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Monomeric and Multimeric Blockers of Selectins: Comparison of *in vitro* and *in vivo* Activity

N. A. Ushakova[1], M. E. Preobrazhenskaya[1], M. I. Bird[2], R. Priest[2], A. V. Semenov[3], A. V. Mazurov[3], N. E. Nifantiev[4], T. V. Pochechueva[5], O. E. Galanina[5], and N. V. Bovin[5]*

[1]Orekhovich Institute of Biomedical Chemistry, Russian Academy of Medical Sciences, ul. Pogodinsksya 10, 119121 Moscow, Russia; E-mail: natalia.ushakova@ibmc.msk.ru
[2]Discovery Research, GlaxoSmithKline Ltd, Stevenage, UK; E-mail: mike.i.bird@gsk.com
[3]Institute of Experimental Cardiology, Cardiology Research Center, Russian Ministry of Health, ul. 3-ya Cherepkovskaya 15A, Moscow, Russia
[4]Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, 117913 Moscow, Russia; E-mail: nen@ioc.ac.ru
[5]Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (7-095) 330-5592; E-mail: bovin@carbohydrate.ru

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Abstract—The potency of the oligosaccharides SiaLe^x, SiaLe^a, HSO_3Le^x, and HSO_3Le^a, their conjugates with polyacrylamide (PAA, 40 kD), and other monomeric and polymeric selectin inhibitors has been compared with that of the polysaccharide fucoidan. The following assay systems were used: 1) a 96-well assay based either on the use of recombinant E-, P-, and L-selectins or an analogous assay with natural P-selectin isolated from human platelets; 2) a platelet-based P-selectin cell assay; and 3) a rat model of peritoneal inflammation. IC_{50} values for the neoglycoconjugate SiaLe^a-PAA were 6, 40, and 85 μM for recombinant E-, P-, and L-selectins, respectively; all monomeric inhibitors were about two orders of magnitude weaker. PAA-conjugates, containing as a ligand tyrosine-*O*-sulfate (sTyr) in addition to one of the sialylated oligosaccharides, were the most potent synthetic blockers *in vitro*. Compared with fucoidan, the most potent known P- and L-selectin blocker, the bi-ligand glycoconjugate HSO_3Le^a-PAA-sTyr displayed similar inhibitory activity *in vitro* towards L-selectin and about ten times lower activity towards P-selectin. All of the tested synthetic polymers displayed a similar ability to inhibit neutrophil extravasation in the peritonitis model (*in vivo*) at 10 mg/kg. The data provide evidence that monomeric SiaLe^x is considerably more effective as a selectin blocker *in vivo* than *in vitro*, whereas the opposite is true for fucoidan and the bi-ligand neoglycoconjugate HSO_3Le^a-PAA-sTyr.

Key words: fucoidan, HSO_3Le^x, HSO_3Le^a, inflammation, neoglycoconjugates, platelets, polyacrylamide, selectins, SiaLe^x, SiaLe^a, tyrosine-*O*-sulfate

Leukocyte trafficking from blood vessels into sites of inflammation is a cooperative multistep process, the first event being leukocyte interaction with the surface of endothelial cells of blood vessels accompanied by blood cell migration along the vessel wall (leukocyte rolling) that is mediated by the selectin family of adhesion molecules (C-type lectins): L-selectin is constitutively expressed on the surface of circulating leukocytes, E-selectin expression on endothelial cells results from cytokine inducible *de novo* synthesis, whilst P-selectin is stored in the membranes of intracellular granules of platelets and endothelial cells and is rapidly translocated to the cell surface upon their activation [1-5]. The selectins share the ability to recognize the tetrasaccharide SiaLe^x. The binding affinities for SiaLe^x are low, being in the micromolar to millimolar range. To date only limited information is available on the nature of the natural ligands that mediate rolling. A number of neutrophil glycoproteins such as leukosialin, LAMPs, CEA-like proteins, and LFA-1 has been reported to act as P-selectin (and/or E-selectin) ligands. However, only one neutrophil glyco-

Abbreviations: SiaLe^x) sialyl Lewis X, Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc; SiaLe^a) sialyl Lewis A, Neu5Acα2–3Galβ1–3(Fucα1–4)GlcNAc; HSO_3Le^x) 3′-sulfo-Lewis X, 3′-HSO_3Galβ1–4(Fucα1–3)GlcNAc; HSO_3Le^a) 3′-sulfo-Lewis A, 3′-HSO_3Galβ1–3(Fucα1–4)GlcNAc; sTyr) tyrosine-*O*-sulfate; PAA) polyacrylamide; ZZ) IgG-binding fragment of protein A; biot) biotin residue.

* To whom correspondence should be addressed.

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protein, PSGL-1, is known to generate high affinity biologically relevant binding with P-selectin. This binding involves a two-site interaction between P-selectin and both $SiaLe^x$ and sulfated tyrosine (sTyr) residues present on the polypeptide backbone of PSGL-1 [6]. L-Selectin can recognize PSGL-1 and GlyCAM-1, a murine mucin-like glycoprotein, bearing sulfate on the $SiaLe^x$-glycan [7]. Candidates for endothelial L-selectin ligands include another mucin-type glycoprotein CD34 [7] and also MAd-CAM-1 [8]. ESL-1, a murine glycoprotein bearing a $SiaLe^x$ motif on a branched N-chain, has been described as a murine ligand for E-selectin [9].

The question of how selectins generate specific recognition remains unsolved, and it is unclear whether selectins exist as monomers or multimers on the cell membrane [10]. In this paper we describe the ability of monomeric oligosaccharides and their polymeric forms, including a polymer containing both $SiaLe^x$ and sTyr, to block selectins. We compare relative activities of the inhibitors *in vitro* and *in vivo* to elucidate role of multivalency in selectin–ligand interactions.

MATERIALS AND METHODS

Materials. Heparin, Tween 20, human immunoglobulin, bovine serum albumin (BSA), and fucoidan from *Fucus vesiculosus* (F5631) were from Sigma (USA). Fucoidan was also isolated by Dr. A. I. Usov (Zelinsky Institute of Organic Chemistry) from *Laminaria saccharina* (it contained 40.5% L-fucose and 26.7% sulfate) [11]. Both preparations demonstrated similar activity in preliminary experiments. Peptone was from Reakhim (Russia) and streptavidin–peroxidase conjugate was obtained from Boehringer Mannheim (Germany). All other chemicals were of analytical grade from Fluka (Switzerland). 96-well microtiter MaxiSorp immunoplates were from Nunc (Denmark). Monomeric $SiaLe^a$, $SiaLe^x$, HSO_3Le^a, and HSO_3Le^x as 3-aminopropyl glycosides were synthesized as described earlier [12, 13]. Polymeric neoglycoconjugates containing 20 mole % of carbohydrate, namely $SiaLe^a$-PAA, $SiaLe^x$-PAA, HSO_3Le^a-PAA, and HSO_3Le^x-PAA, as well as biotinylated samples were obtained from Syntesome GmbH (Germany). Other neoglycoconjugates were prepared according to published methods [14].

Recombinant selectins-ZZ (monovalent) lacking the transmembrane and cytosolic domains were produced by Nicholas Smithers (GlaxoSmithKline, UK) as C-terminal chimeras with ZZ domain of protein A. The ZZ domain binds tightly to human and rabbit IgG. A baculovirus/insect cell expression system was used, and the IgG-purified proteins were characterized by SDS-PAGE and in cell adhesion assays [15, 16].

Murine anti P-selectin mAb CRC81, which does not block P-selectin–ligand interaction, was obtained as described [17]. Affinity-purified rabbit polyclonal antibodies against P-selectin and against platelet glycoprotein IIb–IIIa complex were kindly provided by Dr. Berndt (Beiner Medical Research Institute, Melbourne, Australia).

Platelet membrane P-selectin was purified by affinity chromatography on a column with immobilized CRC81 mAb according to the method described by Skinner et al. [18]. Human platelets were prepared from healthy donor blood by consecutive washing as described [19]. Washed platelets were used as a suspension in Tyrode/HEPES solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 0.1% glucose, 5 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.35).

Selectin-ZZ binding assay. 96-well plates were coated with human IgG (Sigma) (10 μg/ml) in 0.05 M Na-carbonate buffer, pH 9.6, for 1 h at 37°C, and blocked with 3% bovine serum albumin (BSA) in buffer A (20 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$) for 1 h at 37°C. Plates were washed three times with buffer A containing 0.1% Tween 20, and then 100 μl of selectin-ZZ was added at a concentration of 30 (E-) or 60 (P- or L-selectin) ng/ml in buffer A with 0.3% BSA. Plates were incubated for 1 h at 37°C and then overnight at 4°C, followed by washing three times with buffer A containing 0.1% Tween 20. The plates were then incubated with HSO_3Le^a-PAA-biot (10 μg/ml in buffer A with 0.3% BSA) for 2 h at 37°C, washed, and finally incubated with a streptavidin–peroxidase conjugate (1 : 1000). Color was developed by 30-min incubation in 0.1 M sodium phosphate/0.1 M citric acid buffer containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2. The reaction was stopped by addition of 50 μl 1 M H_2SO_4, and the absorbance was read at 492 nm in a microtiter plate reader. A blank reaction was performed by omitting the biotinylated neoglycoconjugate. The background absorbance was subtracted from the final reaction optical density to give net absorbance values.

For the evaluation of inhibitors, the compound under test was added simultaneously with HSO_3Le^a-PAA-biot. Percent of inhibition was calculated as: $(OD_A - OD_I) \cdot 100/OD_A$, where OD_A is the mean value of optical density in absence of inhibitor and OD_I is the mean value of optical density in presence of inhibitor.

Antibody-capture P-selectin assay. Plates were coated with mAb CRC81 in PBS (10 μl, 10 μg/ml) for 1 h at 37°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS/Tween) and blocked for 1 h at 37°C with 150 μl of 2% BSA in PBS/Tween. P-Selectin in PBS containing 0.2% Triton X-100 (100 μl, 5 μg/ml) was added for 1 h at 37°C, and unbound P-selectin was removed with PBS/Tween. $SiaLe^a$-PAA-biot (10-20 μg/ml) alone or together with inhibitor was added in 100 μl PBS containing 1 mM $CaCl_2$ and incubated for 40 min at 20°C. The plates were washed five times, and peroxidase–streptavidin (100 μl/well, dilution 1 : 1500) in

PBS/Tween was added. The plates were incubated for 45 min at 37°C. After washing, plates were developed with *o*-phenylenediamine/H_2O_2 and monitored at A_{492}.

Platelet assay. Washed platelets were coated onto 96-well plates as described previously [19]. Briefly, 10^7 cells (100 μl) per well in Tyrode/HEPES solution were added onto plates, sedimented by centrifugation at 1000*g* for 5 min, and incubated for 30 min at 37°C. Plates were washed three times and blocked for 1 h at 37°C with 150 μl 2% BSA in Tyrode/HEPES solution. The subsequent steps were the same as described for the antibody-capture P-selectin assay, except that Tyrode solution was used instead of PBS/Tween.

Acute peritonitis model. Five milliliters of 4% peptone solution in 0.9% NaCl was administered intraperitoneally to female Wistar rats (about 200 g) under ether anesthesia to induce peritoneal inflammation. An equal volume of 0.9% NaCl (blank) was similarly administered to control rats. Three hours later, the animals were anesthetized and decapitated. Peritoneal lavage was performed by injecting 30 ml of medium containing PBS, 60 units/ml heparin, 0.02% EDTA, and 0.3% bovine serum, vigorously massaging of peritoneal wall for 1 min, and removing the injected liquid. Total cells were counted using a cell counting camera. To count neutrophils the cell suspension was centrifuged at 400*g* for 10 min. The concentrated suspension was diluted with bovine serum (1 : 1), and smears were made and then stained by the Pappenheim method. Neutrophils were counted on two parallel smears, 300-600 cells on each. The neutrophil number in peritoneal lavage was calculated from the neutrophil percent and total cell number. Inhibitors were injected into the rat femoral vein under anesthesia 15 min after peptone injection in 0.25 ml sterile 0.9% NaCl. The control animals were injected intravenously with 0.25 ml sterile 0.9% NaCl.

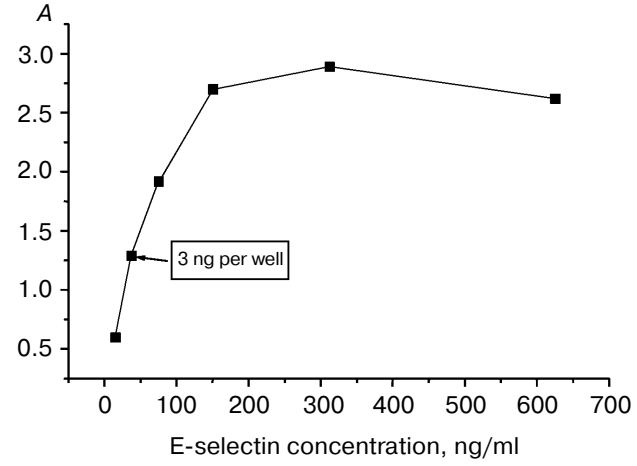

Fig. 1. Optimization of E-selectin loading in the solid phase assay. A 96-well plate was coated with a maximal concentration of IgG (see "Materials and Methods") followed by selectin-ZZ over a range of concentrations of 1-20 ng per well. Concentration of 3 ng per well, which gave 50% of the maximal *A* value, was used in further experiments.

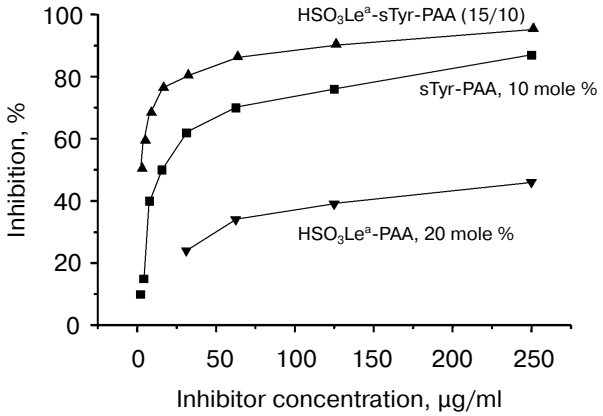

Fig. 2. Effect of tyrosine-*O*-sulfate incorporation into HSO_3Le^a-PAA neoglycoconjugate on its inhibitory potency towards P-selectin. The selectin-ZZ assay is described in "Materials and Methods" section. Inhibition data for HSO_3Le^a-PAA (the conjugate without sTyr) and sTyr-PAA (without HSO_3Le^a) are shown for comparison.

RESULTS

***In vitro* assays.** The inhibitory characteristics of soluble monomeric and polymeric carbohydrate-based selectin blockers were evaluated *in vitro* in three types of assay: 1) an assay employing recombinant human E-, P-, or L-selectins; 2) an assay employing P-selectin purified from human platelets, and 3) a cell-based P-selectin-dependent assay employing immobilized human platelets.

The first assay utilized human IgG as a primary coating reagent to immobilize recombinant E-, P-, or L-selectin via the ZZ-domain of the fusion protein. A working concentration of E-selectin of 3 ng/well was selected based on the data shown in Fig. 1 (see the figure legend). Similarly, an optimal coating of 6 ng/well was selected for P- and L-selectins. To optimize assay conditions, a range of concentrations of selectins (20 ng/ml-20 μg/ml) and the glycoconjugate ligand HSO_3Le^a-PAA-biot (1-50 μg/ml) were evaluated. Of the biotinylated PAA-glycoconjugates tested (HSO_3Le^a-PAA-biot, HSO_3Le^x-PAA-biot, $SiaLe^a$-PAA-biot, and $SiaLe^x$-PAA-biot), the HSO_3Le^a-PAA-biot was chosen for future experiments because it showed more uniform level of binding for all three ZZ-selectins (data not shown). The inhibitory efficacy of soluble monomeric and polymeric compounds in E-, P-, and L-selectin assays is presented in Table 1. The IC_{50} value of 20 μM for $SiaLe^x$-PAA in

Table 1. Inhibition of HSO_3Le^a-PAA-biot binding to ZZ-selectins by multimeric and monomeric compounds (concentration giving 50% inhibition*, μM)**

Inhibitor	E-selectin	P-selectin	L-selectin
Multimeric			
$SiaLe^x$-PAA	20	40	40
$SiaLe^a$-PAA	6	40	85
HSO_3Le^x-PAA	120	250	NI (250 μM)
HSO_3Le^a-PAA	60	120	250
$SiaLe^a$-PAA-sTyr (20/10)***	30	10	150
HSO_3Le^a-PAA-sTyr (15/10)***	25	2	2
$HSO_3OCH_2CH_2$-PAA	NI (600 μM)	300	1200
sTyr-PAA (10)***	NI (300 μM)	20	100
αMan-PAA	NI	100	800
Le^a-PAA	NI	NI	NI
Fucoidan (*L. saccharina*)	NI	0.1 (0.1 μg/ml)****	0.8 (0.8 μg/ml)****
Monomeric			
$SiaLe^x$	NI	NI	NI
$SiaLe^a$	400	>1500	1500
HSO_3Le^x	NI (1.5 mM)	NI (1.5 mM)	NI (1.5 mM)
HSO_3Le^a	NI (1.5 mM)	NI (1.5 mM)	NI (1.5 mM)
αManOR*****	NI	NI	NI
Man	NI	NI	NI

Note: NI, no inhibition.
* Values for inhibition were the means of at least triplicate determinations. Standard deviations (not shown) were less than 10%.
** Molar concentrations were calculated for carbohydrate ligand.
*** Value in brackets indicates molar percent of the given ligand(s) in composition of polyacrylamide conjugate; values divided by a slash indicate a bi-ligand conjugate; if not indicated, molar content is 20 mole %.
**** Molar concentration was calculated assuming that the active unit is a hexasaccharide.
***** For number of aliphatic and aromatic α-glycosides.

the current E-selectin assay (Table 1) was similar to the value (7 μM) obtained previously [16] in a scintillation proximity assay. We also observed comparable inhibitory potency of $SiaLe^x$-PAA in the two P-selectin assays: the P-selectin-ZZ assay and the platelet P-selectin assay (see below). The inhibitory potency of the tri- and tetrasaccharides in their multimeric, neoglycoconjugate form is about 100-fold higher than that of the best monomer ($SiaLe^a$), which is in accordance with other data [16, 20]. The 3′-sulfated Le^x polymer was a poor inhibitor of all three selectins; the most potent polymer was 3′-sialylated Le^a-PAA.

Polymeric α-mannoside Man-PAA (used as a surrogate of $SiaLe^x$-PAA [21]) moderately blocks P-selectin, but not L-selectin, at a concentration of 100 μM, whereas monomeric mannose or α-mannosides do not inhibit at all. Most of the synthetic PAA glycoconjugates, as well as two natural sulfated polysaccharides, were also tested in P-selectin assays either employing P-selectin purified from platelets or in a cell-based assay with adherent platelets. The most potent synthetic inhibitors were the bi-ligand glycoconjugates $SiaLe^a$-PAA-sTyr and HSO_3Le^a-PAA-sTyr (where sTyr represents a tyrosine-*O*-sulfate moiety in the copolymer). Both compounds are much more active blockers of P- and L-selectins compared with the corresponding sTyr-free polymers (see Table 1 and Fig. 2). We observed a similar relationship (4-fold increase in potency with $SiaLe^x$-PAA-sTyr) in a scintillation proximity assay [16].

Fucoidans (polymerized fucose sulfates) are the most potent known inhibitors of P- and L-selectins. The IC_{50} for fucoidan from *L. saccharina* was 0.1 and 0.8 μg/ml for P- and L-selectins, respectively (Table 1). Assuming the repeating fragment of this polysaccharide is a hexasaccharide (the true size has not been defined yet), we can re-calculate the IC_{50} as 0.1 μM (P-selectin) and

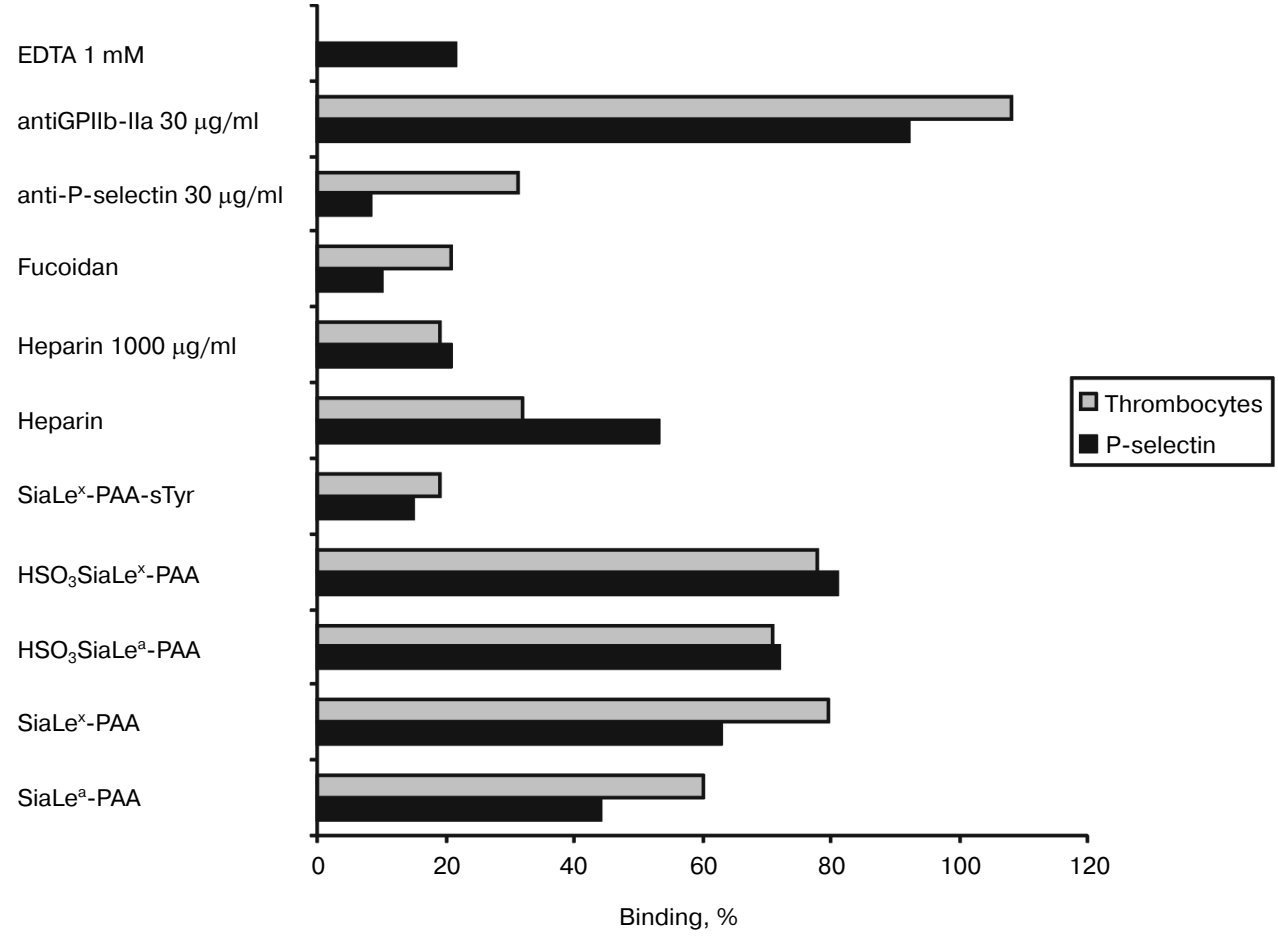

Fig. 3. Comparison of the effects of various compounds on the binding of SiaLe^a-PAA-biot to human platelets or P-selectin purified from human platelets. Unless otherwise specified, inhibitor concentration was 100 μg/ml. Assay components were as described in "Materials and Methods" section. Bars are means of duplicate determinations; control reaction was performed by omitting inhibitor.

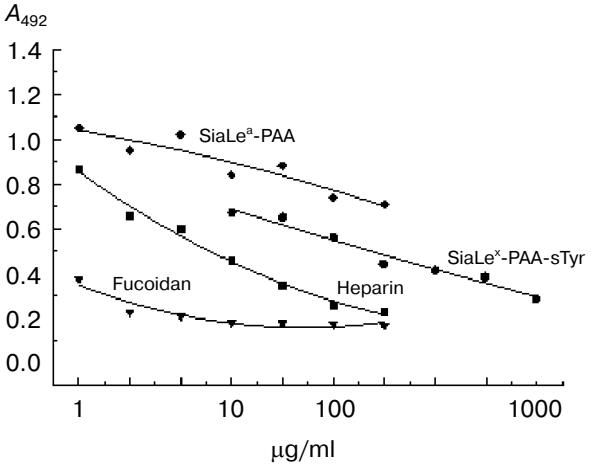

Fig. 4. Inhibition of P-selectin-mediated binding of SiaLe^a-PAA-biot to human platelets by SiaLe^a-PAA, SiaLe^x-PAA-sTyr, heparin, and fucoidan. The assay is described in the "Materials and Methods" section. Representative data are shown of two experiments with similar results.

0.8 μM (L-selectin). On this basis, the potency of HSO$_3$Le^a-PAA-sTyr against L-selectin is similar to that of fucoidan (IC_{50} values are 2 and 0.8 μM, respectively).

Two further assays were developed to determine whether the efficacy of the inhibitors used above was similar when tested against P-selectin from natural sources. First, P-selectin was purified from human platelets and then immobilized on plates (500 ng/well) coated with an antibody to the non-lectin domain of P-selectin. SiaLe^a-PAA-biot was used as the P-selectin ligand. Second, a cell-based assay was used in which human platelets were allowed to adhere to plastic wells. Such adherence results in the activation of platelets and the translocation of P-selectin to the cell surface [17]. Again, SiaLe^a-PAA-biot was used as the P-selectin ligand. An anti-P-selectin antibody and EDTA blocked ligand binding in both assays (Fig. 3). Figure 3 shows that the properties of soluble monomeric carbohydrate inhibitors were similar in these two assays. Inhibition by heparin and fucoidan was also similar in the two assays. The concentration dependence

Table 2. Inhibition of rat peritoneal neutrophil accumulation by sialylated and sulfated glycoconjugates and fucoidan (results are presented as the means ± SD)

Preparation	Dose, mg per rat	Number of rats tested	Inhibition, %
$SiaLe^x$	3.0	12	37 ± 12
$SiaLe^x$-PAA	1.0	9	43 ± 17
HSO_3Le^a-PAA	3.0	3	46 ± 12
$SiaLe^a$-PAA-sTyr (20/10)	3.0	8	50 ± 14
HSO_3Le^a-PAA-sTyr (15/10)	3.0	5	40 ± 20
Fucoidan (*L. saccharina*)	0.3 0.6 1.0	9 5 10	43 ± 17 82 ± 10 94 ± 7

for PAA-glycoconjugates and sulfated polysaccharides is shown in Fig. 4. Fucoidan is the most potent inhibitor, followed by the bi-ligand glycoconjugate $SiaLe^x$-PAA-sTyr, which is more active than heparin. The following compounds were much less potent as inhibitors in the platelet P-selectin assay: $SiaLe^a$-PAA, $SiaLe^x$-PAA, HSO_3Le^x-PAA, and HSO_3Le^a-PAA (data shown only for $SiaLe^a$-PAA).

The results from the above three types of assay show that the IC_{50} values are not dependent on the source of selectin (natural or chimeric), selectin valency (mono- versus di-, as compared from earlier data [16, 22]), the selectin coating concentration, or the manner of presentation of selectin (platelet versus plastic).

***In vivo* assay: inhibition of peritoneal inflammation.** Peptone-induced acute rat peritonitis was characterized by an increase in neutrophil number in the peritoneal cavities from $(0.5 \pm 0.6)\cdot10^6$ ($n = 7$, blank group) to $(46 \pm 24)\cdot10^6$ ($n = 22$, control group). Intravenous injection of selectin inhibitors resulted in considerable reduction of neutrophil extravasation. The results are shown in Table 2. Injection of monomeric $SiaLe^x$ (5-15 mg/kg) led to about 40% inhibition of neutrophil extravasation compared to the control. Comparison of monomeric and multimeric inhibitors at the same dose did not reveal any statistically significant differences in their ability to reduce neutrophil extravasation (range 37-50%, Table 2). The inhibitory effect of $SiaLe^a$-PAA-sTyr (50%) was slightly higher than that of the other synthetic preparations, although fucoidan was still the most potent inhibitor, causing a 90% reduction in neutrophil extravasation at 5 mg/kg.

DISCUSSION

A principal aim of this work was to compare the efficacy of monovalent and multivalent selectin blockers in three types of experimental systems: 1) fully artificial (based on inhibition of the interaction of recombinant selectins with a synthetic carbohydrate-based ligand); 2) cellular (based on inhibition of ligand interaction with P-selectin on platelets), and 3) *in vivo* (based on blocking of rat peritoneal inflammation). Since neutrophil extravasation in the peritonitis *in vivo* model depends mainly on P-selectin [23], the results from the current study will be discussed primarily in relation to P-selectin. The inhibitory profile of monovalent and multivalent compounds was not dependent on the source of P-selectin. Similar results were obtained using either recombinant P-selectin, purified natural P-selectin, or P-selectin expressed on platelets. As expected, the potency of multimeric compounds *in vitro* was much greater than that of the soluble monomeric compounds. However, this relationship was not found in the *in vivo* model. The activity of the polymeric (multivalent) form of $SiaLe^a$ *in vivo* was approximately equivalent to that of the monomeric tetrasaccharide. In fact, all of the tested PAA-conjugates (see Table 2), including the bi-ligand compound containing both the $SiaLe^x$ and sTyr moieties, had similar activities *in vivo*. A similar equivalence of activity *in vivo* of monomeric and polymeric $SiaLe^x$ derivatives was described earlier for E-selectin [20].

To compare *in vitro* and *in vivo* potencies, we calculated an empirical ratio termed E_f (see Table 3, last column), which is the ratio of the approximate IC_{50} value *in vitro* to that *in vivo*. A value $E_f >> 1$ would indicate that the inhibitor is much more active *in vivo* than *in vitro*. Table 3 illustrates that the monomeric tetrasaccharide $SiaLe^x$ has a high E_f value (~100) in our models. A similar relationship has been described for monomeric pentasaccharide $SiaLe^x$, which is much more active *in vivo* in a rat lung injury model [24] and other models [25] than expected from the relatively low activity (mM) *in vitro*. On the other hand, the polymeric inhibitors, such as $SiaLe^x$-PAA, had an E_f value closer to 1 (Table 3). The introduction of additional charged residue sTyr increased inhibitory activity *in vitro* but not *in vivo* ($E_f = 0.1$). Finally, the lowest E_f value of 0.01 was observed for the most active *in vitro* inhibitor, the high molecular weight polysaccharide fucoidan.

The above results pose two important questions. First, why are polymeric inhibitors less active *in vivo* than *in vitro*? Second, why is monomeric $SiaLe^x$ considerably more active as a selectin inhibitor *in vivo* than *in vitro*?

There are a number of possible explanations for the first observation. For example, the binding of charged polymers to blood cells and/or plasma proteins may reduce their free concentration in blood. Such nonspecific binding would reduce their apparent potency. The

Table 3. Comparison of IC_{40-50} for four compounds *in vitro* and *in vivo*. *In vitro* data were taken from this work and other published works [31, 32]. *In vivo* IC values were calculated on the assumption of a uniform distribution of the sample in rat blood (volume 20 ml). The inhibition values are shown relative to mass rather than molarity because the precise molarity of fucoidan is unknown

Inhibitor	$IC^{in\ vitro}$, μg/ml	$IC^{in\ vivo}$, μg/ml	Efficiency, $E_f = IC^{in\ vitro}/IC^{in\ vivo}$
$SiaLe^x$	10000	100	100
$SiaLe^x$-PAA	100	100	1
$SiaLe^a$-PAA-sTyr	10	100	0.1
Fucoidan (*L. saccharina*)	0.1	10	0.01

driving force for these nonspecific interactions could be either ionic interactions with a positively charged receptor (such as the scavenger receptor) or Ca^{2+}-mediated interaction with negatively charged binding partners. The data shown in Table 3 indicates that the increase of total charge in the series $SiaLe^x$-PAA → $SiaLe^a$-PAA-sTyr → fucoidan is accompanied by an inverse change in E_f values. Another possibility is that a rapid clearance of high molecular weight compounds from the circulation would also result in a decrease of the effective blood concentration of inhibitor. However, our results indicated that intravenously administered $SiaLe^x$ was as effective at 120 min as 15 min after injection. Furthermore, fucoidan produced the same inhibitory effect *in vivo* whether administered 15 min after or 3 h before the inflammatory challenge (data not shown). It is also possible that *in vivo* polymeric compounds could have dual effects on neutrophils. For example, a soluble polymeric L-selectin ligand may act as an agonist, triggering signal transduction via L-selectin and the up-regulation of integrin-mediated adhesion. On the other hand, polymeric ligands could also induce L-selectin shedding [26], which potentially could reduce neutrophil capture and rolling. Further work is necessary to evaluate these opposing possibilities.

A further consideration *in vivo* is the influence of compounds on the homotypic aggregation of neutrophils, which are dependent on interactions between PSGL-1 and L-selectin on opposing cells [27]. Aggregated neutrophils, which can appear after an insult to the circulation, have poorer access to the microvasculature. This could result in an impairment of extravasation. Polymeric $SiaLe^x$ appears to inhibit neutrophil aggregation better than the monomeric form, and hence in the presence of the polymer there may be a greater number of cells available for extravasation.

Multimeric compounds may also have a low E_f value through virtue of their binding to soluble P-selectin. Since soluble selectins can modulate selectin-mediated interactions, multivalent compounds could interfere with the anti-inflammatory effect of soluble selectins. Monomeric $SiaLe^x$ has insufficient affinity to modulate any anti-inflammatory activity of soluble P-selectin.

Although the above considerations may help to explain the lower-than-expected apparent activity of polymeric inhibitors *in vivo* ($E_f < 1$), it is more difficult to explain why the inhibitory effectiveness of monomeric $SiaLe^x$ is orders of magnitude greater *in vivo* than *in vitro* ($E_f >> 1$). One possibility is the critical difference between static (*in vitro*) and dynamic (*in vivo*, rolling) adhesion systems. Rolling is a rate-limiting step in the leukocyte recruitment process. It has been shown that rolling is 50% inhibited by fucoidan at a concentration of 10 μg/ml [28], i.e., at the same concentration as observed in the current study for inhibition of peritoneal neutrophil extravasation. The notion of a quantal tethering unit has been invoked in the context of P-selectin-mediated rolling [29]. Under physiological flow conditions, the tethering is thought to involve a mono- or divalent interaction between P-selectin and a PSGL-1 molecule on the neutrophil. On the other hand, under static conditions (*in vitro*) the binding is obviously multivalent. It is conceivable that monomeric $SiaLe^x$ can more readily inhibit a one- or two-point interaction than a multivalent interaction. For example, under static (multivalent) conditions, a steady state is achieved in the presence of an inhibitor with interactions being made and then broken. Relatively high concentrations of an inhibitor would be required to reduce net binding. Under *in vivo* conditions, however, the cell bearing the macromolecule ligand would be rapidly removed by the flow and lower concentrations of inhibitor would be effective in reducing net adhesion.

If tethering under physiological flow conditions does involve only one or two specific interactions at any one time, then a polymeric inhibitor is unlikely to offer significant advantages in terms of inhibitory potency. A polymer will contain many copies of the inhibitory "unit" and yet only one "unit" is effective as an inhibitor at any one time. The other copies are redundant. Therefore, on a molar basis, monomeric $SiaLe^x$ could be 50-100 times more potent than $SiaLe^x$-PAA at inhibiting rolling. Clearly, physiological rolling requires rapid kinetics of attachment and detachment [30]. The interaction of soluble monomeric $SiaLe^x$ with a selectin is a rapid event, whereas available evidence indicates that the "on-rate" for a polymeric ligand is relatively slow.

It is noteworthy that the macromolecular $SiaLe^x$ is not an efficient inhibitor of the rolling process *in vivo*. For example, the concentration of $SiaLe^x$ borne on α_1-acid glycoprotein (AGP) in circulating blood is about 10 μM

(calculated as "monomeric" units). This concentration of free monomeric $SiaLe^x$ would be expected to almost completely inhibit neutrophil recruitment. Therefore, if $SiaLe^x$-borne AGP had the same activity *in vivo* as the $SiaLe^x$ monomer, the rolling process would be severely compromised. Based on these calculations, we speculate that the release of $SiaLe^x$-containing N-chains by the action of an endoglycanase in blood could be one more pathway for the physiological regulation of leukocyte rolling.

In summary, it is likely that many of the factors discussed above will contribute to the differences seen in the effectiveness of monomeric and polymeric selectin ligands as inhibitors *in vitro* and *in vivo*. Further studies are required to identify which factors predominate in influencing the effectiveness of selectin inhibitors *in vivo*. This knowledge is important for the design of selectin blockers as potential therapeutic agents for treating inflammatory diseases.

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