Introduction

Human polymorphonuclear leukocytes-elastase (PMNE) is a 30 kDa serine protease produced by inflammatory cells and various cancer cell lines. It cleaves connective tissue components, among them elastin, collagen, fibronectin and proteoglycans (1-4). In addition, it degrades the plasma proteins fibrinogen and fibrin. The major physiological function of this enzyme is to facilitate the extravasation of PMN to the sites of inflammatory response (5). Normally, the activity of the enzyme is under control of endogenous inhibitors, including α1-antitrypsin, α2-macroglobulin and secretory leucoprotease inhibitor (6). However, during inflammation and tumor cell metastasis, there is an imbalance between PMNE activity and its endogenous inhibitors caused by the release of large amounts of the enzyme and the partial inactivation of the inhibitors (7, 8). This results in excessive elastase activity and crucial tissue damage, facilitating the invasion of either PMN or metastasizing cancer cells, and thus supporting inflammation or tumor cell metastasis. This local imbalance between elastase and its endogenous inhibitors is involved in the pathogenesis of several acute and chronic inflammatory diseases including pulmonary emphysema (9), adult respiratory distress syndrome (10, 11), septic shock (12, 13), cystic fibrosis (14, 15), rheumatoid arthritis (16, 17) and psoriasis (18, 19). Furthermore there is evidence that overexpression of elastase by tumor cells of lung- (20, 21), breast- (22)
and bladder cancer (23) and lymphoma (24) promotes metastasis. Clinical data suggest a strong correlation between the amount of released elastase and the stage of tumor progression. High levels of elastase have been associated with poor prognosis for cancer patients (25).

Therefore, inhibition of elastase represents an interesting option for the treatment of inflammatory and tumor diseases. Due to its strong cationic nature, PMNE binds to several polyanions such as sulfated glycosaminoglycans (26, 27) via electrostatic interactions. Although heparin was shown to inhibit PMNE activity in vitro and in vivo (28, 29), its therapeutic use as an elastase inhibitor is restricted due to its pronounced anticoagulant activity. Moreover, unfractionated heparin (UFH) consists of a heterogeneous mixture of different carbohydrate molecules and there is considerable structural variability between different UFH preparations (30). Accordingly, distinct heparin fractions may widely differ in their interactions with biomolecules such as antithrombin (31). An alternative to heparin may be structurally well defined semisynthetic sulfated carbohydrates, which were shown to exhibit potent antiinflammatory and antimetastatic activity in vivo (32, 33) but to have reduced anticoagulant activity, which can be controlled depending on the respective structure (34, 35). The aim of the present study was to examine whether an inhibitory effect of new semisynthetic SC contributes to their known antiinflammatory and antimetastatic activity. Besides the evaluation of structure-activity relationships, the effects of the compounds in different test systems for PMNE activity should be compared to obtain information on their mechanisms of inhibition.

The following test compounds were included in the study: 1. Structurally defined SC produced by chemical modification of natural neutral polysaccharides, 2. commercial heparinoids and 3. as reference compounds, various heparins as well as cephalothin (36) and ursolic acid (37), two known non-carbohydrate inhibitors of PMNE. The elastase inhibiting activity was investigated in a purified buffer as well as plasma containing chromogenic substrate assay and in two different test systems using the physiological substrates elastin and collagen, resp. Moreover, enzyme-inhibitor binding studies were performed. Finally, a cancer cell-based model was used to examine the effect of semisynthetic SC on cell-mediated elastinolysis.

Materials and methods

Materials

The low molecular weight heparin (LMWH) certoparin and the corresponding unfractionated heparin (UFH) from porcine mucosal origin (147 USP-U/mg) were kind gifts from Novartis AG (Nürnberg, Germany). ursolic acid, cephalothin and all other chemicals, if not indicated were obtained from Sigma (Taufkirchen, Germany). Citrated human platelet poor plasma pooled from at least 8 healthy volunteers was prepared by centrifugation at 3000 g for 20 min at 15°C, transferred into cryovials in aliquots of 1 ml, frozen in liquid nitrogen and stored at -70°C until use.

Tris buffer, if not otherwise defined consisted of 50 mMTris, 155 mM NaCl, pH 8.3. All test substances were dissolved in 0.9% NaCl, except of ursolic acid, which was diluted from a stock solution in DMSO, reaching a final DMSO concentration in the assay of max. 0.1% DMSO. PMNE as well as elastin and FITC-elastin were received from Calbiochem (Bad Soden, Germany). Both common flat bottom microplates and black flat bottom fluorescence microplates (Fluotrac200) were used from Greiner (Frickenheim, Germany), if not otherwise mentioned. All spectrophotometric assays were done in a 96well MRX microplate reader (Dynex, USA). In the case of chromogenic substrate assays, S2484 from Chromogenix (Mölndal, Sweden) was used. The fluorimetric assays were performed using a Tecan fluorescence microplate reader (Tecan, Münndorf, Switzerland). All incubation procedures, if not differently described, were done in a microplate incubator (Oyenx, USA).

All measurements were done in duplicate and repeated at least two times on different days (n = 6) and are presented as mean ± SD.

Preparation and characterization of semisynthetic glucan sulfates

Semisynthetic glucan sulfates were produced by sulfation of the natural polysaccharides phycarin (Goemar Laboratories, St. Malo, France), a β-1,3-glucan, and both genuine and thermally degraded and fractionated pullulan, an α-1,4/1,6-glucan (Wacker Chemie, München, Germany) with SO3/pyridine in dimethylformamide. The phycarin sulfate PS3 was synthesized as described (32). Pullulan sulfates with different molecular weight (MW) and degree of sulfation (DS) were obtained according to Alban et al. (38) by varying the starting pullulan and the conditions of the sulfation process. The degree of sulfation was analyzed by ion chromatography after acid hydrolysis (38). Both the average MW and the MW distribution were determined as the hydrodynamic volume by gel permeation chromatography on a FPLC system (Pharmacia, Freiburg, Germany) using neutral pullulans of known MW as calibration standards (Polymer Laboratories, Shropshire, UK) (38). The distribution of sulfate groups was examined by methylation analysis, followed by gas chromatography/mass spectrometry (39). The structural parameters of the products are shown in Table 1.

Chromogenic substrate assay

PMNE activity was determined in a modified version of the assay described by Kramps et al.(40) as a microplate assay. Briefly, 25 µl of Tris buffer were mixed in a 96well flat bottom microplate with 25 µl of inhibitor. After addition of 25 µl enzyme solution (final concentration 25 nM), the mixture was...
incubated for 5 min at 37°C. The enzymatic reaction was started after addition of 25 μl chromogenic substrate (final concentration 0.5 mM). After 20 min incubation at 37°C, the optical density (OD) at 405 nm was measured versus a blank composed of chromogenic substrate in buffer. The inhibitory activity of the test compounds was determined as decrease (%) of the OD produced by PMNE.

\[ K_M = \frac{([S]_0 - [S]_K)}{100} \]

was found to be 0.6 mM in agreement with literature (40). The \( K_v \) values were determined according to Baici et al. (27): Tris buffer was mixed with inhibitor and substrate solution and equilibrated to 37°C. The enzymatic reaction was started by addition of 25 μl enzyme solution. The \( K_v \) values were obtained using the specific velocity plot.

**Colorimetric proteinolysis assay**

The ability of glucan sulfates to inhibit the PMNE-mediated collagenolysis was studied spectrophotometrically in a microplate assay according to Nethery et al. (41) with minor modifications. After preparation of the collagen plates (Collagen C5533 Sigma), 50 μl of Tris buffer were added, followed by 25 μl of inhibitor. The reaction was started by addition of 25 μl enzyme solution (final concentration 25 nM). Incubation and staining of the wells was performed as described (41), with the following exception: After drying of the wells at RT, the remaining colour was dissolved by adding 200 μl of lysis solution (99 ml DMSO, 1 ml AcOH, 10 g SDS) and the OD at 590 nm was measured. Control experiments include collagen without enzyme digestion as 0% values, respectively.

Fluorimetric elastinolysis assay

The ability of the test compounds to inhibit PMNE-mediated elastinolysis was determined in a modified version of the method described by Wiedow et al. (42). The elastinolytic activity was determined by hydrolysis of fluorescein-conjugated (FITC) elastin from bovine neck ligament. An aliquot of 25 μl of inhibitor was pipetted together with 25 μl of Tris buffer into the wells of 96 well V-plates (Nunc, Denmark). After addition of 50 μl enzyme solution (final concentration 33 nM), the microplates were incubated for 10 min at 37°C. FITC-elastin was suspended in Tris buffer at a concentration of 20 mg/ml, followed by centrifugation at 800 g for 10 min. The supernatant was removed and the substrate was resuspended in assay buffer. After several washes with assay buffer, FITC-elastin was suspended in assay buffer at a concentration of 20 mg/ml. The enzymatic reaction was started by addition of 50 μl of substrate suspension to the assay mixture (final elastin concentration: 6.67 mg/ml). After 60 min incubation with gentle agitation, 50 μl of assay buffer were added and the plates were centrifuged at 800 g for 20 min. An aliquot of 100 μl of clear supernatant was transferred into black flat bottom fluorescence microplates and the fluorescence of solubilized FITC-elastin peptides was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence signals were corrected by enzyme-free controls.

**SPC-EA assay**

Binding studies between PMNE and sulfated polysaccharide-based inhibitors were assessed using the sulfated polysaccharide-coating enzyme activity assay (SPC-EA-assay), a modified version of the SPC-ELISA (43). After coating of the microplates with the resp. polysaccharides, 50 μl of Tris buffer were added to each well, followed by 25 μl of PMNE solution (final enzyme concentration in the assay: 33.3 nM). In the presence of elastin, 25 μl Tris buffer was mixed with 25 μl elastin suspension (5 mg/ml). After incubation for 30 min at room temperature, the plates were centrifuged at 800 g for 10 min and the supernatant was transferred to a 96well flat bottom microplate. After addition of 25 μl chromogenic substrate, the plates were treated as described in the chromogenic substrate assay. The release of p-nitroaniline was used to determine the amount of enzyme by reading as mentioned above. Controls were included by assaying BSA- instead of polysaccharide-coated wells as 100% and blanks composed of chromogenic substrate in buffer. For the determination of \( K_d \) as a measure for the affinity between SC and PMNE, the following equation was used:

\[ SC + PMNE \rightarrow SC-PMNE (I) \]

\[ K_d = \frac{[SC] 	imes [PMNE]}{[SC-PMNE]} (II) \]

**Cancer cell-mediated elastinolysis assay**

To study the influence of cancer cell mediated proteolysis in a direct cell-based model of elastinolysis, sterile flat bottom microplates (Greiner, Frickenhausen, Germany) were coated with FITC-elastin to form a substrate matrix. The substrate containing plates were dried for 24 h under laminar air flow. The PMNE producing human breast cancer cell line MCF-7 (44) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained in 25 cm² plastic cell culture flasks (Greiner, Frickenhausen, Germany) containing DMEM medium, supplemented with 10% FCS (Biochrom, Berlin, Germany). Cells were cultivated at 37°C in a 95% room air-5% carbon dioxide humidified incubator. At 90% confluence, the medium was removed and the cells were detached from the flask by adding sterile DPBS (Biochrom, Berlin, Germany) containing 0.02% EDTA. The cells were resuspended in phenol red-free DMEM without serum supplementation, three times washed and finally adjusted to a concentration of 2 × 10⁶ cells per ml in phenol red-free DMEM without serum supplementation. Trypan blue staining revealed more than 95% of viable cells. An aliquot of 100 μl cell suspension was pipetted into the FITC-elastin-
coated wells, followed by addition of 10 μl calcium ionophore (A2383, ICN Biomedicals, Irvine, USA) in water (final assay concentration 4 μM) and 50 μl inhibitor in sterile DPBS. The plates were incubated in a 95% room air-5% carbon dioxide humidified incubator for 24 h at 37°C and then centrifuged for 20 min at 800 g. To determine the amount of proteolytically cleaved FITC-elastin peptides, 50 μl of supernatant were transferred into black flat bottom fluorescence microplates. After addition of 50 μl water, the fluorescence was measured as described in the FITC-elastinolysis assay. FITC-elastin coated wells without cells served as controls.

Statistical analysis
All results were determined as mean ± SD (standard deviation) of six measurements.

Results

Characterisation of the test compounds
As obvious from Table 1, the test compounds not only differ in their MW, MW distribution and DS, but also in the structural parameters of the basic polysaccharides. Whereas those of PS3 and PulS1-7 consist exclusively of neutral glucose units, UFH, LMWH and PPS contain uronic acids, and the two heparins additionally glucosamine residues. In contrast to the other SC, which are linear hexosans, PPS is a glycosidically branched pentosan. Further, the types of glycosidic bonds are different. As found by GC-MS analysis of the semisynthetic glucan sulfates, this influences the sulfation pattern (38, 39). The reactive primary OH-groups on C6 of PS3 are fully sulfated, whereas the secondary OH-groups on C2 and C4 are only sulfated to about 50% each. In the α-1,4/1,6-glucan pullulan, 30% of the C6-OH-groups are involved in glycosidic bonds. Due to the linkage-blocked C6-OH groups and the different three-dimensional structure of pullulan, the part of sulfated C6 amounts only to about 40% so that mainly the secondary OH groups on C2, C3 and C4 are sulfated. In low-sulfated PulS, C2, C3, and C4 are equally sulfated, but with increasing DS the relative part of C4-sulfate decreases.

Inhibition of PMNE in the chromogenic substrate assay
Figure 1A shows the concentration-dependent elastase inhibition of three different SC. The semisynthetic glucan sulfates PS3 (IC_{50} = 0.18 μg/ml) and PulS1 (IC_{50} = 0.25 μg/ml) are only slightly superior to UFH (IC_{50} = 0.5 μg/ml) in inhibiting PMNE activity. Compared to cephalothin (IC_{50} = 8.9 mM) and ursolic acid (IC_{50} = 33.7 μM) (Fig. 1B) PS3 (IC_{50} = 18.1 nM) turned out to be much more active than these low molecular weight inhibitors. The inhibition of elastase by SC is abolished by the polycationic Polybrene® as shown in Figure 1C. Polybrene® itself shows no significant influence on the activity of PMNE.

Human plasma as a source of endogenous elastase inhibitors exhibits concentration-dependent inhibitory activity on PMNE (Fig. 2A). UFH, as well as PulS1 and PS3 strongly potentiate the inhibitory activity of diluted plasma. As obvious from the

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<table>
<thead>
<tr>
<th>SC</th>
<th>Basic polysaccharide</th>
<th>Monosaccharide units of the basic polysaccharide</th>
<th>Types of glycosidic bonds</th>
<th>MW (kDa) 1</th>
<th>MW range (kDa)</th>
<th>DS 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PulS1</td>
<td>Pullulan, TD</td>
<td>D-Glc</td>
<td>α-1,4 / α-1,6</td>
<td>10</td>
<td>13-7</td>
<td>2.00</td>
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<tr>
<td>PulS2</td>
<td>Pullulan, TD</td>
<td>D-Glc</td>
<td>α-1,4 / α-1,6</td>
<td>10</td>
<td>13-7</td>
<td>1.50</td>
</tr>
<tr>
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<td>Pullulan, TD</td>
<td>D-Glc</td>
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<td>13-3.3</td>
<td>0.60</td>
</tr>
<tr>
<td>PulS4</td>
<td>Pullulan, TD</td>
<td>D-Glc</td>
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<td>0.25</td>
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<tr>
<td>PulS5</td>
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<td>D-Glc</td>
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<td>100-30</td>
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<td>PulS6</td>
<td>Pullulan</td>
<td>D-Glc</td>
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<td>380-50</td>
<td>0.28</td>
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<tr>
<td>PulS7</td>
<td>Pullulan</td>
<td>D-Glc</td>
<td>α-1,4 / α-1,6</td>
<td>250</td>
<td>380-50</td>
<td>1.28</td>
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<td>UFH</td>
<td>D-GlcN(Ac), L-IdoA, D-GlcA</td>
<td>α-1,4 / β-1,4</td>
<td>28</td>
<td>80-4.5</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>LMWH</td>
<td>D-GlcN(Ac), L-IdoA, D-GlcA</td>
<td>α-1,4 / β-1,4</td>
<td>10</td>
<td>13-3.3</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>PPS</td>
<td>Glucuronoxylan, 4-O-Me-D-GlcA</td>
<td>β-1,4 / α-1,2</td>
<td>10</td>
<td>13-3.3</td>
<td>2.00</td>
<td></td>
</tr>
<tr>
<td>PS3</td>
<td>Phycaricine</td>
<td>D-Glc</td>
<td>β-1,3</td>
<td>10</td>
<td>12-7</td>
<td>2.00</td>
</tr>
</tbody>
</table>

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1 average molecular weight of the SC determined by GPC as hydrodynamic volume using neutral pullulan standards
2 MW distribution of the SC
3 degree of sulfation of the SC (sulfate groups per monosaccharide unit)
PMN-elastase inhibiting effects of glucan sulfates
comparison of the activities of the SC in the absence or presence of plasma, resp., the increase of inhibition ranges between 20% and 25% (SD +/- 2.5%) whereas plasma alone causes only 5% inhibition (SD +/- 5%) (Fig. 2B).

The comparison of the PMNE inhibition by semisynthetic pullulan sulfates with MW of 10 kDa but increasing DS (PulS1-PulS4) shows a strong correlation between their inhibitory capacity and their DS (Fig. 3A). Further, the activity is increasing with increasing MW (Fig. 3B). This is demonstrated both for pullulan sulfates with a low DS of about 0.3 (PulS4, PulS6) and for high-sulfated ones (PulS2, PulS5, PulS7). In Figure 3C, the influence of the basic carbohydrate structure on inhibition of PMNE is shown. At a concentration of 0.25 µg/ml, the inhibitory effect of LMWH (DS 1.2, MW 10 kDa) with 10.5% is distinctly weaker than that of PulS2 (DS 1.5, MW 10 kDa) with 37.5%, whereby the somewhat higher DS cannot explain the activity difference (compare Fig. 3A). Further, despite of its

Figure 1: A) Concentration-dependent inhibition of PMNE activity by UFH (circles), PulS1 (triangle) and PS3 (squares) in the chromogenic substrate assay.
B) Concentration-dependent inhibition of PMNE activity by PS3 (squares), ursolic acid (rhombus) and cephalothin (circle) in the chromogenic substrate assay.
C) Neutralisation of the inhibitory activity of PS3 by Polybrene®: PS3 (squares), PS3 and Polybrene® (triangle), Polybrene® (circle). The values represent the mean ± SD (n = 6)

Figure 2: A) Concentration-dependent inhibition (mean ± SD, n = 6) of PMNE activity by human pooled platelet poor plasma.
B) Comparison of the inhibitory activity (mean ± SD, n = 6) of UFH, PulS1 and PS3 in the absence (filled bars) and presence (striped bars) of 0.003 µl plasma (final inhibitor conc.: 0.25 µg/ml). The control represents 0.003 µl plasma without any SC.
identical DS and MW, PPS (30.0% inhibition) is much less active than the glucan sulfates PulS1 (51.8% inhibition) and PS3 (62.0% inhibition). However, the β-1,3-glucan sulfate PS3 and the α-1,4/1,6-glucan sulfate PulS1 only slightly but not significantly differ in their inhibitory capacity.

For a relative comparison, both the $K_i$ values as well as the maximum inhibitory activity of the different SC were determined as described above (Table 2). For compounds with a low MW (PulS1-PulS4), the $K_i$ decreases with increasing DS from 44 nM to 12 nM. The high-MW SC (PulS5-PulS7) have about 10 times lower $K_i$ values despite of their lower DS. The $K_i$ of LMWH (58 nM) and UFH (5.6 nM) as well as those of PulS2 (22 nM), PulS 5 (3.4 nM) and PulS7 (0.8 nM) demonstrate the importance of the MW. In addition, a significant $K_i$ difference between low and high sulfated SC is only found for PulS2 and PulS4 with a MW of 10 kDa, but not for PulS6 and PulS7 with 250 kDa. The $K_i$ of PulS1 (12 nM) and PS3 (13 nM) compared to that of PPS (19nM) confirm the superiority of the glucan sulfates.

Table 2: Comparison of the $K_i$ values and the maximum inhibitory activity of sulfated carbohydrates in the chromogenic substrate assay.

<table>
<thead>
<tr>
<th>SC</th>
<th>$K_i$ values</th>
<th>maximum inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PulS1</td>
<td>12.20</td>
<td>72 % +/- 7 %</td>
</tr>
<tr>
<td>PulS2</td>
<td>21.50</td>
<td>48 % +/- 1 %</td>
</tr>
<tr>
<td>PulS3</td>
<td>36.00</td>
<td>23 % +/- 5 %</td>
</tr>
<tr>
<td>PulS4</td>
<td>44.10</td>
<td>16 % +/- 4 %</td>
</tr>
<tr>
<td>PulS5</td>
<td>3.40</td>
<td>70 % +/- 2 %</td>
</tr>
<tr>
<td>PulS6</td>
<td>1.07</td>
<td>69 % +/- 3 %</td>
</tr>
<tr>
<td>PulS7</td>
<td>0.80</td>
<td>72 % +/- 1 %</td>
</tr>
<tr>
<td>UFH</td>
<td>5.61</td>
<td>60 % +/- 4 %</td>
</tr>
<tr>
<td>LMWH</td>
<td>58.00</td>
<td>45 % +/- 2 %</td>
</tr>
<tr>
<td>PPS</td>
<td>18.50</td>
<td>68 % +/- 1 %</td>
</tr>
<tr>
<td>PS3</td>
<td>13.20</td>
<td>72 % +/- 5 %</td>
</tr>
</tbody>
</table>

1 expressed in nM
2 expressed as % inhibition at 1.25 µg/ml
PMN-elastase inhibiting effects of glucan sulfates

Corresponding to the $K_i$ values, the maximum inhibitory activity of the SC increases with increasing DS for SC with low MW (PulS1-PulS4). In addition, a MW dependent increase is also found for LMWH and UFH as well as for PulS2 and PulS5. However, the SC with MW $\geq 65$ kDa (PulS5-PulS7) exhibit similar maximum inhibitory activities independently of their DS, and in contrast to the $K_i$ values, their maximum effects are not better than that of low MW SC. Also SC with different carbohydrate basic structures but identical DS and MW do not significantly differ in their maximum activities. Whereas the $K_i$ of UFH (5.6 nM) is lower than that of PS3 and PulS, its maximum activity is significantly weaker than that of PS3 and PulS.

Inhibition of PMNE in the colorimetric proteinolysis assay

In the colorimetric proteinolysis assay (Fig. 4A), the influence on the degradation by PMNE of the physiological substrate collagen is examined. PS3 represents a much better inhibitor than UFH. For UFH, a maximum inhibition of about 40% is achieved at 2.5 $\mu$g/ml, which decreases at higher inhibitor concentrations. In contrast, 2.5 $\mu$g/ml PS3 inhibit PMNE nearly totally.

Inhibition of PMNE in the fluorimetric elastinolysis assay

Figure 4B demonstrates that both PulS1 and PS3 also concentration-dependently inhibit the PMNE activity against the physiological substrate elastin. UFH, however, exhibits only weak inhibitory activity with a maximum inhibition of 30% at a final concentration of 100 $\mu$g/ml. The preincubation of PMNE with inhibitor followed by addition of elastin results in a stronger inhibition than the preincubation of enzyme and substrate with subsequent addition of the inhibitor (Fig. 4C).
Enzyme-inhibitor-binding studies

The binding of PMNE to immobilized sulfated polysaccharides is shown in Figure 5A. Both PS3 and PulS1 strongly bind PMNE, whereas UFH exhibits a distinct weaker binding capacity. Up to 70% of the added PMNE are bound by the semisynthetic glucan sulfates compared to at most 40% by UFH. This effect is confirmed by comparison of the dissociation constants (Table 3). In the absence of elastin, PulS1 and PS3 have similar but about 3 times lower $K_d$ than UFH. In the presence of elastin, the $K_d$ values of all three polysaccharides are increased, whereby that of PS3 is the lowest.

Inhibition of cancer cell-mediated elastinolysis

As determined by the degradation of FITC-elastin, the MCF-7 cells release elastin-degrading activity. This potency showed to be increased when the cells were stimulated with calcium ionophore. As presented in Figure 6, coincubation of the cells with PulS1 resulted in concentration-dependent reduced elastinolysis. As determined by LDH-release and MTT-assay, PulS1 does not show any cytotoxic effect (data not shown).

Discussion

Sulfated polysaccharides display manifold biological activities, some of them contributing to their antiinflammatory and antimetastatic effects. The most widely used sulfated polysaccharide with clinical impact is heparin, which is known to inhibit not only blood coagulation, but also PMNE (45). PMNE is a cationic glycoprotein with 19 arginine residues, located on the surface of the enzyme (46, 47). The inhibition of PMNE by heparin derivatives is described to be based on electrostatic interactions (48). The observed neutralisation of the PMNE inhibitory activity of SC by the polycation Polybrene® underlines this. Baici further describes the mechanism as a tight binding, hyperbolic non-competitive inhibition (49). For the interaction between glycosaminoglycans with PMNE, it has been shown that in dependence on the chain length one single inhibitor molecule is able to bind more than one enzyme molecule (26). The importance of the polysaccharide chain length for the inhibitory potency (50) is confirmed by the presented data (Fig. 3B, Table 2). The relatively weak maximum activities of high-MW SC (compared to their low $K_i$) may be due to their decreased tendency to bind more than one PMNE molecule at high concentrations. Further, increase of the charge density of glycosaminoglycans has been described to result in improved activity (50, 51). This is in consistence with the presented strong correlation between the DS and the inhibitory activity (Figure 3A). In addition, the present study demonstrates that also the basic carbohydrate structure plays an important role (Figure 3C). This parameter has already been proven to influ-

<table>
<thead>
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<th>Assay</th>
<th>UFH</th>
<th>PulS1</th>
<th>PS3</th>
</tr>
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<tr>
<td>Inhibition in the chromogenic substrate assay</td>
<td>25 % +/- 1 %</td>
<td>42 % +/- 4 %</td>
<td>58 % +/- 4 %</td>
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<tr>
<td>Inhibition in the FITC-elastinolysis assay</td>
<td>12 % +/- 4 %</td>
<td>32 % +/- 8 %</td>
<td>41 % +/- 7 %</td>
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<td>Dissociation constant in the SPCEA assay without elastin</td>
<td>0.509 $10^6$</td>
<td>0.163 $10^6$</td>
<td>0.138 $10^6$</td>
</tr>
<tr>
<td>Dissociation constant in the SPCEA assay with elastin</td>
<td>1.29 $10^6$</td>
<td>0.801 $10^6$</td>
<td>0.345 $10^6$</td>
</tr>
</tbody>
</table>

1 expressed as % inhibition at 0.25 μg/ml final concentration
2 expressed as % inhibition at 1.5 μg/ml final concentration
3 expressed as molar dissociation constant (M)
4 expressed as molar dissociation constant (M)

Figure 6: Concentration-dependent inhibition (mean ± SD, n = 6) of cancer cell-mediated elastinolysis by PulS1 as measured by the amount of released FITC-elastin peptides.

Table 3: Comparison of the inhibitory activities of sulfated carbohydrates in the functional assays with their binding capacities in the SPC-EA assay.
ence also other activities such as anticoagulant, profibrinolytic and anti-complementary effects of SC (32, 52, 53). Compared to UFH, β-1,3- and α-1,4/1,6-glucan sulfates with the same DS and MW have weaker anticoagulant but much higher anti-complementary activity. Whereas α-1,4/1,6-glucan sulfates are more potent anticoagulants, β-1,3-glucan sulfates are better inhibitors of complement activation. According to structure-activity studies (38, 39), both the conformation of the α-1,4/1,6-glucan chain and the high content of secondary sulfate groups are favorable to anticoagulant activity. Regarding the elastase inhibition, β-1,3- and α-1,4/1,6-glucan sulfates, however, do not significantly differ. But as demonstrated by PulS4 (DS 1.28, 10 kd) and LMWH (DS 1.20, 10 kd), a glucan structure seems to be more suitable for interactions with PMNE than that of a glycosaminoglycan. UFH is not suitable for a comparison, since its MW is higher or lower than that of glucan sulfates with similar DS. Another example for the relevance of the polysaccharide structure is given by the linear hexosan PS3 and the branched pentosan PPS: The elastase inhibiting activity of PPS is lower than that of PS3, but its anticomplementary activity is somewhat higher (unpublished results). In conclusion, the interactions of SC with biomolecules are not exclusively based on charge effects. In dependence on the target, also the distribution of the sulfate groups and the basic structure of the carbohydrate chain with its respective conformational flexibility (54) are crucial parameters. As a consequence, by detailed structure-activity relationships studies the optimal structure parameters can be evaluated for a rational design of a carbohydrate derivative with a specific action profile. The potential of semisynthetic glucan sulfates as PMNE inhibitors becomes obvious from their significant higher activity compared to commercially available low molecular weight PMNE inhibitors (Fig. 1B).

An important feature for the development of enzyme inhibitors is the enzyme specificity, i.e. the effect of SC on other serine proteases. As a result of corresponding substrate assays (55), UFH and PS3 do not directly influence the amidolytic activity of trypsin, chymotrypsin, kallikrein, tPA, activated protein C, thrombin, and factor Xa (unpublished results). As an exception, PS3 concentrations higher than 10 μg/ml slightly inhibit u-PA and stimulate plasmin activity (56). However, due to the limited explanatory power of chromogenic substrate assays (57), more detailed experiments are needed to clarify the enzyme specificity of the glucan sulfates.

Heparin is known to increase the inhibition of PMNE by endogenous inhibitors (58, 59). As source of such inhibitors, we used pooled human plasma (Fig. 2A). Similar to heparin, also semisynthetic glucan sulfates potentiate PMNE inhibitors. The identification of the respective inhibitor, however, needs further studies. This experiment additionally demonstrates that the activity of glucan sulfates is not neutralized by plasma components, which is important regarding their use in vivo.

Although chromogenic substrate assays are widely used for screening methods, these small peptides are considerably different from the physiological substrates. Therefore, potential enzyme inhibitors have also to be tested using physiological substrates. In PMN, elastase is stored in the azurophilic granules and can either be released or expressed on the cell surface (60). The tight binding of PMNE to its different proteinaceous substrates, especially to the hydrophobic elastin (61), makes it difficult for any inhibitor to interfere. Correspondingly, UFH turned out to be only a weak inhibitor in the two proteinolysis assays (Fig. 4A, 4B). In contrast, the semisynthetic glucan sulfates represent potent inhibitors of the PMNE activity against both collagen and FITC-labelled elastin, even when PMNE has already bound to the substrate (Fig. 4C). The different inhibitory capacities of the SC in the three assays may be due to the different nature of enzyme-substrate interactions. The findings in the functional assays correlate well with the affinity of the SC to the enzyme: both, PulS1 and PS3, bind stronger to elastase than UFH (Fig. 5), which is confirmed by the $K_d$ values. Further, the binding studies revealed that the affinity of SC to PMNE is reduced in the presence of elastin (Table 3). This explains their lower activities in the proteinolysis assays compared to those in the chromogenic assay.

In addition to the tight binding of the enzyme to the substrate after recognition, PMNE producing inflammatory cells have several mechanisms to impede the action of endogenous inhibitors. So direct contact of cells with proteinaceous substrate allows local substrate degradation even in the presence of endogenous inhibitors (62). According to our findings in the tumor cell-mediated elastinolysis assay, SC are yet able to inhibit elastinolysis by cell-derived enzymatic activity (Fig. 6). Further studies are needed to evaluate whether this is due to direct inhibition of cell surface-bound enzyme or prevention of enzyme release or even inhibition of the expression of elastase.

In conclusion, the PMNE inhibitory activity of SC may contribute to the in vivo demonstrated inhibitory effects on inflammation and metastasis. As a result of the presented data, semisynthetic glucan sulfates with high DS ≥ 2.0 and a MW of 10-20 kDa are promising candidates for the future development of new antiinflammatory and antimetastatic drugs. In all the PMNE-assays, they are more active than heparins and also the semisynthetic PPS, but have lower anticoagulant activities (53), whereby a certain extent of antithrombotic activity is absolutely valuable for tumor and sepsis therapy (63, 13). Glucan sulfates with higher MW are better PMNE inhibitors, but exhibit anticoagulant activities similar to that of UFH. As shown by PS3 and PulS1, β-1,3-glucan sulfates tend to be superior particularly in the presence of elastin and have lower anticoagulant activity than α-1,4/1,6-glucan sulfates. Based on these structure activity relationships, it has to be established whether an even higher DS or a modification of the sulfation...
pattern of β-1,3-glucan sulfates selectively improves the PMNE inhibitory activity or whether further structural variations such as the substitution with lipophilic residues in analogy to the lipophilic character of elastin improves the affinity to PMNE. Another advantage of the semisynthetic glucan sulfates is their easy economic and reproducible production method. The starting polysaccharides are obtained from fast renewable resources and there is no risk of contamination as in the case of the animal product heparin.

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