P-Selectin Blocking Potency of Multimeric Tyrosine Sulfates
In Vitro and In Vivo

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Abstract—P-selectin blocking potency was investigated using synthetic monomeric and polymeric anionic compounds containing
sulfate groups such as O-sulfotyrosine (sTyr) and/or sulfated Lewis structures. A non-carbohydrate-containing polyacrylamide
conjugate sTyr-P AA (80% mol of sTyr) was a remarkably potent inhibitor of P-selectin binding in vitro, having an IC50 value of
6 ng/mL (equivalent to 10 nM calculated on the basis of sTyr residues or 0.1 nM calculated by the mass of the macromolecule). The
inhibitory effect of sTyr-P AA (80%) towards P-selectin is significantly greater than that of fucoidan (IC50, 100 ng/mL). However,
sTyr-P AA (80%) was less effective than fucoidan at reducing neutrophil extravasation in an in vivo rat model of peritonitis.

Leukocyte trafficking from blood vessels into sites of
inflammation is a co-operative multistep process involving
an initial leukocyte rolling on endothelium mediated via
the selectin family (E-, P- and L-selectins) of adhesion
molecules.1 The selectins share an ability to recognize the
tetrasaccharide SiaLe&, although the binding affinities for
monomeric SiaLe& and its mimetics are relatively low,
being in the millimolar range. A well-characterized physiologically relevant high affinity ligand for P- and L-
selectin is the neutrophil cell surface glycoprotein PSGL-1.
High affinity binding of P-selectin to PSGL is achieved via
interactions at two distinct sites. One interaction occurs
between SiaLe& borne on PSGL-1 and the lectin domain of
P-selectin, whereas the second interaction occurs between
a cluster of sulfated tyrosine (sTyr) residues on PSGL-1
and an anionic binding site on P-selectin.2

It is well established that multimeric negatively charged
molecules such as heparin, inositol hexaphosphate, sul-
fatide, and especially the polysaccharide fucoidan can
inhibit P-selectin-mediated interactions in vitro and in vivo.3–5 We have shown previously6 that a substituted polymeric template (polyacrylamide, PAA) is a valuable tool for exploring the molecular requirements for P-selectin inhibition. For example, the presentation of both SiaLe& and sTyr motifs on the same polymeric template (PAA) produced a synergistic inhibitory effect on P-selectin binding as compared to the effects of sTyr-P AA and SiaLe&–P AA alone or as a mixture. In this paper, we report that SiaLe-free polymers bearing densely situated sTyr residues are the most potent P-selectin blockers in our system, being more active in vitro than fucoidan, the most potent inhibitor described previously.

Materials
All PAA-based neoglycoconjugates (30-40 kDa) were
synthesized by standard methods.7,8 sTyr-P AA (80%)1

1The figure in brackets designates molar percent of the given ligand in
PAA-conjugate.
was synthesized using 200% excess of sTyr over the activated polymer and the degree of sTyr substitution was calculated by the increase in the mass of the conjugate. Recombinant human ZZ-selectin (monovalent) lacking the transmembrane and cytosolic domains was produced by Nicholas Smithers (Glaxo Wellcome, Stevenage) as a C-terminal chimera with the ZZ-domain of protein A. The tripeptide Tyr-Tyr-Tyr (Bachem, Germany) and aminoglutitol (Sigma) were per-O-sulfated with a \( \text{SO}_4/\text{Py} \) complex. Fucoidan was obtained from Sigma (USA).

P-selectin binding assay. In a 96-well plate assay human IgG was used as a primary coating reagent to immobilize recombinant human P-selectin via the ZZ-domain of the fusion protein. The working concentration of selectin was 3 ng/well. HSO\(_3\)Le\(_a\)-PAA-biotin \( \text{I} \) was chosen as the ligand because it showed greater binding efficacy in P-selectin assays than HSO\(_3\)Le\(_a\), HSO\(_3\)Le\(_e\), SiaLe\(_e\), and SiaLe\(_e\)-PAA. It should be noted that the activity series of P-selectin blockers did not depend on the taken ligand, for example, HSO\(_3\)Le\(_e\) versus SiaLe\(_e\). Details of the assay have been published previously.  

**Table 1.** Inhibition of binding of HSO\(_3\)Le\(_a\)-PAA-biotin to P-selectin by sulfate-containing and related compounds

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_{50}), ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multimeric</td>
<td></td>
</tr>
<tr>
<td>1. sTyr-PAA (80)</td>
<td>0.01 (6 ng/mL)</td>
</tr>
<tr>
<td>2. Fucoidan</td>
<td>0.1 (100 ng/mL)</td>
</tr>
<tr>
<td>Multimeric</td>
<td></td>
</tr>
<tr>
<td>3. SiaLe(_a)-polyacrylic acid</td>
<td>2</td>
</tr>
<tr>
<td>4. SiaLe(_e)-PAA-sTyr (20/10)</td>
<td>10</td>
</tr>
<tr>
<td>5. SiaLe(_e)-PAA-sTyr (20/max)</td>
<td>0.1</td>
</tr>
<tr>
<td>6. HSO(_3)Le(_a)-PAA-sTyr (15/5)</td>
<td>25</td>
</tr>
<tr>
<td>7. SiaLe(_e)-PAA</td>
<td>40</td>
</tr>
<tr>
<td>Monomeric</td>
<td></td>
</tr>
<tr>
<td>8. HSO(_3)Le(_e)-PAA</td>
<td>120</td>
</tr>
<tr>
<td>9. HSO(_3)O(_2)CH(_2)CH(_2)-PAA (80)</td>
<td>40 (6 pg/mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC(_{50}), ( \mu \text{M} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10. SiaLe(_a), SiaLe(_e)</td>
<td>&gt;1500</td>
</tr>
<tr>
<td>11. HSO(_3)Le(_a)</td>
<td>NI (1.5 mM)</td>
</tr>
<tr>
<td>12. sTyr</td>
<td>NI</td>
</tr>
<tr>
<td>13. sTyr-sTyr-sTyr</td>
<td>NI</td>
</tr>
<tr>
<td>14. Hexa-O-sulfo-aminoglucitol</td>
<td>NI</td>
</tr>
</tbody>
</table>

*Values for inhibition are the means of at least triplicate determinations. Standard deviations were less than 10%.

High-affinity binding of PSGL-1 to P-selectin requires the N-terminus of the PSGL-1 polypeptide chain to present both a glycan containing SiaLe\(_a\) as well as a cluster of sulfated tyrosine residues. The objective of the current work was to design an sTyr-based polymeric conjugate possessing P-selectin binding activity comparable to that of PSGL-1 and fucoidan. Since the three sulfated tyrosine residues in PSGL-1 are located in close proximity (sTyr\(_{46}\)-X-sTyr\(_{48}\)-X-sTyr\(_{52}\)), we attempted to cluster sulfated residues in a mimetic to achieve a maximal density of negative charge. Two low molecular weight compounds, sTyr-sTyr-sTyr (#13, Table 1) and hexa-O-sulfo-aminoglucitol (#14, Table 1) were synthesized in order to achieve a high density of HSO\(_3\) groups in the absence of a polymeric template. However, neither compounds inhibited P-selectin in the cell-free binding assay (Table 1). These results are consistent with the report that an 18-mer peptide derived from PSGL-1 containing the three sulfated tyrosine residues, but lacking carbohydrate modification, did not bind to P-selectin.

We then investigated the efficacy of P-selectin inhibition of polymeric PAA conjugates modified with an increasing degree of substitution with sTyr. Figure 1 shows that increases in sTyr loading on the PAA dramatically increased P-selectin-blocking potency. Increasing the sTyr substitution from 5 to 80% caused a 600-fold increase in...
potency, namely a decrease in IC₅₀ values from 6 µM to 10 nM, respectively, calculated on sTyr residues. The latter value extrapolates to a value of ~0.1 nM (6 ng/mL) when calculated for the whole macromolecule. It is noteworthy that this highly sulfated (80% sTyr) PAA conjugate is significantly more potent as an inhibitor of P-selectin than PSGL-1 or fucoidan.

As described above, the occupancy of two sub-sites is essential for high-affinity (Kₐ 0.3 µM) binding of P-selectin to PSGL-1: a carbohydrate-binding site (selective for SiaLeᵃ) and an anionic site (with affinity for sulfotyrosines). The synthetic 18-mer peptide derived from PSGL-1 and containing both a natural oligosaccharide and three sTyr residues binds to P-selectin with a Kₐ of 0.65 µM, whereas the same peptide free of sulfotyrosine binds with a Kₐ of 30 µM, and the peptide lacking fucose shows no binding at all. Furthermore, it is evident that the spatial organization of SiaLeᵃ and sTyr on the PSGL-1 polypeptide chain is extremely important. Presentation of the SiaLeᵃ tetrasccharide on the core 1 O-chain results in a much lower binding affinity than when it is presented on the core 2 chain. Previous work has shown that optimal activity of complex inhibitors is achieved when the SiaLeᵃ and sTyr motifs are presented on the same template. The individual mono-ligand moieties have much weaker activity. Fucoidan (whose structure is still debated) should be considered as a bi-functional ligand rather than a mono-functional ligand, because its polysaccharide chain contains both dense O-sulfo groups as well as fucose residues.

We next investigated the influence of the nature of the anionic groups on P-selectin inhibitory activity. Firstly, the conjugate HSO₃Leᵃ-PAA-sTyr (15/5) (#6, Table 1) was found to be 60 times less active than sTyr-PAA (20%) even though the latter compound contains with the same concentration (15 + 5) of sulfated Tyr residues. Furthermore, it is evident that the spatial organization of SiaLeᵃ and sTyr on the PSGL-1 polypeptide chain is extremely important. Presentation of the SiaLeᵃ tetrasccharide on the core 1 O-chain results in a much lower binding affinity than when it is presented on the core 2 chain. Previous work has shown that optimal activity of complex inhibitors is achieved when the SiaLeᵃ and sTyr motifs are presented on the same template. The individual mono-ligand moieties have much weaker activity. Fucoidan (whose structure is still debated) should be considered as a bi-functional ligand rather than a mono-functional ligand, because its polysaccharide chain contains both dense O-sulfo groups as well as fucose residues.

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Fig. 1. The inhibitory potency in vitro of five sTyr-PAA conjugates containing variable amounts of sTyr was compared with the polysaccharide fucoidan. The in vitro assay system is based on the inhibition of the binding of HSO₃OCH₂CH₂-PAA-biotin to recombinant P-selectin: (5%) sTyr-PAA, (10%) sTyr-PAA, (15%) sTyr-PAA, (20%) sTyr-PAA, (no preparation).

These results show that by using polymers highly substituted with sulfotyrosine it is possible to achieve high-affinity binding to P-selectin without the need to incorporate a carbohydrate ligand. This conclusion is exemplified by the conjugate sTyr-PAA (80%), which had an IC₅₀ of 10 nM. The precise nature of the interaction of sTyr-PAA (80%) with P-selectin is unclear, although it is likely that the stoichiometry of the interaction of sTyr-PAA with P-selectin in the solid phase assay is 1:1 and that the high activity is not the result of cross-binding between one molecule sTyr-PAA with several P-selectin molecules. It is possible that a cluster of three sTyr residues in the conjugate bind to cognate

Table 2. Comparison of the inhibition of neutrophil extravasation in a rat model of peptone-induced peritonitis by sialylated and sulfated PAA-conjugates, fucoidan and free SiaLeᵃ tetrasccharide

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Number of rats in group</th>
<th>Number of neutrophils per rat x 10⁻⁶</th>
<th>Mean inhibition (% to control)</th>
<th>Dose, mg per rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>12</td>
<td>41.4 ± 5.4</td>
<td></td>
<td>1.5-3.0</td>
</tr>
<tr>
<td>SiaLeᵃ-PAA-sTyr 20/10</td>
<td>8</td>
<td>19.0 ± 3.8</td>
<td>54 (p &lt; 0.01)</td>
<td>1.0-3.0</td>
</tr>
<tr>
<td>SiaLeᵃ</td>
<td>11</td>
<td>37.0 ± 5.6</td>
<td>11</td>
<td>1.0</td>
</tr>
<tr>
<td>SiaLeᵃ-PAA</td>
<td>9</td>
<td>30.2 ± 6.2</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Series 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>19</td>
<td>32.7 ± 2.9</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>(no preparation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SiaLeᵃ-PAA (80%)</td>
<td>5</td>
<td>11.4 ± 0.6</td>
<td>65 (p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>SiaLeᵃ-PAA (80%)</td>
<td>5</td>
<td>23.5 ± 3.8</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Fucoidan</td>
<td>10</td>
<td>2.5 ± 0.5</td>
<td>92 (p &lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>HSO₃OCH₂CH₂-PAA (80%)</td>
<td>5</td>
<td>17.4 ± 5.8</td>
<td>47 (p &lt; 0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM.
positively charged amino acid residues in the sTyr-binding site of P-selectin and that, in addition, the lectin domain of P-selectin is shielded sterically by the large polymeric conjugate. One cannot exclude the possibility that a key sTyr residue on the polymer interacts with a positively charged residue within the domain, for example the key Arg residue which is thought to bind to the carboxyl group of SiaLe\(^\alpha\) or nearly situated Lys 111 residue. This model, which represents a two-site binding model, would explain the need for both a cluster of sTyr residues and a large polymer to achieve high affinity binding with synthetic inhibitors.

**In vivo model of inflammation.** The in vivo anti-inflammatory efficacy of P-selectin inhibitors was investigated using a rat model of acute peptide-induced peritonitis. The peritonitis was evaluated by quantifying neutrophil extravasation into the peritoneal cavity. Only fucoidan, and three of the synthetic conjugates (SiaLe\(^\alpha\)-PAA-sTyr, sTyr-PAA and HSO\(_3\)OCH\(_2\)CH\(_2\)-PAA) significantly blocked neutrophil extravasation (Table 2). The sTyr-PAA (80) conjugate, which had shown the highest potency for P-selectin inhibition in vitro, was less active in vivo than fucoidan. A similar loss of potency in vivo has been observed with inositol hexaphosphate, which was considerably less active in vivo (IC\(_{50}\) of approx. 70 mg/kg) than expected from the in vitro potency.\(^5\)

To date, only recombinant PSGL-1 at \(\sim 1\) mg/kg has an in vivo activity approaching that of fucoidan.\(^16\),\(^17\) Low activity of synthetic high molecular weight blockers of P-selectin has been observed and discussed previously.\(^13\) Many factors could influence in vivo activity, including compound pharmacokinetics and binding to other proteins, which could reduce the effective free concentration of the inhibitor. In addition, other mechanisms than the interaction of PSGL-1 and P-selectin may contribute to neutrophil extravasation in vivo. Nevertheless, the current study demonstrates that a non-carbohydrate-containing conjugate sTyr-PAA (80) is the most potent reported inhibitor of P-selectin in vitro (IC\(_{50}\) 10 nM) and shows appreciable ability to block neutrophil extravasation in vivo (\(\sim 50\%\) reduction, 2 mg/rat). This work provides a better understanding of selectin-inhibitor interactions which may lead to the development of non-carbohydrate inhibitors for use as therapeutic agents in inflammatory disorders such as asthma and inflammatory bowel disease, but also in sepsis or in the acute respiratory distress syndrome.

**Acknowledgements**

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**References and Notes**