JPP Journal of Pharmacy and Pharmacology

2003, 55: 697–706

Received October 29, 2002
Accepted February 11, 2003
DOI 10.1211/0022357021107
ISSN 0022-3573

Selectin-blocking semisynthetic sulfated polysaccharides as promising anti-inflammatory agents

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Abstract

Selectin-induced leucocytes rolling along the endothelial surface of blood vessels initiate a complex adhesion cascade, which is an essential step in the cellular immune response. Consequently, blocking the binding between the selectins and their ligands represents a promising strategy for suppressing pathological inflammatory reactions. This study describes the effects of an unfractionated heparin and a low-molecular-weight heparin and a series of structurally well-defined semisynthetic glucan sulfates on selectin-mediated cell-rolling with respect to inhibition. To simulate the blood flow characteristics of postcapillary venules, the rolling experiments were performed in a dynamic-flow-chamber system with immobilized selectins and selectin ligand-carrying U937 cells. The influence of the test compounds on cell rolling was measured by the percentage of adherent cells after a certain flow time and the velocity of the rolling cells. Whereas the test compounds displayed no inhibitory effect on E-selectin-mediated cell rolling, they efficiently blocked the rolling induced by P-selectin. The glucan sulfates were much more active than either unfractionated heparin or low-molecular-weight heparin, or the standard inhibitor Sialyl Lewis^X. Their inhibitory potency turned out to be strongly dependent on various structural parameters, such as sulfation pattern and molecular weight. In conclusion, the semisysntheticglucan sulfates represent promising candidates in the development of selectin blocking agents.

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Acknowledgements and funding: This work was supported by the Deutsche Forschungsgesellschaft (Sonderforschungsbereich 197) and Goemar Laboratories (St Malo, France). We are grateful to Dr Christian Gege and Prof. Dr Richard R. Schmidt, from the University Konstanz, for supplying the sLex-based glycolipids. Many thanks go to U. Goder for the excellent technical assistance.

Introduction

The receptor-mediated recruitment of leucocytes to sites of inflammation is essential for the development of an appropriate immune response. The leucocyte adhesion and emigration into the local tissue is a multi-step process triggered by the tethering of cells to the vessel wall in the vascular shear flow. This is followed by rolling along the endothelium, the development of a firm adhesion, and, finally, by diapedesis (Springer 1995). The selectins, a family of three adhesion molecules present on the surface of both endothelial cells and leucocytes, support tethering and rolling by rapid association and dissociation with carbohydrate ligands (McEver 1997). All members of the family are transmembrane glycoproteins and share a highly conserved N-terminal Ca^{++}-dependent (C-type) lectin domain (Vestweber & Blanks 1999). The minimal structures recognized by all selectins are the tetrasaccharide Sialyl Lewis^X (sLex) and its stereoisomers (Phillips et al 1990).

Besides their role in the cellular immune response, selectins are also involved in pathological inflammatory events (Gonzalez-Amaro et al 1998), such as rheumatoid arthritis, ischaemia and reperfusion injury. Furthermore, selectins are important in angiogenesis and cancer metastasis (Krause & Turner 1999). It is therefore of great pharmacological interest to inhibit these processes by antagonizing the binding of the aforementioned saccharides to the selectins. Soluble sLex and its isomers are able to prevent the adhesive function of selectins and display certain anti-inflammatory activity in various models of selectin-dependent inflammation (Tojo et al 1996; Norman et al 1998). However, their very low affinity (K_d 0.1–5.0 mM) (Cooke et al 1994; Jacob et al 1995), hydrolytical cleavage of the glycosides in the bloodstream and the lack of inexpensive and convenient synthesis contradicts their use as potential drug candidates. Due to the proven therapeutic benefit of structures that are capable of mimicking sLex

in the adhesion process, much effort has been made to develop simpler analogues (Simanek et al 1998). However, despite improved characteristics, the above-mentioned drawbacks of sLex could not be eliminated completely by these derivatives, which therefore impeded progression to advanced clinical trials. Further insight into the molecular binding requirements was gained by analysing the crystal structure of a complex of P-selectin with its natural ligand PSGL-1 (Somers et al 2000). These studies revealed that electrostatic forces predominantly contribute to the binding. This led us to search for new structural classes of potential selectin inhibitors, which are easily accessible, are based on carbohydrates similar to the natural ligands and contain charged residues with the ability for strong electrostatic interactions. We therefore focus our search on sulfated polysaccharides such as heparin.

Heparin belongs to the group of vertebrate glycosaminoglycans and is a complex, highly sulfated polysaccharide with a molecular weight between 3000 and 30 000. The linear molecules consist of alternating uronic acid and glucosamine residues. Heparin has been used as an antithrombotic drug for more than 60 years, but it also displays many other biological activities (Linhardt & Toida 1997). A few studies report on selectin binding properties of heparin. According to Skinner et al (1989), heparin, as well as other sulfated glycans such as fucoidin or dextran sulfate, binds to P-selectin. They postulated that the molecular binding mechanism of sulfated polysaccharides is analogous to that of the natural P-selectin ligand PSGL-1 (Skinner et al 1991). Other groups demonstrated the ability of heparins to bind to L-selectin (Norgard-Sumnicht et al 1993). Initial approaches show that the antithrombotic and selectin-binding properties of heparins differ in their structural requirements (Koenig et al 1998, Xie et al 2000). Further reports on the anti-inflammatory activity of heparins in in-vivo models (Yanaka et al 1996; Libersan et al 1998) are partially conflicting, which might be due to the structural variability of these tested compounds of natural origin. Despite the promising findings of heparins acting as potential selectin inhibitors, detailed structure–activity relationships could not be established. A recent study focused on the influence of sulfation of heparin on its selectin-binding and anti-inflammatory activity in-vivo (Wang et al 2002), in which a correlation between both selectin binding and anti-inflammatory activity and the degree of sulfation was found.

The aim of this study was to obtain further information about structural requirements on sulfated polysaccharides for selectin binding activity, which contribute to the anti-inflammatory efficacy of this substance class (Wang et al 2002). In a previous study we introduced a dynamic-flow-chamber system to investigate the molecular characteristics of the selectin-induced cell rolling (Vogel et al 1998; Bakowsky et al 2002). In a further study, we used this dynamic system as an inhibition assay and could show that the simulation of the physiological shear force conditions of the bloodstream is important for the search of selectin inhibitors (Schumacher et al 2002). The inhibitory potency of test compounds was manifested in a reduced number of rolling cells and in an increase of the rolling velocity and turned out to differ significantly under static and dynamic conditions. This is supposed to be due to the changing dominance of different binding forces, putting special emphasis on electrostatic interactions.

Since natural glycosaminoglycans like heparin generally represent poorly defined, complex, polydisperse molecule mixtures (Alban 1997), a series of structurally defined, semisynthetic sulfated polysaccharides was included in the study beside the heparins. Since the expression of both endothelial selectins (E- and P-selectin) is spatially related to inflammation, their inhibition would be most relevant to anti-inflammatory therapy. Therefore, we investigated the capability of five new heparinoids compared with two commercial heparins to block the binding to E- and P-selectin in a parallel-plate flow-chamber system. Results were discussed with respect to the correlation of structural characteristics and inhibitory capacity.

Materials and Methods

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Sigma (Deisenhofen, Germany). 1-Palmitoyl-2-{12-[(7-nitro-2-1,3-benzo xadiazol-4-yl)amino] dodecanoyl}-*sn*-glycero-3-phosphocholine (NBD-PC) was purchased from Avanti Polar Lipids (Alabaster, AL). The free sLex and the sLex-based glycolipid, as well as the fluorescent-labelled NBD-sLex lipid, were synthesized as described previously (Gege et al 2000, 2001). All substances were used without further purification. P-selectin was isolated from outdated human platelets as described before (Schumacher et al 2002) and quantified by an ELISA (Biozol, Eching, Germany).

Preparation of the model membranes

For preparing model membranes of the endothelial surface (i.e., either supported planar bilayers or immobilized selectins), microscopy slides (glass, diameter 18 mm, thickness 0.2 mm) were used as transparent supports. To achieve a highly homogeneous surface the slides were incubated in conc. H_2SO_4–H_2O_2 mixture (7:3) for 30 min at 80 °C under ultrasonic conditions and then rinsed with ultra-pure water for 30 min at room temperature. To increase the density of silanole groups on the surface, a cleaning procedure with NH_3–H_2O_2–H_2O (1:1:5) was performed, followed by a final rinse with ultra-pure water and drying of the slides.

To form a supported bilayer, monochlordimethyloctadecyl-silane (Sigma, Deisenhofen, Germany) was covalently bound to the surface of the slide for 30 min at 50 °C, resulting in a hydrophobic monolayer. A DSPC film containing the desired concentration of glycolipid ligand was pre-formed on the air–water interface of a Langmuir trough. The bilayer on the slide was completed by transfer of this X-type monolayer to the hydrophobic substrate at a lateral pressure of 38 mN m^{-1} and a speed

of 0.5 mm min^{-1}. The transfer ratios were 0.95–1. Freshly prepared supported bilayers were immediately used for experiments in the flow chamber.

For the covalent immobilization of selectins, cyanuric chloride was used as crosslinker between glass and proteins. In a first step, a solution of cyanuric chloride (Sigma) in chloroform reacted with the glass under ultrasonication, followed by drying and incubating with a mixture of selectin with BSA in borate buffer (pH 8.8) for 2 h at room temperature. The glasses were thoroughly rinsed with ultra-pure water immediately before the rolling experiments.

Sulfated polysaccharides

Unfractionated heparin of porcine mucosal origin (147 USP-U mg^{-1}) was purchased from Sigma (Deisenhofen, Germany). The low-molecular-weight heparin Certoparin and the semisynthetic polysaccharide pentosan polysulfate were kind gifts from Novartis (Nürnberg, Germany) and bene-Arzneimittel (München, Germany). The semisynthetic sulfated polysaccharides phycarin (PhyS), curdlan (CurS), selectively sulfated phycarin (2,4-PhyS), and selectively sulfated curdlan (2,4-CurS) were obtained by sulfation of the linear β-1,3 glucans phycarin (Goemar, St Malo, France) and curdlan (Wako Pure Chemical Industries, Osaka, Japan) with SO_3-pyridine in DMF–pyridine as described (Yvin et al 2002; Alban & Franz 1994, 2000).

The degree of sulfation of the test compounds was determined by ion chromatography on an HPLC system and their molecular weight (MW_{HD}) (i.e., their hydrodynamic volume) by gel permeation chromatography on an FPLC system using neutral pullulans of defined MW as standards (Alban et al 2001). The sulfation pattern of the glucan sulfates was established by methylation-ethylation analysis with subsequent gas-liquid chromatography–mass spectrometry of the resulting partially methylated, ethylated alditol acetates (Alban & Franz 1994).

Cell cultivation

Chinese Hamster Ovarian cells transfected with mice E-selectin (E-CHO cells) were grown in MEM-α media containing 10% (v/v) fetal calf serum, 2 mM L-glutamine and 100 nM penicillin/streptomycin (c.c.pro GmbH, Neustadt, Germany). Flasks seeded with 5×10^4 CHO-E cells were incubated at 37 °C in 5% CO_2 for 3–4 days to near confluency. After trypsinization for 3 min with 2 mL of 0.25% trypsin/EDTA, the cell suspension was transferred to slowly rotating plastic tubes. The cells remained in suspension for up to 4 h. Within this time, the rolling experiments were performed in the flow chamber. U937 cells, a human monocytic cell line expressing the P-selectin ligand PSGL-1, were cultivated in RPMI medium (c.c.pro GmbH) containing 10% fetal calf serum, 2 mM L-glutamine, 100 nM mercaptoethanol (Sigma), 30 μg mL^{-1} Refobacin (Merck, Darmstadt, Germany) and 2.5 μg mL^{-1} Fungizone (GibcoGmbH, Germany). The cells were used for the rolling experiments within 4 h of separation, centrifugation and washing.

Laminar flow experiments

The parallel-plate flow chamber used in these studies has been described in detail in our previous investigations (Vogel et al 1998). The flow apparatus was mounted onto the inverted fluorescence microscope Axiovert 135 of a Laser Scanning Microscope (LSM 410 invert, Carl Zeiss).

Adhesion experiments were performed in a temperature-controlled environment at 37 °C. The indicated cell medium was used as flow medium at a shear rate of about 200 s^{-1} driven by hydrostatic pressure. For the flow experiments 10^6 fluorescent-marked CHO-E or U937 cells (Calcein AM, Molecular Probes, Leiden, The Netherlands) in 100 μL pure medium or 100 μL medium containing 25 μg of test compound were injected into the streaming medium without dilution. The flow was stopped for 5 min to allow interaction of the cells with the supported membrane. After this period, shear force was applied and the adhesion behaviour of the cells was monitored by a sequence of images taken every 2 s. To characterize the cell movement, 50–150 cells within an area of 630×630 μm were analysed throughout a period of 20 s. Only those cells that adhered to the membrane without contact to other adhered cells were counted and analysed. The experiments for the presented data were repeated at least four times.

Quantification of cell adhesion and rolling velocity

To quantify the amount of adhered cells, the first image from each experiment and the image taken after 30 s of flow were analysed. The number of cells after 30 s was divided by the number of cells on the start image and the result was expressed as a percentage.

For the quantification of the rolling velocity, subsequent images taken at intervals of 2 s were assigned to different colours and overlaid using the software from LSM 410 invert (Carl-Zeiss, Jena). The distance was measured and divided by the time interval, resulting in the rolling velocity of one cell.

Statistical analysis

The statistical evaluation of the inhibition data of the tested heparinoids was performed according to the Kruskal–Wallis test followed by the Nemenyi post-hoc test. Differences were accepted to be significant at $P = 0.05$. All experiments were repeated at least three times; the number of control experiments was $n = 18$ for E-selectin and $n = 11$ for P-selectin.

For each test compound, the data were statistically compared among themselves and to the control experiments. The number of analysed rolling cells for E-/P-selectin was: control 787/1661, unfractionated heparin

85/451, low-molecular-weight heparin 124/217, PPS 104/36, PhyS 256/16, CurS 63/25, 2,4-PhyS 206/15, 2,4-CurS 7/no rolling.

For calculating the mean values and standard deviations expressed in the figures, data from identical experiments were pooled.

Results and Discussion

Introduction of the test system

The dynamic inhibition assay can be performed in two different ways, either by rolling of ligand-carrying cells along a surface with immobilized selectins or by rolling of selectin-expressing cells on a model membrane containing sLex glycolipid ligands. Both ways were applied for E- and P-selectin tests but, for practical reasons, the rolling of the promyelotic cell line U937 that expresses the P-selectin ligand PSGL-1 along immobilized P-selectin, as well as the rolling of CHO cells expressing E-selectin along a ligand-containing model membrane, was preferred for the current study. In the latter case, the lateral organization of the sLex-glycolipids in the membrane matrix was shown to be important, since clustered ligands seem to be essential to increase the binding affinity by enabling multivalent binding (Vogel et al 1998). The lateral separation of the very low ligand concentrations (0.1–0.005 mol%) is not easily detectable. Using a fluorescent-labeled sLex lipid (manuscript in preparation for a detailed study), we could prove by confocal laser scanning microscopy that the ligands in this study (0.005 mol% sLex lipid in a DSPC matrix) display a clustered arrangement in the low-μm range as an essential prerequisite for rolling (Figure 1).

Structures of the sulfated polysaccharides

Few studies report on heparin binding to P- and L-selectin. The heparins used in these studies were of natural

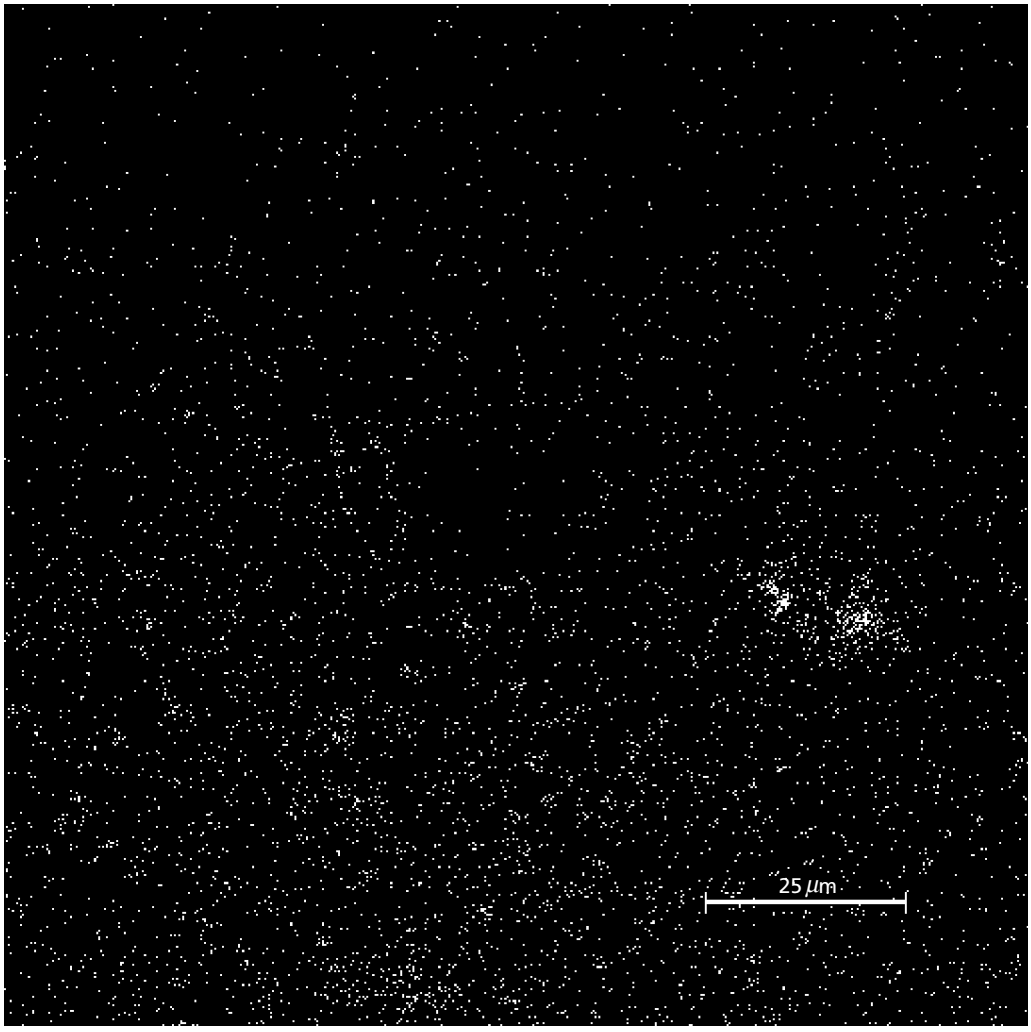


Figure 1 Fluorescence microscopic image of the lateral distribution of the NBD-labelled sLex lipid (0.005 mol%) within a DSPC-matrix. Bar represents 25 μm.

origin, therefore structural differences might be the reason for some controversal interpretations. Furthermore, heparin consists of three different monosaccharide units, which complicates strategies concerning semisynthetic modifications. Clear structure–activity relationships could not be derived, which should be essential in the search for selectin inhibitors.

To establish heparin activity in the new in-vitro flow-chamber model, an unfractionated heparin and Certoparin (a low-molecular-weight heparin) were included in the study (Table 1).

In addition, the semisynthetic pentosan polysulfate, which is applied as an antithrombotic drug, was tested for its inhibitory activity. Pentosan polysulfate consists of (1,4)-β-D xylopyranose chains, branched in position 2 with 4-*O*-methyl-a-D-glucuronic acid, in an average proportion of 1 uronic acid to 9 xylose units (Alban 1997). With a degree of sulfation of 1.9, 95% of the free hydroxyl groups of pentosan polysulfate are sulfated. It has a mean molecular weight of 4.7 kD (mean MW_{HD} 10 kD).

Furthermore, a series of structurally well-defined semisynthetic sulfated polysaccharides was included in the inhibition assay (Table 1). Phycarin sulfate (PhyS) and curdlan sulfate (CurS) are linear β-1,3-glucans only differing in their molecular weight, which are mainly sulfated in position 6 of the glucose units. Protecting the primary hydroxyl group in position 6 (Alban & Franz 1994) resulted in 2,4-PhyS and 2,4-CurS, which are sulfated to more than 90% in position 2 and 4.

Inhibition of E-selectin-mediated cell rolling

In a first set of experiments, the influence of the sulfated polysaccharides on the E-selectin-mediated cell adhesion or rolling was checked. The reference values are represented by the binding extent and the rolling velocity of CHO cells expressing E-selectin along a model membrane containing 0.005 mol% sLex-glycolipid in the absence of inhibitor. According to the literature (Kunkel & Ley 1996; Puri et al 1997), the E-selectin-mediated rolling of about 2 μm s^{-1} is much slower than that mediated by the other selectins, which reflects the lower binding kinetics of E-selectin.

Unfractionated heparin and low-molecular-weight heparin neither influenced the cell adhesion (Figure 2A) nor the cell rolling velocity (Figure 2B). This is consistent with former investigations, which revealed the inability of heparin to bind to E-selectin (Xie et al 2000). In contrast to this, all the semisynthetic sulfated polysaccharides increased the cell adhesion and reduced their rolling velocity (Figure 2A, B). The increased cell binding in the presence of the test compounds might be explained by a possible insertion of sulfated polymers into the model membrane, thus acting as additional immobilized ligands. In this case, insertion should also occur in ligand-free model membranes. Inhibition studies using DSPC model membranes without sLex ligands display, either in the absence or in the presence of the compounds, no cell binding. Consequently, an unspecific insertion of the inhibitors into the model membrane can be excluded. Obviously, the presence of the sLex ligands in the membrane is a prerequisite for the enhanced binding effect by the sulfated polysaccharides, which might thus act as crosslinking agents. To establish whether this crosslinking function is selectin dependent, we used CHO cells lacking an expression of E-selectin. Whereas these cells did not adhere to the ligand containing model membrane in the absence of inhibitors, and only 3% of cells adhered in the presence of the unfractionated heparin, CurS caused an increase of binding to more than 18% of cells. Consequently, the semisynthetic sulfated polysaccharides interact with other cell surface epitopes than E-selectin. The interaction with both the cells and the sLex ligands might explain the strong increase in cell binding in the E-selectin inhibition experiments. The identification of the target molecule and the molecular mechanism of the interaction need further investigation.

Consequently, the obvious conflict with the distinct inhibitory activity of these compounds concerning the adhesion of U937 cells to E-selectin found under static conditions (Alban et al 2001) might be explained by structural peculiarities of the E-CHO cells and is independent of E-selectin.

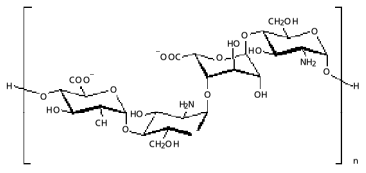
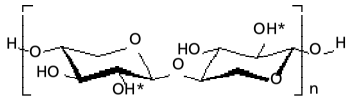
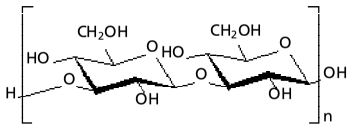
Nevertheless, it can be concluded that neither heparins nor the semisynthetic sulfated polysaccharides show any inhibition on the E-selectin-mediated cell rolling and adhesion to sLex ligands.

Inhibition of P-selectin-mediated cell rolling

The P-selectin inhibition assay is based on the rolling of U937 cells along surface-immobilized P-selectin. The density of the surface-immobilized selectins is an important factor of adhesion. Whereas high concentrations mediate an unphysiological firm attachment of the cells and no interactions occur at very low selectin densities, cell rolling requires a specific concentration, which can be obtained by a balanced mixture of P-selectin and albumin. Albumin suppresses unspecific binding to the glass surface and shows no interactions with the cells. A P-selectin concentration resulting in a rolling fraction of 30% of the cells and a velocity of about 18 μm s^{-1} was defined as optimal, since this value represents the physiological rolling velocity of leucocytes. Compared with E-selectin, the P-selectin-mediated rolling movement is faster, which can be explained by either the intrinsic binding kinetics or the more-extended and flexible binding components (Patel et al 1995).

The inhibitory activity of the sulfated polysaccharides was analysed and compared with that of the standard inhibitor Sialyl Lewis^X. Because of the strong differences in the molecular weight between the polymers and the standard inhibitor, the mass was used for comparison. The addition of 25 μg of sLex per 100 μL cell suspension, which is regarded as internal standard, reduced the rolling cell fraction (Figure 3A), though not significantly, by about one-fifth. Unfractionated heparin had no inhibiting effect—the increase turned out to be not significant.

Table 1 Structural characteristics of the sulfated polysaccharides used in this study.

	Structural elements	Molecular weight	Degree of sulfation per monosaccharide unit	Position of sulfation
Unfractionated heparin		28 kD	1.2	2 of the uronic acid N, 6 and 3 of the glucose amine
Certoparin		10 kD	1.2	
Pentosan polysulfate		10 kD	1.9	2* and 3 of the β-D-xylose
Phycarin sulfate		19 kD	1.6	6, 2 and 4 of the β-D-glucose
curdlan sulfate		170 kD	1.6	
Selectively sulfated phycarin sulfate		14 kD	1.1	2 and 4 of the β-D-glucose
Selectively sulfated curdlan sulfate		170 kD	1.6	

Molecular weights are presented as hydrodynamic volumes (MW_{HD}) as determined by gel permeation chromatography. Branched with 4-*O*-methyl-α-D-glucuronic acid (average proportion: 1 uronic acid to 9 xylose units).

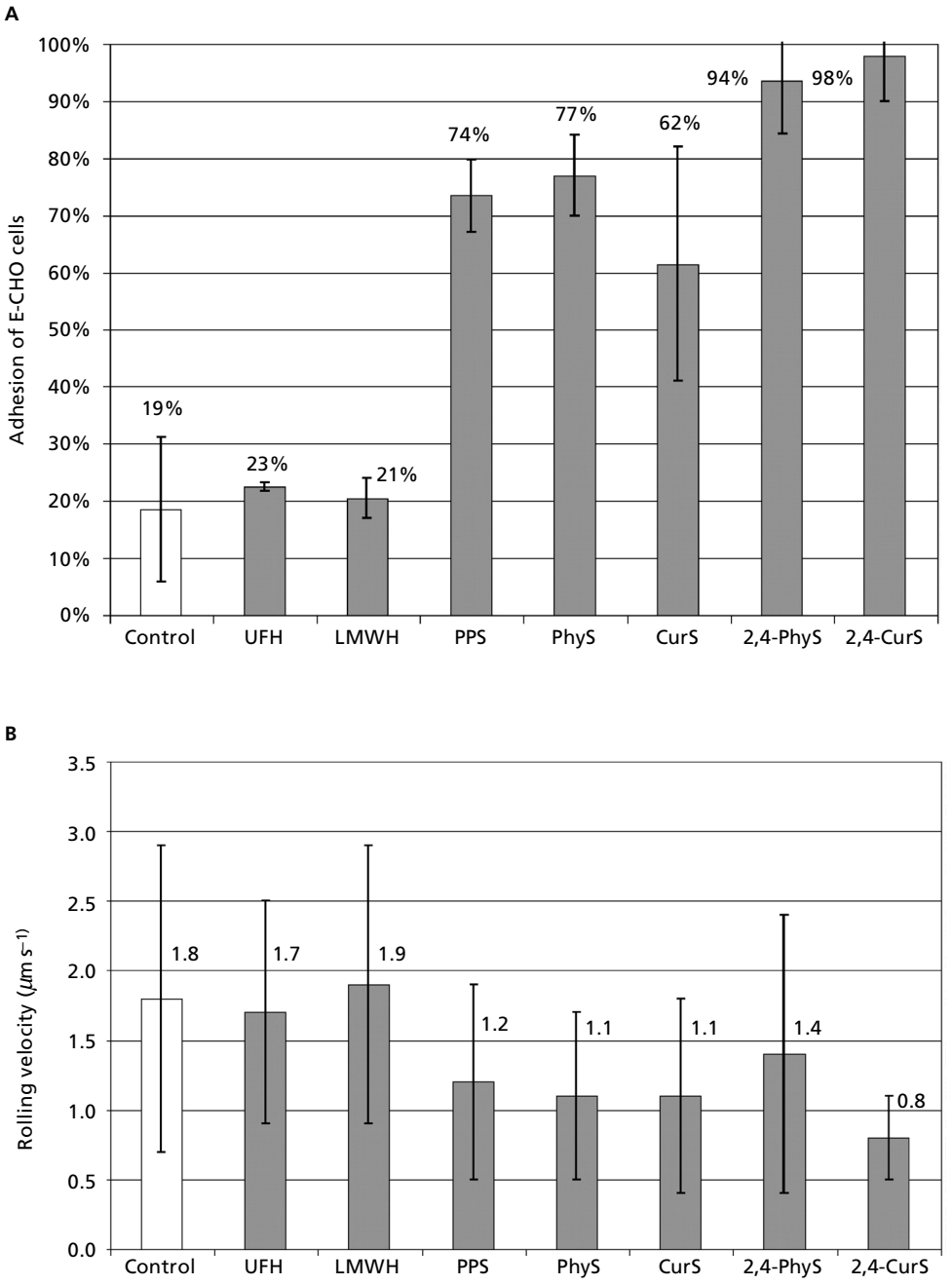


Figure 2 Influences by sulfated polysaccharides on E-selectin-mediated cell rolling. A. Effects on adhesion of E-CHO cells to a 0.005% sLex glycolipid containing model membrane. B. Changes in the rolling velocity of E-CHO cells along a 0.005% sLex glycolipid containing membrane. UFH, unfractionated heparin; LMWH, low-molecular-weight heparin; PPS, pentosan polysulfate; PhyS, semisynthetic sulfated polysaccharide phycarin; CurS, semisynthetic sulfated polysaccharide curdlan; 2,4-PhyS, selectively sulfated phycarin; 2,4-CurS, selectively sulfated curdlan. Data are means ± s.d.

Low-molecular-weight heparin blocked P-selectin slightly (not significantly). In contrast, the semisynthetic compounds pentosan polysulfate, PhyS, CurS, 2,4-PhyS and 2,4-CurS showed a very high and significant blocking capacity for P-selectin by completely suppressing the cell rolling within the time range of detection (30 s) at a concentration of 25 μg/100 μL cell suspension.

In correlation, the rolling velocity (Figure 3B) was nearly unaffected by unfractionated heparin, whereas low-molecular-weight heparin and sLex caused a slight increase. With the exception of the PhyS, all polysaccharides induced a considerable faster rolling movement and thus strongly inhibited the interactions between cells and P-selectin. 2,4-CurS reduced the number of rolling cells to

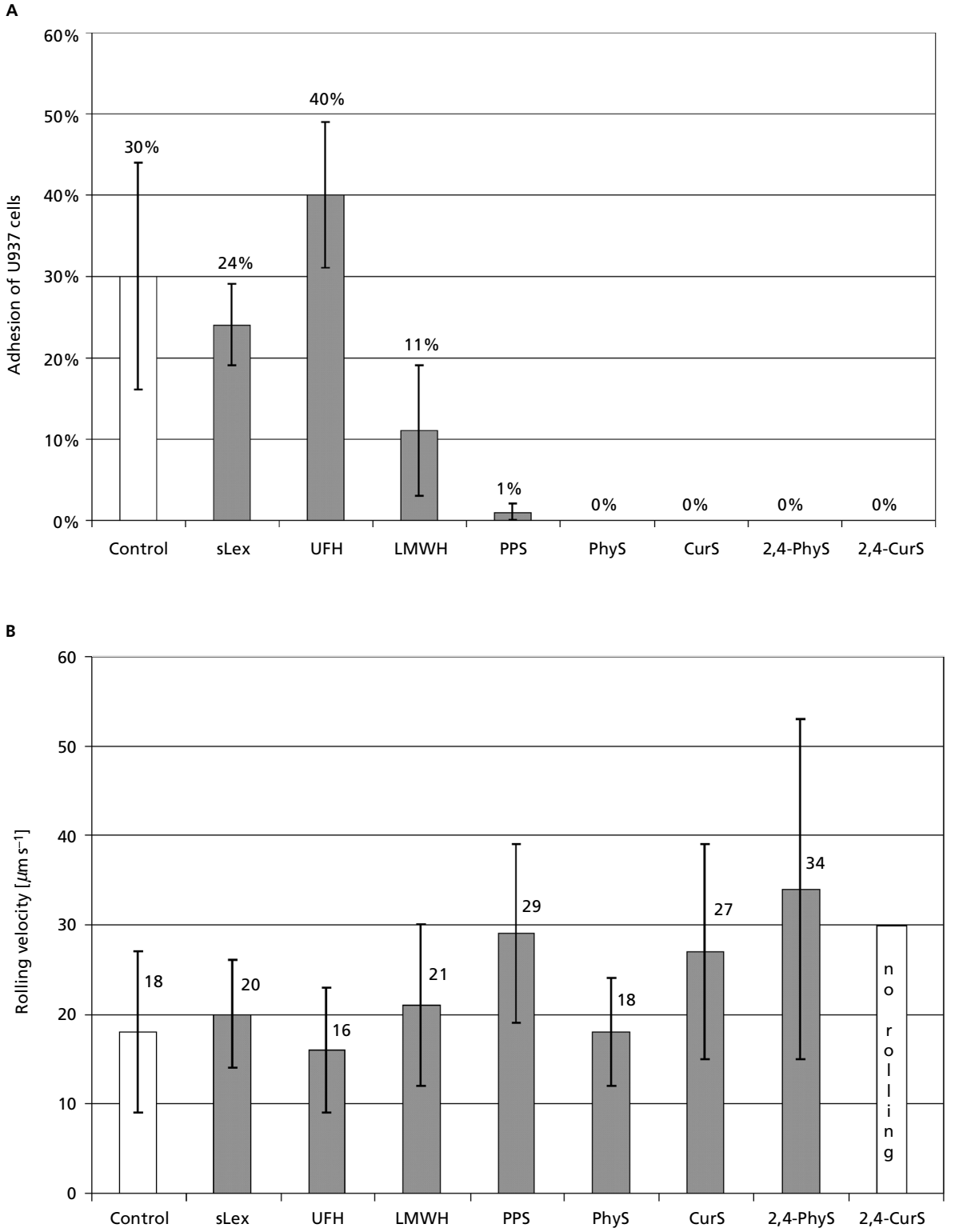


Figure 3 Inhibition of the P-selectin-induced cell rolling by sulfated polysaccharides. A. Adhesion of U937 cells to a P-selectin layer. B. Rolling velocity of U937 cells along a P-selectin layer. sLex, Sialyl Lewis^{X}; UFH, unfractionated heparin; LMWH, low-molecular-weight heparin; PPS, pentosan polysulfate; PhyS, semisynthetic sulfated polysaccharide phycarin; CurS, semisynthetic sulfated polysaccharide curdlan; 2,4-PhyS, selectively sulfated phycarin; 2,4-CurS, selectively sulfated curdlan. Data are means ± s.d.

such an extent that it was impossible to evaluate an exact rolling velocity of the remaining cells.

In conclusion, both cell adhesion and rolling velocity clearly indicate that, in contrast to unfractionated heparin and low-molecular-weight heparin, the five semisynthetic sulfated polysaccharides are efficient P-selectin inhibitors, exhibiting much higher activity than the standard compound sLex.

Comparing the activity of the test compounds, some structure–activity relationships should be derived with respect to molecular weight and degree of sulfation. It is conceivable that electrostatic interactions of the negatively charged polysaccharides with the positively charged target molecules are favoured with increasing charge density and chain length.

The pairs PhyS/CurS and 2,4-PhyS/2,4-CurS, respectively, demonstrate that the inhibitory activity rises with increasing molecular weight. In contrast, low-molecular-weight heparin proved to be superior to unfractionated heparin. However, taking account of their relative small mass difference and structural inhomogenity, a dependence between inhibitory efficiency and molecular weight is likely.

In analogy to the natural selectin ligand PSGL-1, which contains sulfated tyrosines arranged in negatively charged clusters (Pouyani & Seed 1995; Sako et al 1995), a higher degree of sulfation should imply better inhibitory properties. Wang et al (2002) found natural heparin to be active in selectin blocking and demonstrated a clear dependence of selectin binding on the degree of sulfation.

Pentosan polysulfate, with a degree of sulfation of 1.9, seems to be more active than low-molecular-weight heparin, with a degree of sulfation of 1.2 and identical MW_{HD}. But in view of the different polysaccharide basic structure, this conclusion might be unlikely.

Regarding the activity of pentosan polysulfate (degree of sulfation = 1.9) and 2,4-PhyS (degree of sulfation = 1.1), the basic structure seems to be an important parameter. Despite its considerably lower degree of sulfation and only a slightly higher MW_{HD}, 2,4-PhyS tends to be more active. Furthermore, 2,4-PhyS proved to be a much better P-selectin-antagonist. Consequently, the basic polysaccharide structure and the detailed molecular structure (e.g. the sulfation pattern) may be of greater importance for P-selectin binding than the degree of sulfation and the molecular weight.

This is clearly supported by the finding that the selectively sulfated 2,4-PhyS and 2,4-CurS exhibit higher inhibitory activity than the corresponding mainly 6-sulfated analogues. 2,4-PhyS was even more active than CurS despite its lower degree of sulfation and lower molecular weight.

Therefore, selective sulfation seems to be most efficient in obtaining high P-selectin binding capacity using β-1,3 glucans as starting polymers.

These results open the question of the molecular basis of the ligand/P-selectin interactions. The sLex standard represents a monomer-binding epitope which enables a direct equimolar relation with the selectins and correlation between amount and activity (i.e., each molecule binds to one P-selectin molecule only). With respect to multivalent binding and the importance of electrostatic forces, which were postulated to increase the binding affinity (Somers et al 2000), sulfated polysaccharides should interact in a different way. To get further insight into these mechanisms, we repeated the inhibition experiments with subsequently diminished amounts of 2,4-CurS, starting from the original concentration of 25 μg/100 μL cell suspension. It became obvious that upon reducing the amount down to 0.06 μg/100 μL cell suspension, the total blocking capacity was retained; a further reduction to 0.02 μg/100 μL cell suspension led to a total loss of inhibition and the system came close to the blank. Consequently, we could not find a linear correlation between amount and activity of the heparinoid, which is inconsistent with a monomer interaction. Obviously, the polymers are able to block a dominant and functionally relevant fraction of the selectins, whereby the interaction is driven by electrostatic attraction; below a critical concentration, a sufficient number of selectins is free to mediate cell rolling.

Conclusions

To search for new classes of selectin-inhibiting compounds, which could interfere with these adhesion receptors in the course of various pathological situations, we analysed a series of semisynthetic sulfated polysaccharides for their E- and P-selectin blocking potency. To get an insight into the molecular binding characteristics and, eventually, to derive structure–activity relationships, we applied a series of well-defined semisynthetic polysaccharides differing in molecular size, type of glycosidic backbone and sulfation. To simulate the physiological shear-force conditions of the blood flow and to focus on the molecular binding properties, we performed these inhibitions studies in a parallel-plate flow chamber.

The semisynthetic sulfated polysaccharides did not show any inhibitory effects toward E-selectin, but they blocked P-selectin much better than the standard binding epitope sLex. This reveals different affinity to E- and P-selectin.

Our data emphasize that modifying the structure of sLex leads to a total drop in E-selectin affinity. Consequently, the molecular structure of the sulfated polysaccharides does not meet the requirements for tight binding to E-selectin. In contrast, the ideal binding conditions for P-selectin display, in addition to a glycosidic basis, several optimally disposed residues that participate in binding by electrostatic interactions (Somers et al 2000). These requirements are met by the structural characteristics of the highly charged sulfated polysaccharides.

Considering the total inactivity of the tested unfractionated heparin as leading compound, the importance of using structurally well-defined semisynthetic compounds becomes evident. Both degree of sulfation and molecular weight were found to be less important than the position of the sulfate groups in the glucose units. Consequently, the 2,4-CurS seems to offer the best binding properties.

In summary, semisynthetic β-1,3 glucan sulfates represent, due to their inexpensive and reproducible accessibility, a promising class of P-selectin inhibitors.

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