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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–PAA (80% mol of sTyr) was a remarkably potent inhibitor of P-selectin binding in vitro, having an IC_{50} 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–PAA (80%) towards P-selectin is significantly greater than that of fucoidan (IC_{50}, 100 ng/mL). However, sTyr–PAA (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 $SiaLe^x$, although the binding affinities for monomeric $SiaLe^x$ 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 $SiaLe^x$ 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, sulfatide, and especially the polysaccharide fucoidan can inhibit P-selectin-mediated interactions in vitro and in vivo.[3–5] We have shown previously[6] 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 $SiaLe^x$ 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-PAA and $SiaLe^x$–PAA 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-PAA (80%)[†]

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[†]The figure in brackets designates molar percent of the given ligand in PAA-conjugate.

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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.[9] The tripeptide Tyr-Tyr-Tyr (Bachem, Germany) and aminoglucitol (Sigma) were per-*O*-sulfated with a SO_3/Py complex.[10] 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_3Le^a-PAA-biot[11] was chosen as the ligand because it showed greater binding efficacy in P-selectin assays than HSO_3Le^a-, HSO_3Le^x-, $SiaLe^a$-, and $SiaLe^x$–PAA. It should be noted that the activity series of P-selectin blockers did not depend on the taken ligand, for example, HSO_3Le^a versus $SiaLe^x$. Details of the assay have been published previously.[6,12,13]

Rat peritoneal inflammation model. The acute rat peritonitis model was performed as described earlier[14] with some modifications. 10 mL of PBS containing 10% peptone was injected intraperitoneally into female Wistar rats (180–220 g). After 3 h, the rats were sacrificed and their peritoneal cavities were lavaged with 30 mL PBS containing 60 units/mL of heparin, 0.02% EDTA and 0.03% bovine serum. The total cell number in the lavage fluid was counted and the cell suspension was concentrated by centrifugation at $400\times g$ for 10 min. After 1:1 dilution with bovine serum smears were prepared and stained by the Pappenheim method. Neutrophils were counted on two parallel slides, and the total neutrophil number per rat was calculated. The inhibitors were administrated intravenously as a single dose in 0.3 mL of sterile 0.9% NaCl at 15 min after peptone injection. The same volume of NaCl solution was injected to the control animals. A difference between the control and tested groups was analysed for statistical significance based on Student's *t*-test. *p*-values <0.05 were considered as significant.

The current results and previous data with synthetic multivalent inhibitors[12] indicate that the degree of negative charge on P-selectin ligands has a major effect on their inhibitory potency. Highly anionic compounds represent some of the most potent known P-selectin inhibitors. For example, a polyacrylamide conjugate presenting $SiaLe^a$ inhibits P-selectin with IC_{50} of 40 μM (calculated by $SiaLe^a$ residues) or 0.4 μM (calculated using the mass of the whole macromolecule).[12] Sulfatide (3-*O*-sulfated galactocerebroside) and some of its synthetic analogues have IC_{50} values within the range 0.1–1 μM[4], whereas the highly sulfated polysaccharide fucoidan is one of the most potent known inhibitors, having an IC_{50} value of 0.1 μM (calculated on the assumption that fucoidan is a regular polysaccharide with a repeating hexasaccharide unit).[12,15] The potency of fucoidan as an inhibitor of P-selectin binding in in vitro assays is similar to the binding affinity of the natural ligand for P-selectin, PSGL-1 (K_d, 0.3 μM).[16,17] It has also been reported that a synthetic sulfated polymer, a soluble fraction of Amberlite IRC-84 resin displayed good inhibition of P-selectin, having an IC_{50} value of 0.3 μM.[18] Relatively weak low molecular weight inhibitors of P-selectin have also been described, including myoinositol hexaphosphate (IC_{50}, 160 μM), myoinositol pentaphosphate (IC_{50}, 260 μM), and myoinositol hexasulfate (IC_{50}, 2.8 mM).[5]

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 $SiaLe^x$ 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-X-$sTyr_{51}$)[2], 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.[2]

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

Table 1. Inhibition of binding of HSO_3Le^a-PAA-biotin to P-selectin by sulfate-containing and related compounds

Inhibitor	IC_{50}, μM[a]
Multimeric	
1. sTyr–PAA (80)	0.01 (6 ng/mL)
2. Fucoidan	0.1 (100 ng/mL)[b]
3. $SiaLe^a$–polyacrylic acid	2
4. $SiaLe^a$–PAA–sTyr (20/10)[c]	10
5. $SiaLe^a$–PAA–sTyr (20/max)[d]	0.1
6. HSO_3Le^a–PAA–sTyr (15/5)	25
7. $SiaLe^a$–PAA	40
8. HSO_3Le^a–PAA	120
9. $HSO_3OCH_2CH_2$–PAA (80)	40 (6 μg/mL)
Monomeric	
10. $SiaLe^a$, $SiaLe^x$	>1500
11. HSO_3Le^a	NI (1.5 mM)
12. sTyr	NI
13. sTyr-sTyr-sTyr	NI
14. Hexa-*O*-sulfo-aminoglucitol	NI

[a]Values for inhibition are the means of at least triplicate determinations. Standard deviations were less than 10%.
[b]Calculation of molar concentration based on the assumption that a hexasaccharide is the active unit.
[c]The figure in brackets designates a mol.% of the given ligand in the polyacrylamide conjugate; two figures via '/' mean two ligands; no sign represents 20 mol.%.
[d]'max' means that after introduction of 20 mol.% carbohydrate ligand to the polymer it was subjected to the action of excess sTyr resulting in maximum possible content of sTyr, which equates to about 60%.

potency, namely a decrease in IC_{50} 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 (K_d 0.3 μM) binding of P-selectin to PSGL-1: a carbohydrate-binding site (selective for $SiaLe^x$) and an anionic site (with affinity for sulfotyrosines).[2] 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 K_d of 0.65 μM, whereas the same peptide free of sulfotyrosine binds with a K_d of 30 μM, and the peptide lacking fucose shows no binding at all.[2] The binding affinity of the monosulfated peptide ($sTyr_{48}$) was ten fold lower than that with peptide containing three sulfated Tyr residues.[2] Furthermore, it is evident that the spatial organization of $SiaLe^x$ and sTyr on the PSGL-1 polypeptide chain is extremely important. Presentation of the $SiaLe^x$ tetrasaccharide on the core 1 O-chain results in a much lower binding affinity than when it is presented on the core 2 chain.[2] Previous work has shown that optimal activity of complex inhibitors is achieved when the $SiaLe^x$ and sTyr motifs are presented on the same template. The individual mono-ligand moieties have much weaker activity.[6,12,19] 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 HSO_3Le^a-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 groups (see Fig. 1). Secondly, the PAA-derivative of $HSO_3OCH_2CH_2$- (#9, Table 1) was less active by three orders of magnitude than the corresponding multivalent derivative of sTyr

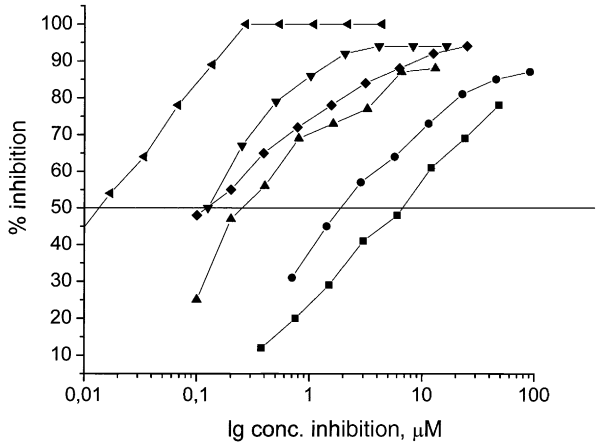

Figure 1. The inhibitory potency in vitro of five sTyr–PAA conjugates containing variable amounts of sTyr was compared with the polysaccharide fucoidan. The in vitro assay system is based on the inhibition of the binding of HSO_3Le^a-PAA-biotin to recombinant P-selectin. ■ 5% sTyr–PAA, ● 10% sTyr-PAA, ▲ 15% sTyr–PAA, ▼ 20% sTyr–PAA, ◆ fucoidan, ◀ sTyr(max)–PAA.

(#1, Table 1). This result suggests that the interaction of P-selectin with sTyr-PAA is not exclusively charge-based, but is influenced considerably by the presence of an aromatic tyrosine residue. Thirdly, the $SiaLe^a$-polyacrylic acid derivative (#3, Table 1) containing 20 mol% of oligosaccharide and 80 mol% of carboxyl groups was 20 times less potent than a similar conjugate containing sulfotyrosine, $SiaLe^a$–PAA–sTyr (#5, Table 1).

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 IC_{50} 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 $SiaLe^x$ tetrasaccharide

Preparation	Number of rats in group	Number of neutrophils per rat $\times 10^{-6}$	Mean inhibition (% to control)	Dose, mg per rat
Series 1				
Control group (no preparation)	12	41.4 ± 5.4		—
$SiaLe^a$–PAA–sTyr (20/10)	8	19.0 ± 3.8	54 ($p < 0.01$)	1.5–3.0
$SiaLe^x$	11	37.0 ± 5.6	11	1.0–3.0
$SiaLe^x$–PAA	9	30.0 ± 6.2	27	1.0
Series 2				
Control group (no preparation)	19	32.7 ± 2.9		—
sTyr–PAA (80)	5	11.4 ± 0.6	65 ($p < 0.001$)	2.0
sTyr–PAA (80)	5	23.5 ± 3.8	28	1.0
Fucoidan	10	2.5 ± 0.5	92 ($p < 0.001$)	1.0
$HSO_3OCH_2CH_2$–PAA (80)	5	17.4 ± 5.8	47 ($p < 0.05$)	2.0

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 $SiaLe^x$ 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 peptone-induced peritonitis. The peritonitis was evaluated by quantifying neutrophil extravasation into the peritoneal cavity. Only fucoidan and three of the synthetic conjugates ($SiaLe^a$–PAA–sTyr, sTyr–PAA and $HSO_3OCH_2CH_2$–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 ~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 (~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.

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