairment of the Physiologic Host Response. Another Important Issue with Heparin Used to Interfere with Inflammation is Dosing, which can be Either Based on Effects on Coagulation (Unit Based), or on the Gravimetric Dose. The Later Seems in our Opinion the Better Choice, as the Anti-Inflammatory Effects are Distinct from the Anticoagulant Activity [26,27]**

| Experimental / study design | Treatment protocol | Main outcome | Reference |
|---|---|---|---|
| Examination of the impact of LWMH in a model of murine colitis | 500 U dalteparin per kg bodyweight daily vs. placebo | Significant reduction of clinical disease activity index and mucosal damage | [111] |
| Examination of the impact of LWMH in a model of murine colitis | 100 U or 200 U of UFH per kg bodyweight daily vs. placebo | No changes observed in severity of infiltration, which was scored histologically | [112] |
| Effect of UFH in a rat model of arthritis | s.c. treatment with UFH at a daily dose of 1, 20 or 40μg per mouse | 20μg prevented arthritis and was capable of reducing symptoms if treatment was initiated after manifestation of disease, lower (1μg) or higher (40μg) doses had no effect | [113] |
| Effect of UFH in a rat experimental autoimmune encephalomyelitis (EAE) | s.c. treatment using UFH at a daily dose of 1, 20 or 50μg per mouse | Prevention of EAE using 20 μg. Lower (1μg) or higher (50μg) doses had a much less pronounced effect. | [113] |
| Effect of UFH on thioglycollate-induced peritonitis in mice | Single i.v. dose of UFH at 0.5 or 1.25 mg/kg bodyweight | Significant reduction of neutrophil extravasation into the peritoneal cavity | [114] |
| Impact of UFH in a murine skin allograft rejection model | s.c. treatment using UFH at a daily dose of 1, 5 or 20μg per mouse | 5μg prolonged graft survival, the 1 and 20μg doses were less effective | [113] |
| Effect of UFH on cutaneous delayed type hypersensitivity (DTH) response | Single i.v. UFH treatment at 1mg per mouse 30 minutes after antigen challenge | Significant decrease in cutaneous inflammation measured as ear swelling response | [114] |
| Effect of LMWH on cutaneous DTH response in mice | Continuous treatment with dalteparin at 1mg/kg bodyweight initiated before sensitization | Significant reduction of DTH response, which could be due to an effect on sensitization- as well as effector-phase of DTH | [110] |
| Effect of UFH on generation of immunity | 5μg of UFH per mouse | Inhibition of generation of an immune response in a model of DTH | [115] |
| Effect of LMWH on IgE-dependent cutaneous reactions in mice | 5μg enoxaparin injected s.c. | Treatment lead to a significant reduction in mast cell induced ear swelling | [116] |
| Effect of LMWH on *S. aureus* induced arthritis in mice | Continuous treatment with dalteparin at 1mg/kg bodyweight initiated before i.v. injection of *S. aureus* | Increase in numbers of bacteria in the spleen and aggravation of arthritis | [110] |

**Table 2. Effect of Heparin in Clinical Trials of Inflammatory Diseases. In Line with the Observation from Animal Models, the Impact of Heparin Treatment in Colitis is Discussed Controversially. A Clear Benefit of Heparin Treatment has However been Established for Asthma and Allergic Rhinitis. In Addition, Two Reports also Shows Effectiveness of Heparin Treatment in Cutaneous Inflammatory Responses**

| Study design | Treatment protocol | Patients (n) | Main outcome | Reference |
|---|---|---|---|---|
| Randomized comparison of UFH with corticosteroids in inflammatory bowel disease | Full i.v. heparinization using UFH for 5 days followed by s.c. 10.000 U UFH daily for 3 weeks, followed by 5000 U UFH daily for 2 weeks<br>*vs*<br>200 mg hydrocortisone (HC) i.v. for 5 days, followed by an oral dose of 40mg HC, which was reduced by 5mg per week | 20 | Similar outcomes of both regimes with regard to clinical activity index, stool frequency, Endoscopic and histological grading | [117] |

**(Table 2. Contd….)**

| Study design | Treatment protocol | Patients (n) | Main outcome | Reference |
|---|---|---|---|---|
| Prospective, double-blind, randomized and placebo controlled evaluation of the effects of tinzaparin on mild to moderately active ulcerative colitis | Tinzaparin at 175 U/kg/day s.c for 14 days followed by 4.500 U tinzaprarin s.c per day for 28 days *vs* isotonic NaCl s.c. for 42 days | 100 | No change of colitis activity, stool frequency rectal bleeding or histology scores were observed between the two groups | [118] |
| Effect of LMWH on epicutaneous patch testing | Patients with confirmed positive epicutaneous test reactions were exposed to the same allergen after a single s.c. dose of enoxaparin (3mg) | 11 patients and 21 positive reactions | After s.c. enoxaparin: - 8 / 21 reactions negative - 4 / 21 reactions decreased | [119] |
| Effect of UFH on skin prick testing | UFH (25U/kg bodyweight i.v.) | 12 [120] plus 10 [121] | Signifiant decrease of skin test reactivity | [120,121] |
| Effect of UFH on methacholine induced bronchoconstriction (double-blind, placebo-controlled, crossover randomised trial) | Immediately after nebulized UFH (20.000 U) methacholine challenge was performed | 12 | Increase of methacholine induced PC20 value, but no protective effect in airway resistance | [120] |
| Effect of UFH on dust mite extract induced bronchospasm in patients with asthma and dust mite allergy (double-blind, placebo-controlled, crossover randomised trial) | Immediately after nebulized UFH (20.000 U) challenge with dust mite extract was performed | 15 | Significant inhibition of bronchospasm | [121] |
| Evaluation of nebulized UFH on exercise-induced asthma (single-blind, randomised, crossover trial) | Nebulized UFH at 1000 U/kg bodyweight prior to allergen challenge | 12 | Inhaled heparin prevents exercise-induced asthma | [122] |
| Effect of UFH on methacholine-induced bronchoconstriction (single-blind, crossover, randomised trial) | Nebulized UFH (1000 U/kg) prior to challenge | 13 | Heparin inhibits methacholine-induced bronchial hyperreactivity | [123] |
| Effect of UFH on nasal response in patients with allergic rhinitis (double-blind, placebo-controlled, crossover randomised trial) | 4 ml of inhaled intranasal UFH at 3750U/ml | 10 | Reduction in symptom scores, eosinophil count and eosinophilic cathionic protein in nasal lavage in UFH treated patients | [124] |

complex process of leukocyte extravasation (Fig. **1A**-**E**, Table **3**). The influence on heparin on leukocyte extravasation will be discussed in detail in the following paragraphs:

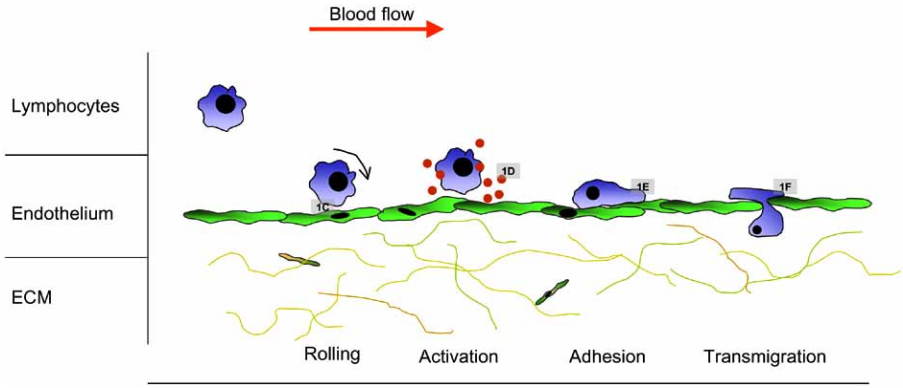
- Selectin inhibition,
- Chemokine inhibition
- Inhibition of leukocyte integrins and
- Inhibition of leukocyte transmigration

## SELECTIN INHIBITION

The selectin adhesion molecule family is comprised of P-, L- and E-selectin. Each selectin shows an individual expression profile and ligand binding (Fig. **1A** and **1B**). They are C-type lectins, which bind to carbohydrate moieties such as the tetrasaccharide sialyl Lewis X (sLex – Fig. **2**) as present e.g. in PSGL-1, the major receptor for P-selectin. But in the case of PSGL-1, the high avidity binding to the C-type lectin domain of P-selectin involves not only sLex containing O-glycans, but also tyrosine sulfate residues both located at its N-terminal tip [13,14]. Direct binding experiments with structurally defined oligosaccharide sequences have revealed, that modifications of the sLex sequence lead to changes in binding affinity to the individual selectin adhesion molecules [14].

Therefore, sLex, but not heparin (Fig. **3A** and **B**), was initially regarded as a lead structure for development of anti-adhesive drugs, as it has been identified as the minimal binding structure recognized by all three selectins. Furthermore, sLex [15] inhibits selectin binding and displays anti-inflam-matory activities in a number of animal models [16,17]. However, due to the low binding affinity and complex structure of sLex and structural variants, which hinders an economic synthesis, so far very few clinical trials with these compounds has been initiated [18,19,20,21]. Two recently published proof-of-concept exploratory trials demonstrate that bimosiamose, a synthetic sLex glycomimetic with potent inhibitory effects on all three selectins, improves the symptoms of asthma and psoriasis [22,23].

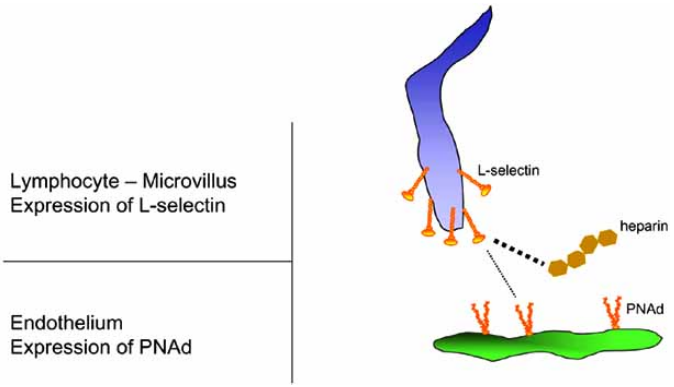
**1A**



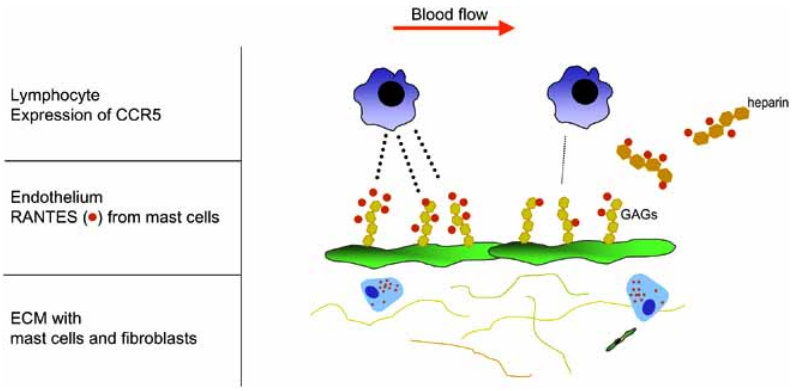

**1B**

| Lymphocyte | Targeted tissue | Endothelial adhesion molecule | Lymphocyte adhesion molecules *teathering/rolling – adhesion -- transmigation* |
|---|---|---|---|
| naive T-cells | Lymph nodes | PNAd<br>ICAMs<br>JAMs | L-selectin<br>LFA-1<br>JAMs / VLA-4 / Mac-1 |
| naive T-cells | Peyer's patches | MadCAM-1<br>MadCAM-1<br>ICAMs<br>JAMs | L-selectin<br>α4β7–integrin<br>LFA-1<br>JAMs / VLA-4 / Mac-1 |
| skin homing T-cells | Skin | P-selectin / PNAd<br>E-selectin<br>VCAM-1<br>ICAM-1<br>JAMs | PSGL-1<br>CLA<br>VLA-4<br>LFA-1<br>JAMs / VLA-4 / Mac-1 |

**1C**




**1D**



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**1E**

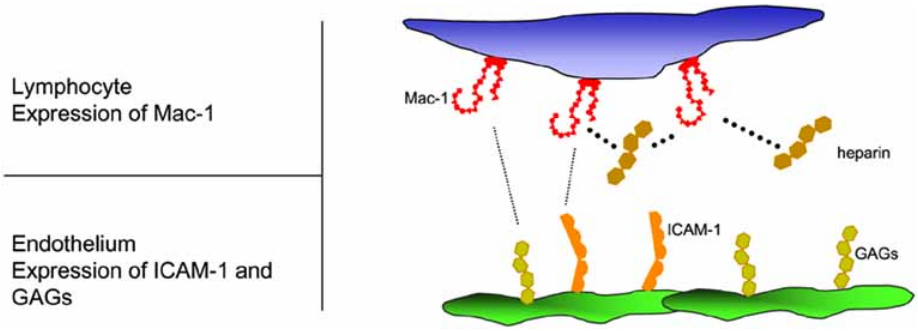


**Fig. (1). Heparin interferes with all steps of leukocyte extravasation.** Leukocyte extravasation, the process of leukocytes leaving the blood stream and entering tissues, is vital for normal immune surveillance. Therefore this process is a hallmark of the physiologic inflammatory response. On the contrary, inappropriate leukocyte homing is a common feature of chronic inflammatory diseases. Hence, targeting leukocyte extravasation is a good target for the treatment of chronic inflammatory diseases. **A** Overview of the steps involved in leukocyte transmigration. The initial contact leads teathering and rolling of leukocytes along postcapillary venules. These interactions are predominately mediated by selectins binding to a diversity of carbohydrate scaffolds. Rolling slows down the leukocytes and allows communication *via* chemokines. This crosstalk between leukocytes and endothelial cells may lead to activation of either cell type, leading for example a higher avidity state of leukocyte integrins, allowing firm adhesion. The final step of this cascade is transmigration in-between endothelial cells. This is the only step, which has so far not been proven to be impaired by heparin. **B** There is a large number of different adhesion molecules. Their respective involvement in the extravasation of leukocytes is highly distinct and depends both on the cell type (e.g. T-lymphocytes, B-lymphocytes, neutrophils, basophils, eosinophils, monocytes), the targeted tissue or organ, resp. (e.g. skin, intestinal tract, lung and airways, central nervous system, synovium) and the stage of the adhesion process. The table exemplary shows the specific adhesion molecules, which are expressed by T-lymphocytes and different targeted tissues to allow organ-specific homing. (for further details see corresponding reviews [126]. **C** Heparin binds to both L- and P-selectin, thus decreasing rolling interactions. Therefore leukocyte extravasation is inhibited at sites where these adhesion molecules are important, e.g. lymph nodes and skin. **D** Through inhibition of chemokine synthesis (not illustrated) and binding of soluble heparin to chemokines, heparin and related molecules inhibit the activity of many chemokines. Under physiological conditions (right part of image) GAGs expressed by endothelial cells are crucial for generation of chemokine gradients. **E** Mac-1, a leukocyte integrin, binds to a variety of endothelial ligands, including ICAM-1 and surface bound GAGs. These interactions allow leukocytes to firmly adhere to the endothelium. In contrast to surface bound GAGs, soluble heparin inhibits adhesion through competitive binding.

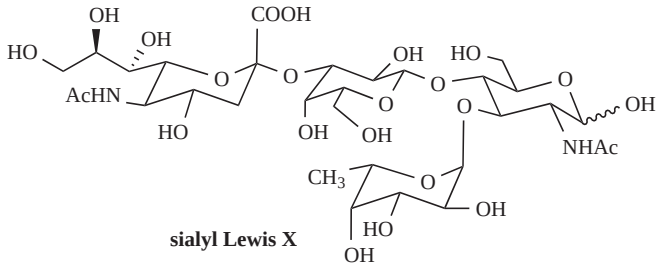


**Fig. (2). Structure of sialyl Lewis X (sLex).** sLex is the tetrasaccharide N-acetylneuraminic acid-α2→3-galactose-β1→4(fucose-α1→3)-N-acetylglucosamine. It thus consists of the trisaccharide Lewis X [Galβ1→4(Fucα1→3)GlcNAc], which is substituted on C3 of the galactose by a sialic acid residue. sLex is present in two O-glycans of PSGL-1. Together with tyrosine sulfate residues within the core protein, sLex is responsible for the interaction of PSGL-1 with P-selectin.

Heparin also binds to L- and P-selectin (Fig. **1C**), and is able to impair the P- and L-selectin functions. It shows a much higher affinity than sLex [21,24,25,26,27,28]. For example, in a competition ELISA, unfractionated heparin (UFH) inhibited the binding of P-selectin- and L-selectin-Ig fusion proteins to sLex with $IC_{50}$ of 0.4±0.3 μg/mL and 3.1± 0.5 μg/mL, resp. [21]. In addition, to a low molecular weight heparin (LMWH) with a molecular weight (MW) of 3,000Da, defined oligosaccharides generated were tested in the same competition ELISA. $IC_{50}$ values increased from 28±9 (L-selectin) and 105±20 (P-selectin) for LMWH to up to 1,000 (L-selectin) or undetectable inhibitory activity (P-selectin) when using a disaccharide. This reflects that the L- and P-selectin inhibitory capacity of heparin declines with decreasing chain length. In addition, comparing the L-selectin inhibitory capacity of a tetrasaccharide with a hexasulfated tetrasaccharide, the later has been shown to be almost fourfold more effective [21]. Hence, for L- and P-selectin inhibition, both molecular weight and degree of sulfation (DS, i.e. the number of sulfate groups per monosaccharide) were viewed important structural features of heparin and related glycosaminoglycans [21]. In line with these findings, a critical role of both MW [29], as well as negative charges in the form of sulfate- and carboxyl-groups [29,30,31,32] was confirmed thereafter.

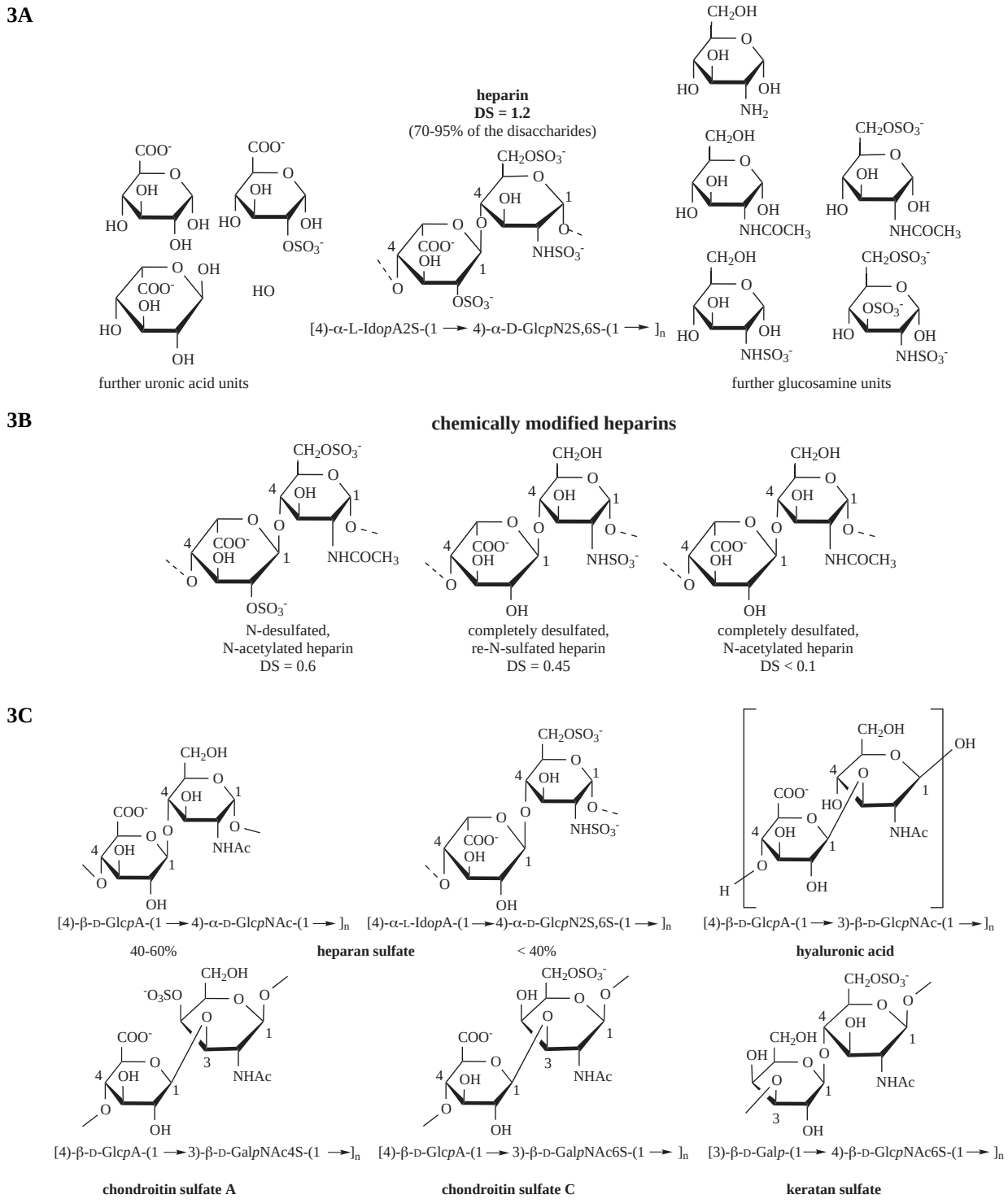


**Fig. (3). A. Main disaccharide unit of heparin and further monosaccharide units present in heparin.** Heparin molecules consist only to 70-95% of the main disaccharide and may contain variable amounts of further monosaccharides. Each heparin preparation therefore represents a complex mixture of different molecules. This structural complexity is even increased by the high polydispersity of heparin (i.e. MW distribution ranging from 5,000 to 40,000). Consequently, structure-activity relationships evaluated by using heparin fractions or chemically modified heparins represent rather rough estimates and do not allow conclusions on a molecular level. In contrast, the two prime examples, where details on the molecular interactions with heparin and heparan sulfate are known, are antithrombin and fibroblast growth factor [127,128] **B.** The shown three chemically modified heparins with reduced DS have often been used to investigate their effects on mechanisms being involved in inflammation. Partial or complete desulfation of heparin generally leads to strongly reduced anticoagulant activity, as hereby the specific antithrombin binding pentasaccharide unit is destroyed. On the contrary, other activities are not mandatory impaired by

**(Legend Fig. 3. contd….)**

partial desulfation, so that the overall activity profile may be shifted to the anti-inflammatory activity. Although sometimes suggested, these modified heparins give only limited information about the influence of the sulfation pattern (i.e. N- or C-sulfation). This is only possible, if the compared modified heparins have identical DS and MW. **C.** Characteristic disaccharide units of glycosaminoglycans (GAG) other than heparin. In general, they have a lower DS than heparin. From its biosynthesis, heparan sulfate is related to heparin. Hyaluronic acid is the only GAG without sulfate groups and consisting of real repeating units. Keratan sulfate is the only GAG without uronic acids.

In contrast to the binding of heparin to antithrombin (AT), its interactions with L- and P-selectins seem to be independent of a specific oligosaccharide sequence. Accordingly, also other sulfated polysaccharides like fucoidans were shown to interfere with selectin-mediated processes [33]. Using a series of chemically defined glucan sulfates (Fig. **4**), we characterized the influence of some structural parameters of sulfated polysaccharides on their L- and P-selectin blocking effects [34,35,36]. For critical inhibition of either selectin function a certain minimum chain length is of importance. Additional determinates are: (a) Type of the glycosidic linkage between the monosaccharides, with α-1,4/1,6 glucan sulfates displaying superior inhibitory capacity compared with β-1,3-linked polysaccharides. (b) DS, with non-sulfated and low-sulfated glucan sulfates being unable to inhibit P-selectin-mediated cell interactions, and a DS-dependent increase in inhibitory activity, reaching a maximum at about a DS of 2.0. (c) Comparison of the L- and P-selectin inhibitory capacity of 2,4-sulfated- with 6-sulfated- β-1,3 glucan sulfates revealed that position of the sulfate-group is another important structural requirement, with the 2,4-sulfated compounds being more potent inhibitors.

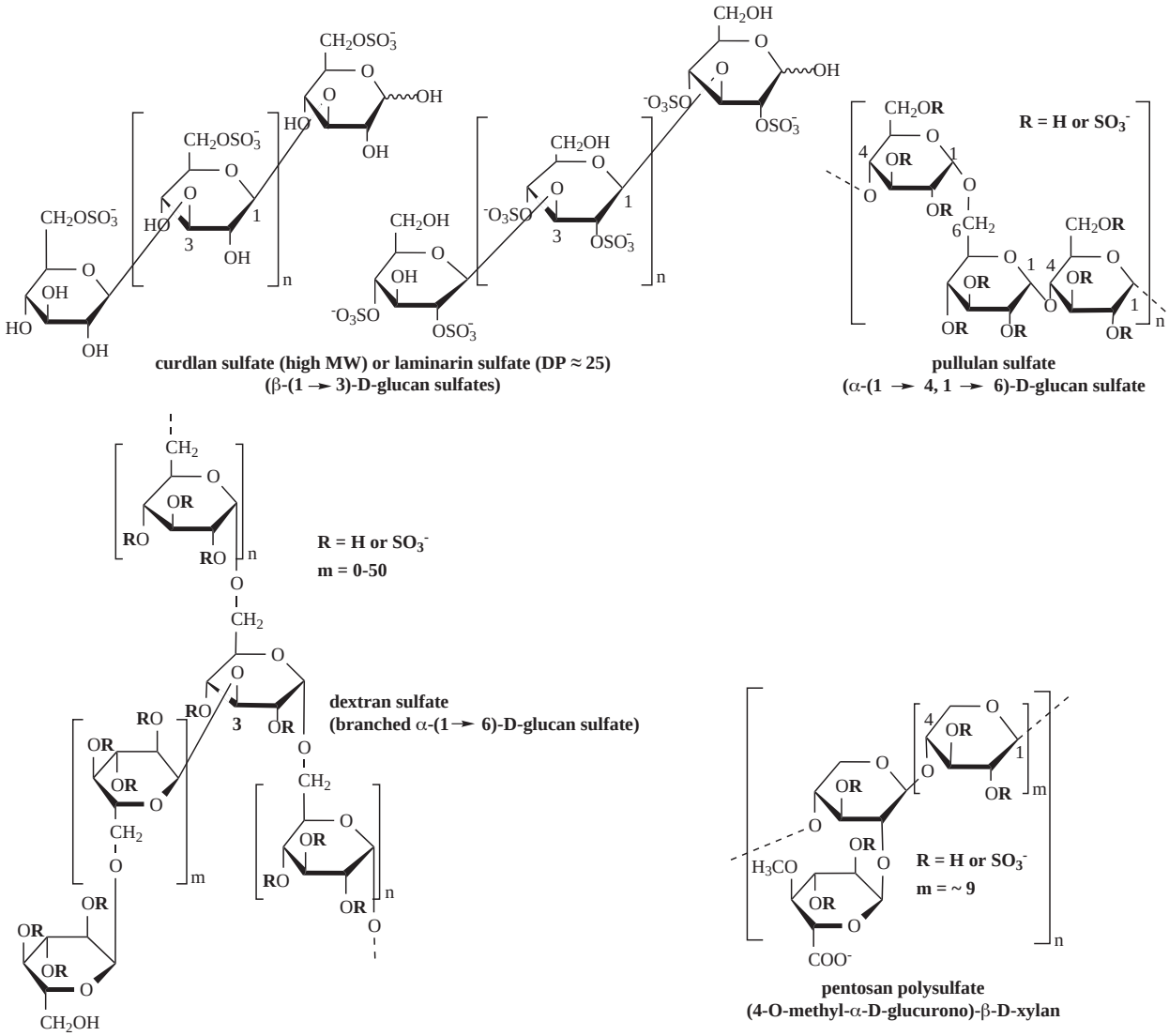


**Fig. (4). Exemplary semisynthetic sulfated polysaccharides.** In the case of β-1,3-glucan sulfates, the sulfation pattern has been specifically modified by introducing protection groups before the sulfation.

In summary, heparins and semi-synthetic oligo- and polysaccharides require both a critical MW and DS to bind to L- and P-selectin (Table **3**). In conjunction with MW and DS, additional parameters, like type of the carbohydrate backbone and sulfation pattern influence the binding to, and function of L- and P-selectin. Accordingly, the interaction of these compounds with L- and P-selectin is not only due to unspecific ionic interactions, but rather depends on the three-dimensional structure of the carbohydrates and the arrangement of the negative charges along the carbohydrate.

The lack of E-selectin inhibition is not considered a hindrance for treatment of chronic inflammatory diseases. The observation from a clinical trial investigating the effect of anti E-selectin antibody treatment in patients with moderate/severe psoriasis is in support of this concept: Despite the clearly documented role of E-selectin in mediating Th1 cell extravasation to the skin [37], and despite increased expression of this adhesion molecule in lesional psoriatic skin [38,39], the study failed to detect an effect of antibody mediated blockade of E-selectin in patients with psoriasis [40].

## CHEMOKINE INHIBITION

Chemokines, cytokines with chemoattractant activity, aid to selectively recruit leukocytes during inflammatory processes. As chemokines bind to glucosaminoglycans, interactions of cell surface bound heparan sulfate proteoglycans (Fig. **3C**) and chemokines drive the formation of haptotactic or immobilized gradients of chemokines at sites of inflammation [5,41,42]. On the contrary, soluble heparins and related molecules compete with the surface bound GAG for chemokines, and thus impair their proper presentation to the targeted cells (Fig. **1D**). This is supported by several reports, identifying heparin to inhibit leukocyte responses, such as chemotaxis, $Ca^{2+}$-release and elastase-secretion, initiated by a number of different chemokines [43,44,45]. On the molecular level, these heparin activities can be attributed to at least two activities: Inhibition of chemokine synthesis / release, as well as binding to the GAG-binding site of chemokines (Table **3**).

### Inhibition of Chemokine Synthesis

It is meanwhile well-known that GAG (Fig. **3C**) and proteoglycans play an important role in cell-matrix- and cell-cell-interactions and in the regulation of cell development and functions by interacting with membrane components or ligands and that they influence the functions and activities of many biomolecules. Correspondingly, it is possible to interfere with these processes by applying heparin or other sulfated polysaccharides, which compete with the endogenous GAG for their binding partners. Analogous to the physiological macromolecules, the effects by exogenous sulfated polysaccharides mostly increase with increasing MW (see below). However, the importance of the GAG is obviously not limited to the polysaccharides, but also degradation products, i.e. oligosaccharides, seem to exhibit regulatory functions [46,47,48]. This principally offers the option to

**Table 3. Summary of Heparin Actions on Leukocyte Extravasation**

| Inhibition of | Structure-activity relationships | References |
|---|---|---|
| P- and L-selectin (figure 1C) | Correlation of inhibitory activity with MW<br>Composition of glycosidic backbone<br>DS of approx. 1.8 optimal for inhibitory activity<br>Position of sulfate group | [21,29,34,35] |
| cytokine synthesis | Disaccharides are effective inhibitors of NF-κB<br>Tri-, but not mono-sulfated compounds are active | [46,48] |
| RANTES (figure 1D) | Correlation of inhibitory activity with MW, tetrasaccharides represent the smallest active polysaccharide to inhibit RANTES<br>DS more important than sulfation pattern | [41,50] |
| MCP-1 (figure 1D) | DS at the N- and O-terminus | [41] |
| IL-8 (figure 1D) | sulfation pattern more important than DS<br>Correlation of inhibitory activity with molecular weight; disaccharides are the smallest active compound. | [41,54,57] |
| MIP-1α (figure 1D) | DS at the N- and O-terminus; as well as N-acetylation | [41,60] |
| CXCR4 (figure 1E) | Disaccharides desensitize CXCR4<br>Tri-, but not mono-sulfated compounds are active | [46] |
| Mac-1 (figure 1E) | N-sulfation one, but not the only determinant for Mac-1 inhibitory activity | [61,64,63] |
| PECAM (CD31) | Contradictionary reports on the PECAM inhibitory activity of heparin and thus its influence on transmigration (figure 1A)<br>No structure-activity relationships investigated | [71]<br>[72]<br>[70] |

develop corresponding oligosaccharides, which either support or inhibit the functions of the physiological GAG.

As an example, Hecht and colleagues recently described a sulfated disaccharide, which inhibits NF-κB activation of anti-CD3 activated T-lymphocytes. As a consequence of NF-κB-inhibition activated T-lymphocytes showed a marked decrease in secretion if IFN-γ and TNF-α if treated with the disaccharide. However, only trisulfated, but not monosulfated disaccharides had this effect, indicating that the DS is an important structural feature for NF-κB inhibition of modified disaccharides [46]. This *in vitro* inhibitory effect of trisulfated disaccharides on cytokine release may explain their anti-inflammatory properties observed in T-cell-dependent, inflammatory animal disease models: Trisulfated, but not mono- or unsulfated, disaccharides inhibited cutaneous hypersensitivity reactions in mice [47,48], as well as joint swelling in a rat model of arthritis [48]. This observation is of particular interest, as these trisulfated disaccharides are generated by inflammation-induced degradation of the extracellular matrix. Thus, these compounds may act as naturally occurring regulators of immune function [47].

**Inhibition of Chemokines**

With regard to heparin binding to chemokines, a wide variation in heparin affinity among chemokines has been described. Eluting chemokines bound to a heparin-sepharose column showed, that the affinity of heparin to various chemokines decreases in the order RANTES > lymphotactin > IP-10 > MCP-3> IL-8> MCP-1> NAP-2 > MIP-1α [41]. While RANTES and the other chemokines evaluated are basic proteins, MIP-1α has an acidic isoelectric point. Its relatively weak affinity demonstrates that electrostatic forces represent an important perquisite, but not all determinants for the interaction between heparin and related molecules with GAG [41,49]. In support of a specific binding of heparin to chemokines, mutation studies of RANTES identified the BBXB motif as the principal site for heparin binding, which is different from other cytokines [49,50]. However, the interactions between heparin and the various chemokines are not only dependent on the structure of the chemokine, but also on that of heparin. Between the various chemokines, considerable differences were found regarding the influence of structural changes of heparin on its affinity to any chemokine.

A study investigating the binding of **RANTES** to CCR1 and CCR5 showed that RANTES had a higher affinity to CCR1. Heparin derived polysaccharides also bound to RANTES, and affinity increased with the chain length of the polysaccharides. This dependence of MW is also critical *in vivo*: RANTES-induced leukocyte recruitment to the peritoneal cavity was inhibited by tetra-, hexa- and octasaccharides, but not disaccharides. Again, the longer the chain length, the greater the inhibitory effect on leukocyte recruitment to the peritoneal cavity [50]. Next to MW, the DS is critically implied in binding of polysaccharides to RANTES. Compared to heparin (Fig. **3A**), partially desulfated heparins (DS = 0.45 – 0.60) (Fig. **3B**) displayed an about 300-700 times lower inhibitory capacity. Completely desulfated, N-acetylated heparin (Fig. **3B**) poorly bound RANTES, and hardly inhibited binding of RANTES to immobilized heparin or HUVEC. However, the sulfation pattern does scarcely affect the RANTES-inhibitory activity of heparin: The completely desulfated, re-N-sulfated heparin (DS = 0.45) was only 3 times less active than the N-desulfated, N-acetylated heparin (DS = 0.60), which could also be due to lower DS. In contrast, in the case of MCP-1, the desulfated, re-N-sulfated heparin (DS = 0.45) sulfation was 10-times less active than the N- desulfated N-acetylated heparin (DS = 0.60), suggesting a stronger dependence on sulfation pattern than on the overall DS [41]. The latter is supported by the observation that the binding of MCP-1 to heparin (DS = 1.2) is only 6 times more active than that to partially desulfated heparin (DS = 0.6)

Compared to RANTES, **MCP-1** shows a lower binding affinity to heparin [41]. Yet, similar to RANTES, luminal expressed heparin sulfates are believed to retain MCP-1, thus generating haptotactic chemokine gradients [51,52]. Binding of heparin and heparin like molecules to MCP-1 requires the presence of a specific GAG binding site on MCP-1, which has recently been identified [52]. While *in vitro* GAG-binding site deficient MCP-1 mutants have the same effect as the original protein, it's ability to induce cell migration *in vivo* is lost [52]. The DS at the N- and O-terminus has been reported to be a structural requirement for MCP-1 binding [41]. The essential binding of MCP-1 to cell surface GAG was underlined by the finding that the chemotactic response by MCP-1 was almost completely antagonized by addition of heparin [53].

**IL-8** also binds heparin [41]. Treatment of colitis in rats with the LMWH dalteparin leads to a significant improvement of colitis, which was accompanied with a reduced IL-8 serum concentration. The authors however did not observe an effect on TNF-α expression in the mucosa [54]. According to own *in vitro* and *in vivo* experiments, heparins are able to inhibit IL-8 induced PMN chemotaxis and calcium mobilization. In addition, this effect may be due to the observed reduction of calcium mobilization in GAG-treated, IL-8 stimulated cells [41]. In addition, heparin has been implied to enhance immune responses to IL-8, as heparin and heparan sulfate have been shown to increase calcium response and chemotaxis of neutrophils induced by IL-8 [55,56]. N- and O-sulfation [41], as well as DS at glucosamine residues (Fig. **3A**) [57] significantly contributed to the binding of IL-8 to heparin and other GAG. Despite the chain-length dependency for heparin binding to IL-8 has been established [41], MW seems to be of minor importance, as disaccharides bind IL-8 with high affinity [57,58]. In addition, heparin derived disaccharides are also capable to inhibit IL-8 (and IL-1β) secretion, presumably through interference at post-transcriptional stages [59].

Albeit a relatively weak binding affinity to **MIP-1α**, heparin also inhibits MIP-1α binding to HUVEC. In analogy to RANTES, $IC_{50}$ values decrease with increasing chain length of the polysaccharides. For MIP-1α these is however a maximum inhibition observed at 18-polymers, with no decrease in $IC_{50}$ values observed by further increase of the MW [41]. Again, similar to RANTES and IL-8, N- and O-sulfation were important determinants for the inhibitory activity of MIP-1α. In contrast to RANTES, where loss of N- and O-sulfation lead to a 2000-fold increase in $IC_{50}$ values, this effect was at least 10-fold less pronounced [41]. In addi-

tion to sulfation, N-acetylation is another important structural feature for inhibition of MIP-1α induced effects by heparin [60].

In addition to the described function-altering interactions between GAG and chemokines, specific disaccharides, generated by enzymatic degradation of heparin, have been shown to desensitize **CXCR4**, leading to inhibition of CXCL12 induced migration through fibronectin. Comparing trisulfated with monosulfated disaccharides (Fig. **3A**), This effect was only observed using the trisulfated disaccharides [46].

## INHIBITION OF LEUKOCYTE INTEGRINS

The interaction of leukocyte integrins with their endothelial ligands leads to firm adhesion of the cells (Fig. **1E**). Integrins are heterodimeric transmembrane proteins consisting of non-covalently associated α– and β– subunits. The human integrin family includes at least 18 α-subunits and eight known β-subunits. Some α-subunits contain an inserted I domain, which is a major ligand-binding site. Each α-subunit may interact with more than one β-subunit, resulting in 24 different heterodimers identified to date. The leukocyte integrin Mac-1 ($\alpha_M ß_2$) has been shown to bind to several cell surface and soluble ligands including ICAM-1, fibrinogen, iC3b and factor X. In addition, Mac-1 is also capable of binding heparin and heparan sulfate. *In vivo* the interaction of Mac-1 with heparin sustains Mac-1-depedent neutrophil adhesion [61]. This interaction is specific, as Mac-1 does neither interact with chondroitin sulfate, keratan sulfate, nor hyaluronic acid (Fig. **3C**) [61]. For example, heparan sulfate proteoglycans constitutively expressed on epithelial and endothelial cell, lacking ICAM-1 expression, function as receptors for Mac-1. Furthermore, heparin chains of serglycan, which is translocated from the granules to the cell surface after activation, are discussed to represent a counter-receptor on neutrophils that participates in Mac-1 dependent homotypic adhesion. From this data it can be concluded, that heparin sulfate and heparin moieties expressed by inflamed endothelial cells may complement other receptors such as ICAM-1 in the Mac-1-mediated neutrophil extravasation process.

The ability of heparin to bind to leukocyte Mac-1 was confirmed in subsequent studies [62,63,64]. In contrast to the work by Diamond *et al.* [61], the authors focused on the inhibitory capacity on Mac-1 dependent adhesion of soluble heparin. Soluble heparin was found to inhibit Mac-1 dependent binding to ICAM-1 [61,63,65,66], and to reduce TNF-α induced leukocyte rolling, adhesion and migration in rat mesenteric venules [64]. Structure-activity relationships of heparin binding to Mac-1 suggested an essential role of the sulfate groups. In contrast to chemokine-heparin interactions partially desulfated heparin, e.g. exclusively either N- or O-sulfated (Fig. **3B**), display nearly the same affinity as native heparin. This observation corresponds to the fact that the major natural ligand is heparan sulfate, which has a lower DS compared to heparin [61].

## INHIBITION OF LEUKOCYTE TRANSMIGRATION

The final step in the cascade leading to leukocyte extravasation is migration from the bloodstream to the tissue. While most data indicates that leukocytes leave the circulation at the borders of endothelial cells [67], one report suggests, that leukocytes can also pass through endothelial cells [68]. This process of transmigration is governed by mostly homophilic interactions of adhesion molecules expressed at the junctional zones and basolateral areas of endothelial cells and the transmigrating leukocytes. Platelet-endothelial cell adhesion molecule-1 (PECAM-1, CD31), a member of the immunoglobulin family, expressed by endothelial cells and leukocytes is one of the adhesion molecules regulating leukocyte transmigration [69]. There are however conflicting data on heparin binding to PECAM-1: While one report states that "PECAM-1 is not a heparin-binding protein" [70], two other groups demonstrate the opposite [71,72]. This discrepancy may result from different experimental conditions. In summary, this leads to the assumption that interference with transmigration may not represent an important component for heparin's anti-inflammatory activity.

## INHIBITION OF CELL GROWTH AND ANGIOGENIC FACTORS

Inflammatory processes, such as psoriasis and rheumatoid arthritis are also characterized by altered angiogenesis [73], regulated by a number of angiogenic growth factors, e.g. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF). In concert with other cytokines, these growth factors stimulate angiogenesis *via* interactions with their high affinity receptors on endothelial cells [74,75]. The impact of growth and angiogenic factors on inflammation has recently been shown by work from Xia and colleagues, who transgenically delivered VEGF into mouse skin, leading to a severe inflammatory condition resembling psoriasis [76]. Hence, targeting growth and angiogenic factors seems a valid approach for development of novel anti-inflammatory substances. The angiogenic growth factors bind to heparan sulfate proteoglycans that are present on the endothelial cell surface and extracellular matrix (ECM). Soluble heparins compete with these heparan sulfates for the binding of these growth factors, and may cause release of these proteins from the ECM. Indeed in man, therapeutic doses of UFH lead to an increase in plasma levels of growth factors such as bFGF [77]. Another important role of heparin sulfate proteoglycans is their function as low-affinity receptors for angiogenic growth factors. The initial binding to heparin sulfates promotes the optimal interaction of the growth factors with their high affinity receptors, leading to optimal presentation [78]. In the presence of soluble heparin, opposite effects can be observed, which strongly depending on the heparin chain length. This is an unique phenomenon, as in the most other cases of heparin-biomolecule interactions, the respective effect more or less progressively improves with increasing chain length, but does not change into the opposite one. Exemplary, some of the effects of heparin on growth and angiogenic factors are summarized in Table **4**.

In the case of **VEGF**, the chain length of heparin is the major determinant whether the activity of VEGF is inhibited or promoted. Heparin fragments of less than 18 saccharide residues reduce VEGF activity, while fragments larger than 22 sugar units as well as native heparin and heparan sulfate potentiate the binding of VEGF to its receptor [79]. It is suggested that heparin modulates the binding of VEGF to the

**Table 4. Summary of Heparin Action on Growth and Angiogenic Factors**

| Inhibition of | Structural requirements | References |
|---|---|---|
| VEGF | Heparin fragments with a DP<18 inhibit the binding of VEGF to its high-affinity receptor<br>Heparin chains with a DP>22 potentate this binding<br>DS controversially discussed | [79,80] |
| bFGF | UFH promotes bFGF-induced dimerisation of bFGF receptor<br>Heparan sulfate promotes bFGF-induced dimerisation of bFGF receptor<br>LMWH impairs bFGF-induced dimerisation of bFGF receptor<br>Heparin fragments with a DP < 10 inhibit bFGF | [81,125] |
| PDGF | Different PDGF isoforms display distinct binding affinities towards heparin<br>No structure-activity relationships investigated | [85,86] |

VEGF receptors by interaction with cell surface heparin binding molecules. This bridging function requires a certain minimum chain length. On the contrary, the DS only modulates the potentiation: Over-sulfated heparin turned out to be a better potentiator of VEGF binding than native heparin, whereas O-desulfated and N-desulfated heparin (Fig. **3B**) were weaker potentiators [79]. Based on the knowledge that only small heparin fragments exhibit inhibitory activity, Pisano *et al.* recently developed an interesting heparin derivative with negligible anticoagulant activity, but potent inhibitory activity on VEGF-induced neovascularization in the chick embryo chorioallantoic membrane. [80]. They introduced regular sulfation gaps along the prevalently N-sulfated regions of native heparin, followed by glycol-splitting of all nonsulfated uronic acid residues and thus destroying the monosaccharide ring structure. The resulting undersulfated glycol-split heparin was then degraded to a MW of 5.800.

The role of heparan sulfate and heparin in the binding of **bFGF** to its receptors has been investigated in great detail. Activation of the high-affinity bFGF receptor (bFGFR) on endothelial cells requires its dimerization. This bridging of two receptors involves the binding of both FGF and heparan sulfate [81]. The assembly of two bFGF, two bFGFR and two heparan sulfate chains comprises the active signal transduction complex that results in cell replication. In analogy to VEGF, LMWH as well as heparin fragments with a degree of polymerisation (DP) < 10, but not UFH, inhibit the activation of bFGFR by preventing its dimerization [82].

**PDGF**, which is released from the α-granules of platelets, is involved in the immune response for several activities: PDGF is chemotactic for fibroblasts, vascular smooth muscle cells and monocytes. In addition, PDGF primes eosinophils to produce superoxide anion [83]. Heparin has long been recognized to inhibit PDGF-induced cell proliferation [84] by binding to PDGF [85]. According to recent findings, different isoforms of PDGF display distinct binding affinities toward heparin [86]. However, there is still no data are available on structural requirements on heparin inhibiting PDGF-mediated effects.

## INHIBITION OF COMPLEMENT

The complement system is part of the innate immune system and comprises a family of at least 20 plasma and membrane proteins that react in a regulated cascade [87]. Its major functions are recognition of non-self material, non-specific host defense and mediation of inflammation. Its activation, occurring either antibody-dependent *via* the classical pathway or antibody-independent *via* the lectin and the alternative pathway, leads to opsonization and phagocytosis, chemotaxis of neutrophils, release of inflammatory mediators by mast cells and basophils and cell lysis.

Already in 1929, Ecker and Gross demonstrated the capacity of heparin to interfere with complement activation [88]. Numerous subsequent studies indicated that heparin and structurally similar GAG and oligosaccharides regulate multiple steps in the complement cascade. The most important effects of heparin on the complement system are the following [89,90] (a) Inhibition of the first step of the classical pathway activation by binding to C1q, and preventing the formation of the enzymatically active C1 complex. (b) Inhibition of the formation of the C3 convertase of the classical pathway, i.e. C4bC2a, by non-competitively inhibiting the cleavage of C2 and C4 by C1s. (c) Increasing the inhibitory activity of the C1 esterase inhibitor (C1INH) on C1 complex by bridging the active C1 complex with C1INH (d) Inhibition of the formation of the C3 convertase of the alternative pathway, i.e. C3bBb, by impairing the interaction between factor B and C3b. (e) Inhibition of the terminal cell lysis by interfering with incorporation of terminal components into the membrane attack complex (MAC), i.e. C5bC6C7C8).

But the complex interrelationships between complement and heparin and their relevance *in vivo* are not yet completely understood [90]. The same is true for the structure-activity relationships of heparin regarding its effects on the complement system. Although studies indicate the importance of O-sulfation and a minimum chain length, specific binding sites for the various complement factors as for AT have not yet been identified [90].

Concerning the MW-dependency of anti-complementary activity of heparin several studies have been performed: Ekre [91,92] found a MW-independent (MW range 4,800-17,000 Da) inhibition of both human complement-induced hemolysis, which has been confirmed in experimental inflammation in human skin [91,92]. Corresponding to this, Sharath *et al.* [93] reports that heparin fragments < 1000 Da the fragments were essentially inactive and those > 3500 Da have the same activity on a weight basis as UFH (mean MW about 13,000 Da). Whereas these results were observed in global hemoly-

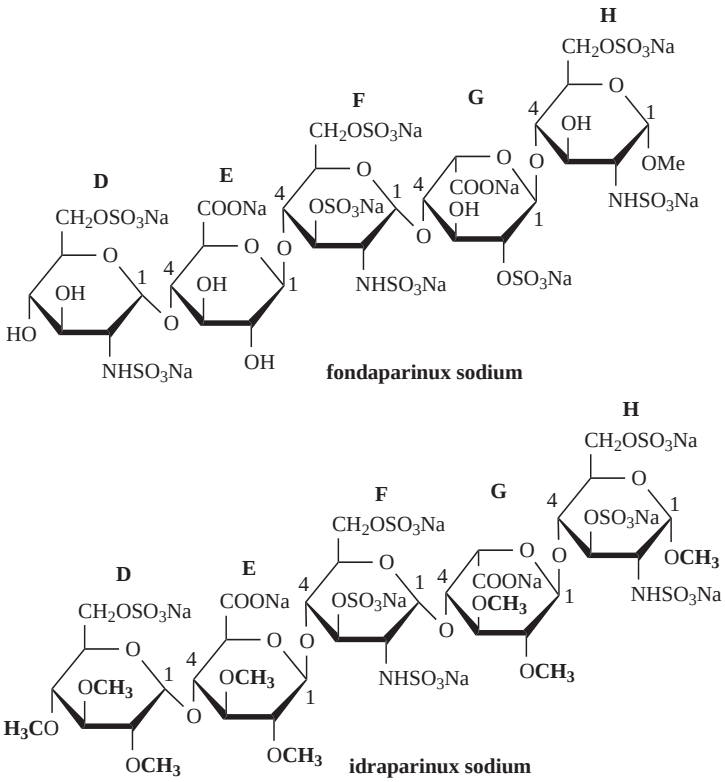


**Fig. (5).** The defined chemical structure of the two synthetically produced antithrombin binding pentasaccharides fondaparinux sodium and idraparinux sodium. Fondaparinux corresponds to the original pentasaccharide sequence in heparin, whereas idraparinux is a N-free, partially methylated analogue.

sis assays, Linhardt *et al*. [90] examined the effect of heparin and heparin-oligosaccharides (OS) (DP 2-16) on a more specific part of the complement cascade, i.e. the alternative amplification pathway of complement, i.e. C3 convertase generation. In principle, the revealed correlation between MW and inhibitory activity confirmed the earlier studies: Short oligosaccharides with DP 2-6 (max. ~1,900 Da) exhibited only marginal effects, OS with DP 6-12 (1,900-3,900 Da) showed a strong MW-dependent activity increase, whereas OS with DP 12-16 (3,900-5,300 Da) demonstrated up to 136% of the activity of an UFH (14,000 Da) on weight basis and up to 54% on molar basis in inhibiting the alternative amplification pathway of complement. These results demonstrate that the apparent MW-independence is incorrect and that the inhibitory effect of a heparin molecule on the alternative complement activation increases with its chain length.

Another structural requirement for anti-complementary activity is the presence of sulfate groups. As demonstrated with tetrasaccharides, the higher the DS the higher is the anti-complementary activity [93]. In contrast to the anticoagulant activity, N-sulfation is, however, not necessary [94,95,96]. Moreover, also the presence of carboxyl groups is not required for anti-complementary activity of heparin derivatives [95], which is also confirmed by semi-synthetic sulfated polysaccharides like dextran sulfates, pentosan polysulfate and β-1,3-glucan sulfates (Fig. **4**) [36]. Finally, several studies showed that non-anticoagulant heparins or OS exhibit pronounced anticomplementary activity and consequently proved the complete separation of these both heparin activities with regard to the respective structural requirements [90,92,94-96].

Despite the limited knowledge about the relevant mechanisms and the structure-activity relationships, there is evidence that heparin-coated membranes and other medical devices have enhanced biocompatibility, i.e. a reduced risk to induce excessive inflammatory reactions [97]. Moreover, its anticomplementary activity may contribute to its overall benefit in situations like cardiovascular surgery or stent implantation.

## ADDITIONAL EFFECTS OF HEPARIN ON IMMUNE RESPONSES

In addition to the above described numerous mechanisms of actions, heparin exhibits additional activities contributing to its overall anti-inflammatory activity. These include (a) inhibition of chemotaxis induced by other chemoattractants than chemokines e.g. the complement fragment C5a, the bacterial formylpeptide fMLP, platelet factor 4 (PF4) and thrombin, (b) inhibition of enzymes like elastase, cathepsin G, hyaluronidase and heparanase, as well as (c) inhibition of the respiratory burst of neutrophils.

Like activities of heparin described above, all these further effects are independent on the presence of the AT-binding site. A general requirement is the presence of sulfate groups enabling electrostatic interactions with the target protein. As at a given DS a longer chain offers more negative charges and thus potential points of interaction, the activity

mostly increases with increasing MW. The identification of the AT-binding site and the minimal binding sequence to bFGF [98] encourage to look for further specific binding sequences, which might serve as lead structures for future development of carbohydrate-based drugs.

Finally, its anticoagulant activity disposes heparin to interfere also with inflammatory processes, since there are manifold mechanistic connections between inflammation and coagulation [99,100]. For example, the anticoagulant activity of heparin is mainly to due its AT-mediated inhibition of factor Xa and thrombin, which are both also known to exhibit proinflammatory activity. The Factor Xa and thrombin inhibition are strongly dependent on the presence of the specific AT-binding pentasaccharide unit as proven by the clinically used antithrombotic fondaparinux, a synthetic analogue of the AT-binding site of heparin [101]. Furthermore, heparin mobilizes tissue factor pathway inhibitor (TFPI) from the vessel wall and in dependence on its MW is in favorite of the circulating of the active free form of TFPI [102]. This activity of heparin is independent of the AT-binding site and the *in vivo* comparison of various heparins showed that there is no correlation between the *ex vivo* anti-factor Xa activity and the TFPI-releasing potency [103]. TFPI is the endogenous inhibitor of tissue factor (TF), which is not only triggers coagulation, but also inflammation. TF has strong pro-inflammatory properties mediated predominantly by monocytes and their release of chemokines [104].

## PERSPECTIVE

In summary, for most of the mechanisms contributing to the anti-inflammatory activity of heparin and related polysaccharides, the presence of sulfate groups is essential and a certain minimum chain length is required. Depending on the target, several structural parameters more or less modulate the activity. Besides the chain length and the charge densitiy, these include the sufation pattern, i.e. the distribution of the sulfate groups along the molecule and within the monosaccharides. Studies with sulfated polysaccharides different from heparin demonstrate that most of the effects are not limited on the heparin structure. Further, as shown for the binding to L- and P-selectin, also the type of glycosidic linkage between the monosaccharide units influences the activities of sulfated polysaccharides. But it has to be critically remarked that despite the multitude of studies with heparin and heparin derivatives, clear structure-activity relationships on a molecular level, as known for the AT-pentasaccharide interaction, are still limited. Nevertheless, certain heparin preparations and related polysaccharides, often called heparinoids, with reduced anticoagulant activities have proven therapeutic efficacy in inflammatory disease models [30,31, 35,50,54,64]. Hence, structurally modified heparins and (semi-) synthetic analogues offer interesting options for the development of novel anti-inflammatory therapeutics.

However, successful strategies will probably not be based on the animal-derived heparin for several reasons: In addition to the already mentioned variability of its heterogeneous composition the natural resources are limited and the officially applied precautionary principle demands to replace animal-derived drugs by alternatives wherever possible [93]. In principle, there are two options for further carbohydrate-based drug development: Sulfated polysaccharides or sulfated oligosaccharides. As proven by fondaparinux, the latter may offer the option to create specifically acting agents being optimal for respective forms of inflammatory diseases. In contrast, a polysaccharide might be structurally optimized regarding one target (e.g. P-selectin, FGF, RANTES), but it will generally exhibit additional activities as recently reviewed in this journal [105]. In our opinion, such a multivalent mode of action is however not a disadvantage, but corresponds to the generally applied combination therapy in many diseases to utilize additive effects and to reduce the side effects.

It is in our opinion however not necessary to search for the "best" anti-inflammatory polysaccharide. Ideally, one should have a variety of compounds at hand, which exhibit distinct activity profiles for treatment of different inflammatory diseases. As similar molecules, e.g. RANTES, P-selectin, growth- and angiogenic factors are also implied in cancer metastasis [106,107,108,109], optimized polysaccharides may be beneficial for cancer patients. This is underlined by numerous clinical trials using heparin in cancer patients, showing a prolonged survival independent of the antithrombotic effect. Hence, in the near future, more effective heparin-related substances will possibly become available for clinicians to be used in a spectrum of difficult to treat diseases such as cancer and chronic inflammation.

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