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Oligosaccharide Recognition by Selectins: Synthesis and Biological Activity of Multivalent Sialyl Lewis-X Ligands

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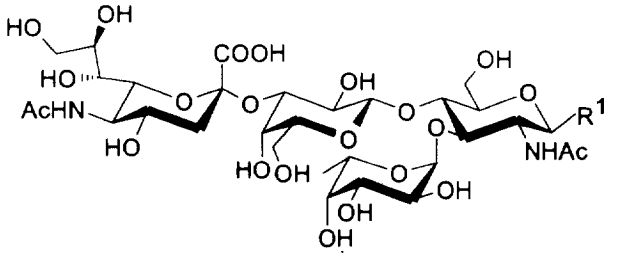
Abstract: Trivalent sialyl Lewis-X ligands **6-8** anchored onto flexible templates have been synthesized and evaluated as inhibitors of E-selectin and P-selectin mediated cell adhesion in cell culture assays and *in vivo*. Biological activities *in vitro* correlated with spacer length and lead to ligands with 3-fold (E-selectin) and 5-fold (P-selectin) improved receptor binding avidity per single tetrasaccharide moiety.

INTRODUCTION

The selectin family of leukocyte adhesion molecules is recognized to play key roles in the recruitment of neutrophils and other leukocytes to sites of inflammation and tissue injury, and to mediate trafficking of lymphocytes to peripheral lymph nodes during recirculation between the blood and lymph.[1] Glycoproteins and -lipids bearing the sialyl Lewis-X tetrasaccharide **1a** are the primary ligands for this selectin mediated cell adhesion.[2]



1a R^1 = OH

1b R^1 = $O(CH_2)_6NH_2$

Figure 1

The selectins are transmembrane glycoproteins expressed on platelets (P-selectin), leukocytes (L-selectin) and on endothelial cells (E- and P-selectins) which mediate early rolling interactions on the endothelial vessel wall in normal and in excessive inflammatory response to cytokines and to other inflammatory mediators. This rolling

represents the most transient cell-cell interaction known and ultimately leads to leukocyte extravasation (diapedesis) at sites of inflammation[1,3], as illustrated in Figure 2.

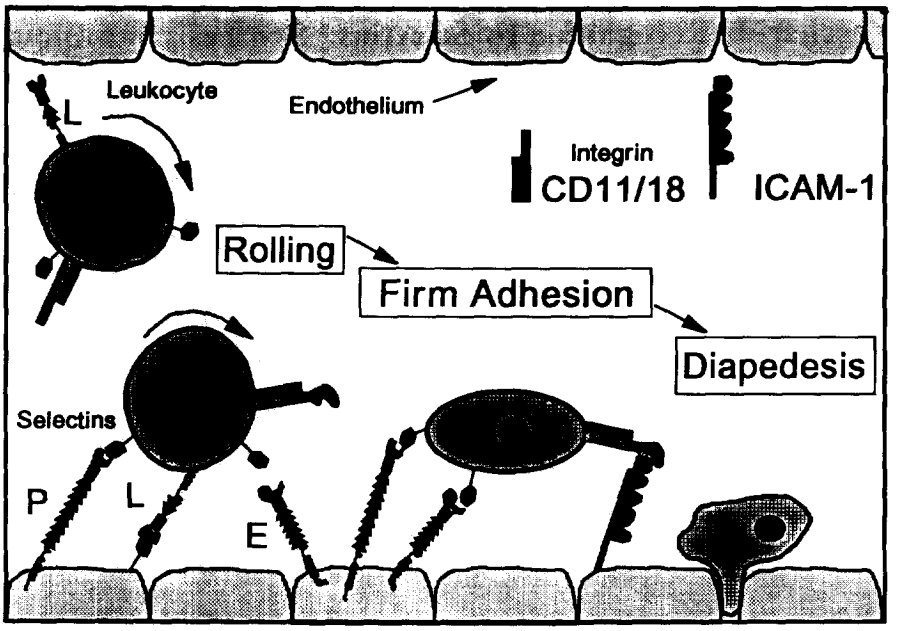


Figure 2: *Illustration of rolling, adhesion and extravasation of leukocytes in blood vessels.*
The selectins (E, P and L) slow down the leukocytes in blood vessels which then roll along the endothelium by binding to glycoproteins and glycolipids bearing sialyl Lewis-X tetrasaccharide ligands **1a**. Subsequent firm adhesion involves a cascade of macromolecular interactions at the cell surfaces. One stage shown is the binding of further adhesion proteins, the leukocyte integrins and the intercellular cell adhesion molecules (ICAMs). The earliest steps in leukocyte recruitment (carbohydrate-protein interactions) as well as the subsequent steps (protein-protein interactions) are considered to be attractive targets for pharmaceutical intervention.[4]

The tetrasaccharide ligand **1a** is now frequently taken as the natural lead structure for the development of glycomimetics that structurally resemble and functionally mimic the natural oligosaccharide. These compounds, designed as selectin receptor antagonists, are currently being evaluated as potential anti-adhesive and anti-inflammatory drugs.[5-8] Some derivatives of **1a** have already shown promising efficacy in animal models of acute inflammation of the lung[9] and of myocardial ischaemia and reperfusion injury.[10]

Issues of basic concern for drug development include efficacy, pharmacokinetics and biodistribution which may prevent oligosaccharides from being developed as orally available drugs against various chronic inflammatory diseases. Inherent weak binding of selectins to the carbohydrate ligand **1a** is well documented in different assay types with half-inhibition concentrations (IC_{50}) approximately in the 1 mmolar range. The IC_{50} value of one of

the tightest binding derivatives of **1a** - the glucose derivative with Glc in place of GlcNAc - is about 0.1 mM which is in reasonable agreement with the thermodynamic dissociation constant K_d, as measured by fluorescence polarization with a value of 0.12 mM.[11] Natural protein associated sialyl Lewis-X carbohydrates of higher complexity have been isolated and characterized and may account for high-affinity binding and selectivity *in vivo*.[12] Based on the X-ray structure of E-selectin[13] and in comparison with 3D-models derived from the X-ray structure of mannose binding protein (MBP),[14] several research groups have identified protein and carbohydrate residues critical for recognition and speculated about the ligand binding site in the selectins.[15,16]

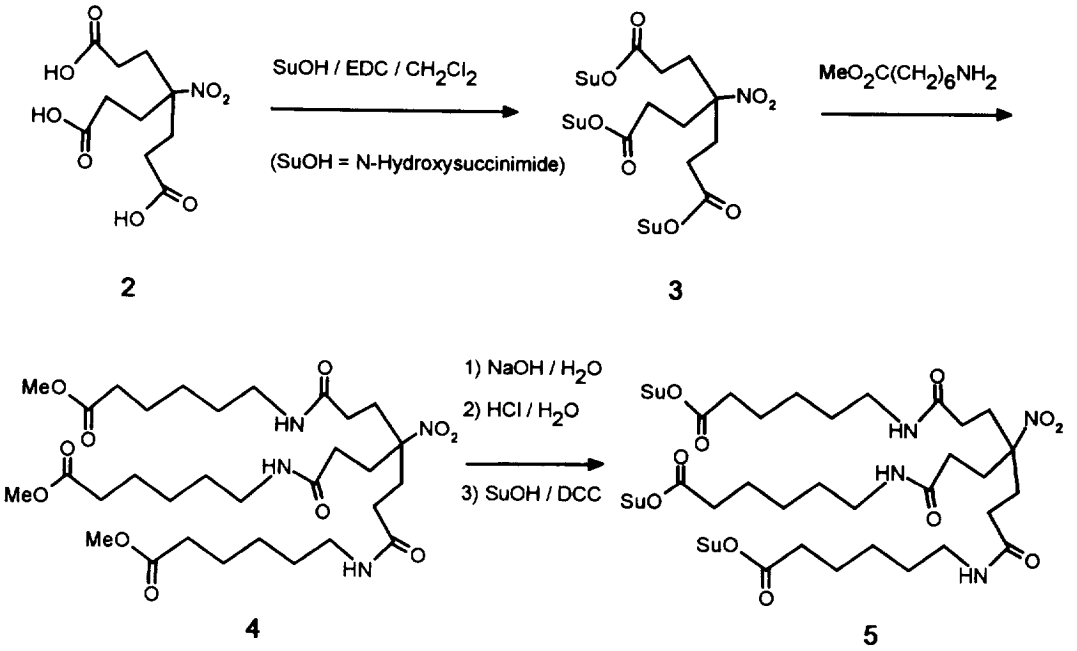
The true functional binding situation, however, remains unclear. One issue of basic concern addressed here is the postulated *multivalency* of binding[17]. Several endogenous selectin ligands are mucins with multiple, closely spaced O-linked sugars which may present oligosaccharides at very high surface densities.[1,18] Multivalent binding is expected to enhance the efficacy of binding. Such avidity effects in carbohydrate-protein interactions have been examined in several studies, e.g. in the inhibition of influenza virus binding to cellular ligands by its sialic acid binding protein (hemagglutinin HA) with mono- und multimeric sialosides[19,20]: The inhibitor potencies were 8 to 30000 times higher than those of the corresponding monovalent sialic acid derivatives.[21,22]

As part of our interest in obtaining a deeper understanding of the nature of selectin-ligand recognition, we report here a concise synthesis of effective selectin antagonists which present three sialyl Lewis-X ligands per molecule, as well as evaluation of their efficacy in a cell-based bioassay and in animals.

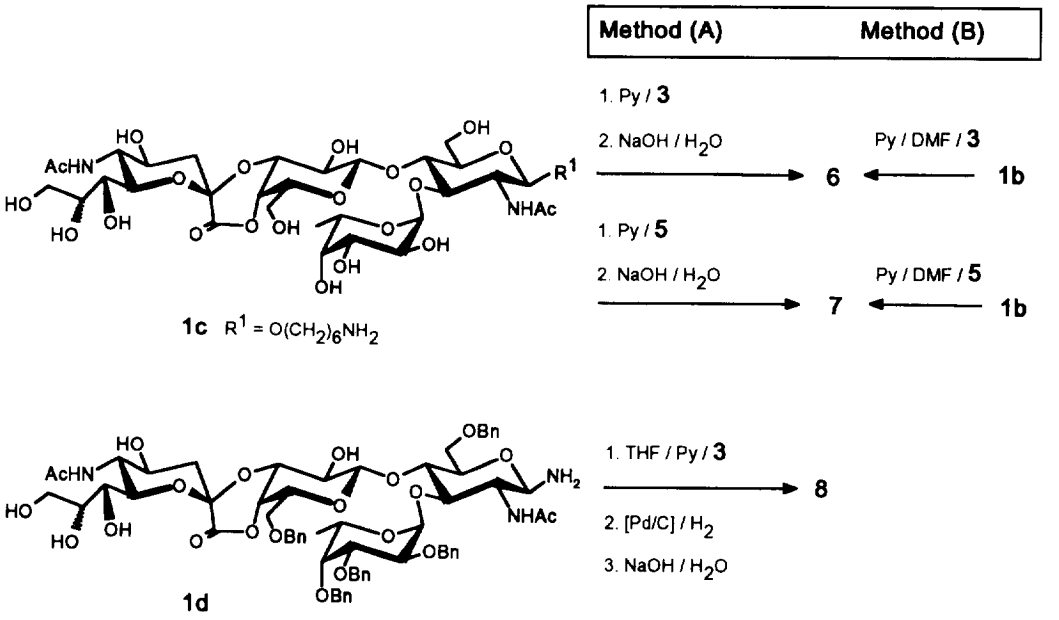
PREPARATIVE RESULTS

A trivalent template similiar to the widely used 2-amino-2-hydroxymethyl-1,3-propanediol ("Tris") precursor for small cluster ligands,[23] the nitromethane-trispropionic acid **2**, was chosen as a suitable starting material for the preparation of trimeric sialyl Lewis-X conjugates. This template was expected to present the saccharide ligands free from any backbone constraints. The triacid **2** was converted to the crystalline succinate **3** with N-hydroxysuccinimide and N-ethyl-N′-dimethylaminopropylcarbodiimide-hydrochloride (EDC) in 86% yield, as indicated in Scheme 1. Further chain elongation involved straightforward chemistry leading from **3** to the triple activated template **5** in 3 steps and 74% overall yield.

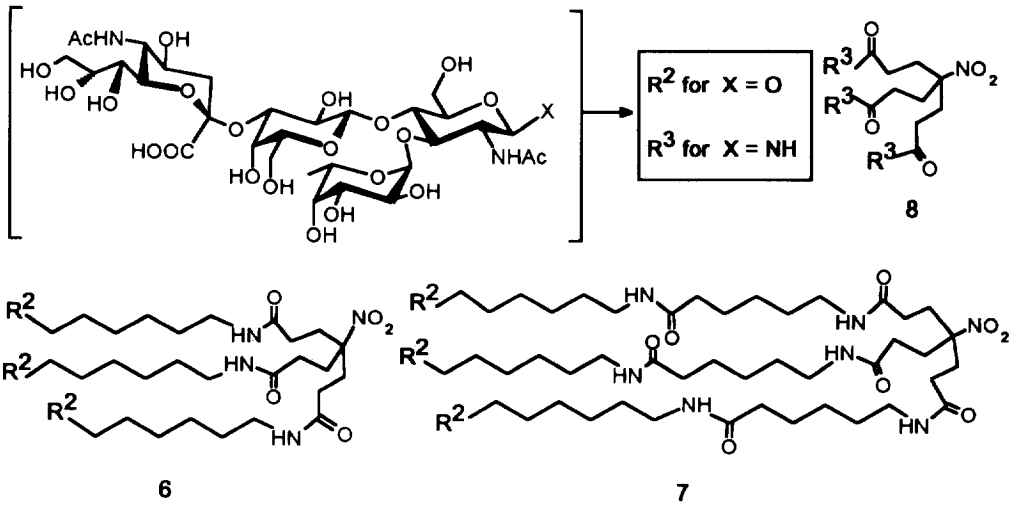
Two alternative methods (A) and (B), as specified in Scheme 2, were applied for the preparation of medium- and long-chain conjugates **6** and **7**, respectively (structures see Scheme 3): According to method (A), 3.3 equivalents of the already described lactone precursor **1c**[6,7] (derived from glycoside **1b**) and one equivalent of **3**



Scheme 1: **Preparation of Activated Templates 3 and 5**



Scheme 2: **Synthesis of Sialyl Lewis-X Conjugates 6, 7 and 8**



Scheme 3: Structures of the Trivalent Sialyl Lewis-X Conjugates Prepared According to Scheme 2

or **5** were stirred in pyridine at 60°C until intermediate mono- and bis-coupling products were completely converted into the more polar tris-coupled product. The reactions were monitored by thin layer chromatography. After saponification of the lactone intermediates, products **6** and **7** were isolated in pure form by column chromatography on Biogel P4 (water) in 85% and 15% yield, respectively.

Alternatively, compounds **6** and **7** could be prepared directly in one step by reaction of the totally unprotected tetrasaccharide **1b** with **3** or **5**, respectively, in pyridine/DMF (method B). Using this direct coupling method, the saccharide **6** was prepared on a 380 mg scale in 83% yield (based on **3**; 71% based on **1b**). For the preparation of the short-chain ligand **8** which carries the three tetrasaccharide moieties by virtue of an anomeric glycosylamide linker, method (A) using the already described[8] partly protected precursor **1d** proved to be adequate: Saccharide **1d** was coupled with **3** in pyridine/THF at ambient temperature to give **8** in 82% overall yield after hydrogenolytic cleavage of the benzyl groups, saponification of the lactone and chromatography on Biogel P2 (water). This reaction sequence proceeded with retention of the β-anomeric configuration at the N-glycoside bond. A similar strategy has been successfully pursued in the convergent synthesis of a sialyl Lewis-X peptide conjugate from the partly protected building block **1d**.[8]

The structures of **6-8** were confirmed by ^{1}H- and ^{13}C NMR spectroscopy (Figure 3) and by electrospray ionisation mass spectrometry (ESI-MS). The NMR data of the tetrasaccharide moieties were in excellent agreement with the spectrum reported for the O-allylglycoside of sialyl Lewis-X:[24] Only a single set of signals was obtained for each of the three Fuc, Gal and NAcNeu residues due to the coincidence of all sugar resonances.

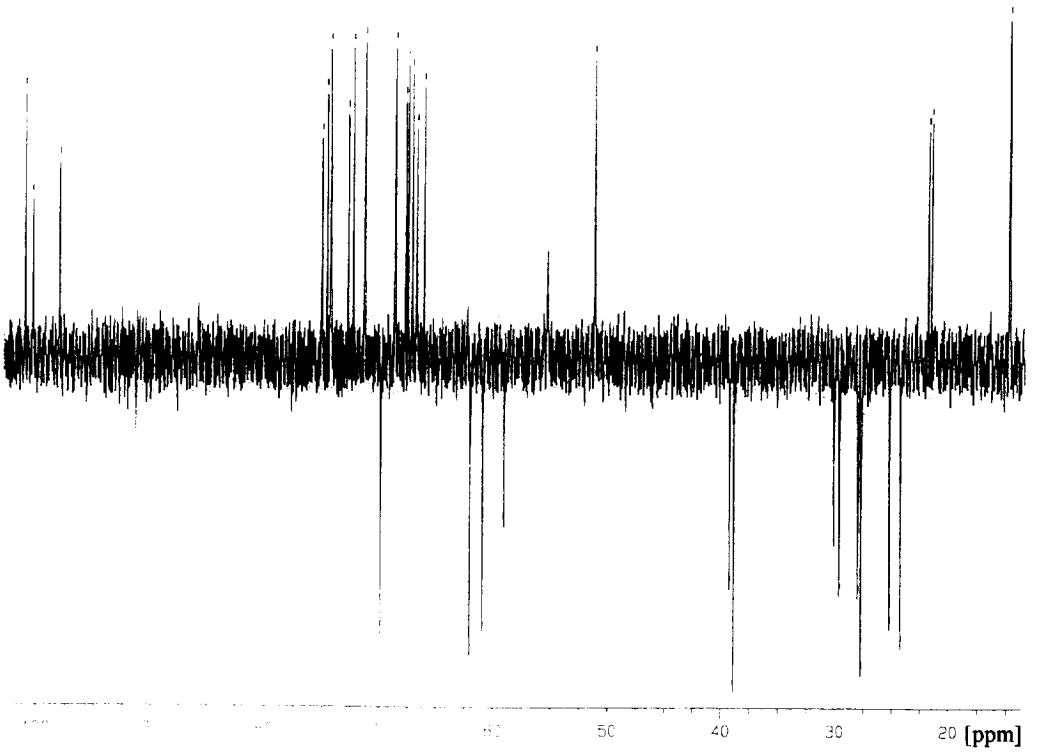


Figure 3: *DEPT-^{13}C-NMR Spectrum of compound 6 at 125.8 MHz in D_2O.*

BIOLOGICAL ACTIVITY OF SIALYL LEWIS-X MULTIMERS

The inhibitory potency of 6, 7 and 8 on HL60 cell binding to immobilized E- and /P-selectin-IgG

The preparation of soluble E- and P-selectin-IgG fusion proteins, composed of the extracellular portions of human selectins E and P and the human immunoglobulin (IgG) heavy chain hinge, CH2 and CH3 regions, has been described.[25,26] These recombinant proteins contain the signal sequence, the lectin-like domain, the EGF (epidermal growth factor) repeat domain and six (E-selectin) and two (P-selectin) of the CR-like (complement regulatory) domains. The bioassay for cell binding to immobilized selectin receptor globulins was performed as described in Figure 4. Briefly, the recombinant proteins obtained from transfected COS cells according to standard procedures[27] were adsorbed on anti-human-IgG-antibodies immobilized on ELISA (enzyme-linked immunosorbent assay) plates. Adhesion of labelled HL60 tumor cells was quantitatively measured in a cytofluorometer. The specific cell binding in the presence of inhibitors **1b**, **6**, **7** and **8** was calculated compared

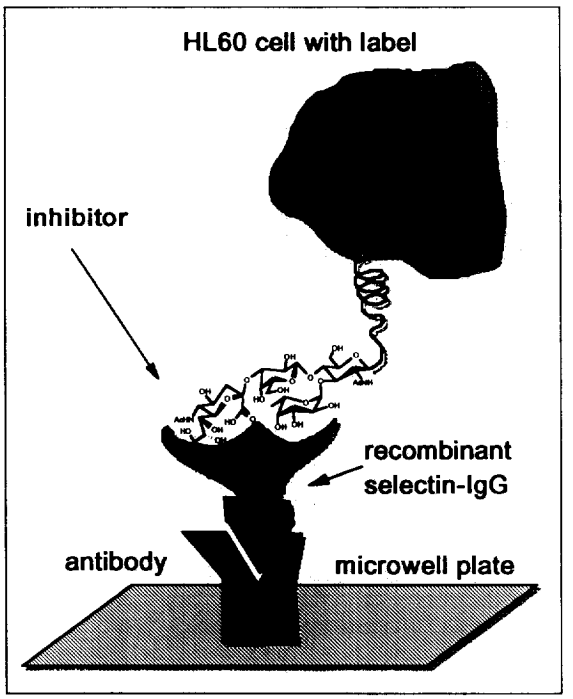


Figure 4: *Schematic illustration of the bioassay used to determine the affinities (IC_{50} values) of trivalent sialyl Lewis-X conjugates* ***6****,* ***7*** *and* ***8*** *to immobilized recombinant E- and P-selectin-IgG fusion proteins*
In this assay the competitive binding of inhibitory compounds relative to sialyl-Lewis-X presenting HL60 tumor cells is determined. Quantitative analysis of binding is performed by measurement of the amount of labelled cells binding to the receptor coated microtiter plates.

with nonspecific binding to the CD4-IgG fusion protein.[28] The concentrations of the inhibitors required to block adhesion of 50% of the HL60 cells ranged from 0.13 mM to >1mM, as is shown in Table 1. Trivalent compounds **6**, 7 and **8** were each significantly more potent than the monomer standard **1b** in blocking adhesion to E-selectin, the trend being **6** > **8** ≥ 7. Essentially the same trend was observed with P-selectin, again compound **6** with medium spacer length exhibiting the highest level of inhibitory activity. The binding to the P-selectin receptor seemed to be slightly more sensitive to the spacer length, possibly suggesting a more pronounced multivalency effect compared with the monovalent standard **1b**.

inhibitor	IC_{50} [mM] E-selectin	IC_{50} [mM] P-selectin
1b	1.0	2.0
8	0.38	>0.8
6	0.13	0.14
7	0.40	>1.0

Table 1: *Inhibition of HL60 cell adhesion to recombinant E- and P-selectin-IgG fusion proteins on plates*
IC_{50}- values are concentrations of inhibitors required to block adhesion of 50% of the cells compared with the nonspecific binding to the CD4-IgG fusion protein[28] as negative control.

Inhibitory effects of compounds 6-8 on lipopolysaccharide (LPS) induced leukocyte adhesion in the microcirculation of rats

The effect of lipopolysaccharide (LPS, 15 mg/kg i.v.) induced leukocyte adhesion in rat mesenteric venules was measured by intravital microscopy and an analogous video image processing system. This *in vivo* model for the assessment of inflammatory effects of endotoxins on the adherence of leukocytes on the endothelium and the study of inhibitory effects by potential anti-inflammatory agents has proven to deliver excellent reproducibility and high sensitivity to endotoxin in the circulation, well below the lethal dose.[29,30,31] The mesentery of the rat was chosen because the nearly two-dimensional connective tissue is about 15-30 μm thick and therefore allows high-resolution intravital microscopy with video analysis and image processing for accurate measurements of leukocyte adhesion, as described in more detail in the experimental part below.

The results shown in Figure 5 suggest that multivalent presentation of the sialyl Lewis-X tetrasaccharide ligand may *not* be required in this animal model since inhibition of leukocyte adhesion by the monovalent standard **1b** is not increased as predicted from the improved selectin binding found in the cell based assay. Actually the data suggest that the tetrasaccharide ligands may not be sufficiently well arranged in order to improve binding *in vivo*.

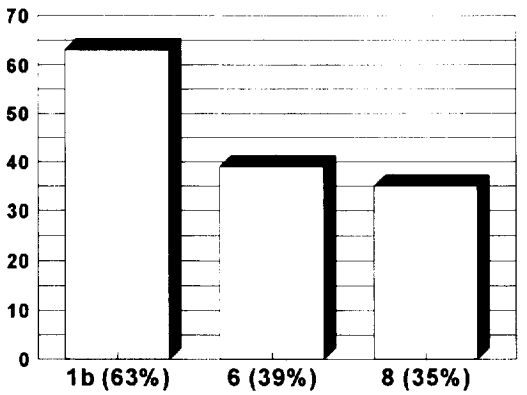


Figure 5: *Inhibition of leukocyte adhesion*

The inhibition of leukocyte adhesion in rat mesenteric venules by application of a 10 mg/kg (i.v.) dose of compounds **1b**, **6** and **8**, respectively, in response to lipopolysaccharide (LPS) induced adhesion.

DISCUSSION AND CONCLUSIONS

Three types of sialyl Lewis-X trimers anchored onto spacers of different length have been prepared by convergent synthesis in a straightforward manner. This procedure is applicable to the preparation of gram-amounts of material sufficient for further *in vitro* and *in vivo* evaluation. Alternative procedures which involve biosynthetic reaction steps are considered to be less convenient with regard to flexible and rapid variation of templates, spacers and functional linker groups. In this (biosynthetic) way, a mixture of 67% mono-fucosylated,

32% difucosylated and 1% (4 nmol of material) trifucosylated NeuAcα2→3(LacNAc)$_3$-Me has been prepared.[32] Furthermore, five sialyl Lewis-X dimers anchored onto galactose templates or attached to 1,4-butanediol and 1,5-pentanediol have been prepared chemoenzymatically and evaluated as inhibitors of the adhesion of HL60 cells to recombinant soluble E-selectin coated plates.[33,34] The IC_{50} value of the best of these dimeric ligands was 0.2 mM. The corresponding IC_{50} value of 0.8 mM for the pentasaccharide monomer indicates that an effect of polyvalency *per ligand* of 2 could be achieved.

Our equally well defined trivalent sialyl Lewis-X ligands **6**, **7** and **8** partly show further improved binding to the selectins with an essentially conserved selectivity to both receptors, compared with the monovalent ligand **1b**. Examination of the NMR spectra of either trivalent compound confirmed that the sialyl Lewis-X domains exhibit equivalent conformations, because of perfect coincidence of each of the three Fuc, NeuNAc and Gal signals. This indicates full flexibility of the oligosaccharides with respect to the trivalent template moiety. The binding enhancement *per ligand* by a factor of 3 and 5 for E- and P-selectin, respectively, observed in the cell based assay with compound **6** (medium spacer length), may support the concept of multivalent interaction between selectins and sialyl Lewis-X. Our inhibition analysis *in vitro*, however, does not reflect the significantly lower inhibition of leukocyte adhesion *in vivo* achieved using the trivalent ligands, compared with the biological efficacy using the monovalent ligand **1b**.

The concept of multivalency was established from the long known recognition of specific carbohydrate sequences by lectins, which is believed to involve polyvalent low affinity carbohydrate-protein interactions.[17] For instance, sialylated neoglycoproteins and polymers presenting multivalent epitopes have been prepared in order to compensate for the low affinity of individual α-sialosides. Well defined dendritic di-, tetra-, octa- and hexadecavalent α-thiosialosides inhibited hemagglutination of human erythrocytes by influenza A virus (strain X-31) at 625, 312, 156 and 91 μM compared with monosialosides (3 mM).[36] Clearly, these results cannot be attributed to the effects of polyvalency by virtue of clustered ligand presentation. Corrected for the number of ligands presented per dendrimer, inhibition potency increased only about 2.5-fold *per ligand*. On the other hand, anti-agglutination assays performed in this as well as in other studies[37] revealed strong cluster effects using (co)polymer sialosides. However, the mechanisms by which these polymers influence hemagglutination remain to be defined.

The biophysical examination of neutrophil-selectin interactions in hydrodynamic flow systems has shown that transient tethers have characteristics suggesting a unimolecular interaction between P-selectin and its glycoprotein ligand (PSGL-1), and possibly multivalent tethering at relatively high receptor densities. This multivalent tethering may be required to maintain the rolling by distributing the shear forces over multiple selectin-ligand bonds.[43] Based on the assumption that the rolling (and subsequent adhesion) phenomena in our animal

model predominantly depend on P-selectin, the data suggest that much larger arrays of weakly binding, multivalent ligands like the sialyl Lewis-X tetrasaccharide may be required to block these multiple interactions efficiently under hydrodynamic flow conditions. The high affinity recognition sites of P-selectin glycoprotein ligands probably derive from a "clustered saccharide patch" of sialylated and fucosylated O-linked oligosaccharide sequences.[39] Multivalency effects with selectins may only become effective in larger structural assemblies more resembling such clustered saccharides than our simple model compounds. This suggestion is in accord with the results obtained using complex, chemically less well defined sialyl Lewis-X protein conjugates like $[SLeX]_{16}BSA$, which inhibited binding of HL60 cells to immobilized E-selectin with IC_{50} ca. 1 μM.[35]

In summary, our results confirm that the endogenous high affinity ligands for E-selectin[12] and P- selectin[38] on myeloid cells contain structural features not provided by the tetrasaccharide or by simple cluster derivatives alone. On the other hand, our results do not exclude that high-affinity ligands for selectins applicable to the development of anti-adhesive and anti-inflammatory drugs may be designed in the near future.[8,44]

EXPERIMENTAL PART

General: 4-(2-Carboxyethyl)-4-nitroheptane 1,7-dicarboxylic acid was purchased from commercial sources and used without further purification. Thin layer chromatography (TLC) with unprotected sugar derivatives was carried out on precoated Kieselgel 60 F_{254} plates (0.25 mm thickness, E. Merck) with the solvent mixture ethyl acetate/ methanol/ water/ acetic acid (5/ 3/ 3/ 0.5 ml) and spots were visualized by spraying the plates with sulfuric acid/ anisaldehyde reagent, followed by heating. E.Merck silica gel (60, particle size 0.040-0.063 mm) was used for flash column chromatography. Yields refer to chromatographically (TLC) and spectroscopically (NMR) homogeneous materials. Melting points were determined using a Gallenkamp MPD 350 apparatus and are uncorrected. Optical rotations were measured using a Perkin Elmer 241 polarimeter. NMR-spectra were recorded on a Bruker WT 300 (300 MHz) and on a Bruker ARX 500 (500 MHz). NMR chemical shifts are given as δ-values with reference to tetramethylsilane (TMS) as internal standard, if not otherwise noted. The spectra recorded in D_2O as solvent were locked to deuterium, unless otherwise stated. NMR spectra were in part not strictly assigned for each proton and each carbon, due to the almost unchanged resonances of the sugar moieties compared with the spectra of **1a,b**[6,24] and some other Sialyl Lewis-X glycoconjugates.[8,33] Mass spectra were recorded on a Finnigan MAT 95 Q-MR.

Preparation of 4-(2-carboxyethyl)-4-nitroheptane-1,7-dicarboxylic acid-tris-(N-hydroxysuccinimide) ester (3): 10.0 g (36.1 mmol) 4-(2-carboxyethyl)-4-nitroheptane-1,7-dicarboxylic acid, 31.1 g (162 mmol) N-ethyl-N′-dimethylaminopropylcarbodiimide hydrochloride (EDC) and 18.7 g (162 mmol) N-hydroxysuccinimide were suspended in 250 ml dichloromethane and stirred for 12 h at ambient temperature. The crystalline,

colourless product was filtered, washed twice with 100 ml dichloromethane and dried to yield 17.7 g (86.2 %) of **3** with mp.189-190°C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 2.18 (m, 6H, C$\underline{H}_2$CH$_2$C=O), 2.74 (m, 6H, CH$_2$C$\underline{H}_2$C=O), 2.80 (s, 12H, O=CC$\underline{H}_2$C$\underline{H}_2$C=O).

Preparation of 4-(5-methoxycarbonylpentylamidoethyl)-4-nitroheptane-1,7-dicarboxylic acid-di-(5-methoxycarbonylpentyl)-amide (4): A mixture of 2.0 g (3.5 mmol) tris-succinimide **3**, 2.11 g (14.6 mmol) 6-aminohexanoic acid methyl ester, 50 ml dry pyridine and 1.5 ml triethylamine was stirred for 5 h at 50°C. After evaporation of the solvent, the residue was treated twice with 50 ml toluene and evaporated to dryness. The crude product was dissolved in ethyl acetate and washed with saturated aqueous sodium hydrogencarbonate solution and brine. The solution was dried ($MgSO_4$), concentrated and then used without further purification in the next reaction step. Yield: 1.95 g (85 %, syrup). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.10-1.60 (m, 18H, NHCH$_2$C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$CH$_2$C=O), 2.02 (m, 12H, C$\underline{H}_2$C$\underline{H}_2$-C=O), 2.30 (t, 6H, C$\underline{H}_2$COOMe), 3.00 (dt, 6H, C$\underline{H}_2$NH), 2.57 (s, 9H, Me), 7.85 (t, 3H, NH).

Preparation of 4-(5-succinimidyloxycarbonylpentylamidoethyl)-4-nitroheptane-1,7-dicarboxylic acid-di-(5-succinimidyloxycarbonylpentyl)-amide (5): 1.95 g (2.9 mmol) **4** dissolved in 10 ml methanol und 4 ml 1M aqueous sodium hydroxide solution was stirred for 72 h at ambient temperature. After acidification with aqueous hydrochloric acid (pH 2) the product was extracted with diethyl ether. The solution was dried ($MgSO_4$), concentrated and then used without further purification in the next reaction step. Yield: 1.7 g (93 %) of the syrupy 4-(5-carboxypentylamidoethyl)-4-nitroheptane-1,7-dicarboxylic acid-di-(5-carboxypentyl)-amide. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.10-1.60 (m, 18H, NHCH$_2$C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$CH$_2$C=O), 2.02 (m, 12H, C$\underline{H}_2$-C$\underline{H}_2$C=O), 2.19 (t, 6H, C$\underline{H}_2$CO$_2$H), 3.00 (dt, 6H, C$\underline{H}_2$NH), 7.85 (t, 3H, NH).

235 mg (0.38 mmol) triacid intermediate, 218 mg (1.90 mmol) N-hydroxysuccinimide and 281 mg (1.4 mmol) dicyclohexylcarbodiimide were dissolved in 10 ml dry tetrahydrofuran (THF) and stirred overnight at ambient temperature. The filtrate was dissolved in cold ethyl acetate and the precipitate separated by filtration. The solution was concentrated to give 325 mg (94%) **5** as a colourless solid. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.15-1.70 (m, 18H, NHCH$_2$C$\underline{H}_2$C$\underline{H}_2$C$\underline{H}_2$), 2.02 (m, 12H, C$\underline{H}_2$C$\underline{H}_2$C=O), 2.65 (t, 6H, C$\underline{H}_2$CO$_2$H), 2.80 (s, 12H, OSu), 3.00 (dt, 6H, C$\underline{H}_2$NH), 7.86 (t, 3H, NH).

Preparation of trimeric sialyl Lewis-X (6) (method A): 30.0 mg (0.033 mmol) tetrasaccharide lactone **1c**[6] and 5.4 mg (0.01 mmol) **3** in 7 ml pyridine were stirred at 60°C until the reaction was complete (ca.7 h), as monitored by TLC. The solvent was evaporated and the residue dissolved in 3 ml water and adjusted to pH 12 with aqueous 1 N NaOH solution. After 1 h the solution was neutralized with ion exchange resin (H^+-form).

Chromatography on Biogel P4 with water as eluent gave 24 mg (84%) pure **6** as colourless solid. **Method B**: 500 mg (0.54 mmol) tetrasaccharide **1b**[6] and 88.1 mg (0.155 mmol) **3** in 2.5 ml pyridine and 25 ml N,N-dimethylformamide were stirred at 60°C until the reaction was complete (ca.7 h), as monitored by TLC. The solvents were evaporated to deliver 383 mg (83% based on **3** and 71% based on **1b**) of product **6** after chromatography as described above. $[\alpha]_D^{20}$ = -39.6° (c = 1; water). Electrospray ionization MS (ESI-MS): m/e = 993.3 $[M-3H]^{3-}$, 1490.4 $[M-2H]^{2-}$; $C_{121}H_{204}N_{10}O_{74}$ (2983.0). ^{1}H-NMR (500 MHz, D_2O): δ = 1.17 (d, 9H, J = 6.5 Hz, 6-H_{Fuc}), 1.28-1.38 (m, 12H, $NCH_2CH_2C\underline{H}_2C\underline{H}_2CH_2CH_2O$), 1.45-1.59 (m, 12H, $NCH_2C\underline{H}_2CH_2CH_2$-$C\underline{H}_2CH_2O$), 1.80 (pseudo-t, J = 12 Hz, 3H, 3-$H_{Nana/ax}$), 2.02, 2.04 (2s, 18H, 2 NAc), 2.20-2.30 (m, 12H, $C\underline{H}_2C\underline{H}_2CNO_2$), 2.77 (dd, 3H, J = 4.5, J = 12.5 Hz, 3-$H_{Nana/equ}$), 3.16 (t, 6H, J = 6.5 Hz, $CH_2C\underline{H}_2NHCO$), 3.53 (dd, 3H, J = 7.5, J = 9.5 Hz, 2-H_{Gal}), 4.09 (dd, 3H, J = 3.0, J = 9.5 Hz, 3-H_{Gal}), 4.53 (2d, 6H, 1-H_{Gal}, 1-H_{GlcNAc}), 4.83 (m, 3H, H-5_{Fuc}), 5.11 (d, 3H, J = 4.0 Hz, 1-H_{Fuc}). ^{13}C-NMR (125.7 MHz, D_2O): δ = 175.06, 174.14, 174.12, 173.93 (C=O), 101.66 (1-C_{Gal}), 101.01 (1-C_{GlcNAc}), 99.70 (2-C_{Nana}), 98.65 (1-C_{Fuc}), 75.68, 75.29, 74.92, 74.89 (3-C_{Gal}, 5-C_{GlcNAc}, 3-C_{GlcNAc}, 5-C_{Gal}), 73.39, 72.94 (4-C_{GlcNAc}, 6-C_{Nana}), 71.94, 71.88 (4-C_{Fuc}, 8-C_{Nana}), 70.52 (Spacer-$\underline{C}H_2O$), 69.30, 69.22 (2-C_{Gal}, 3-C_{Fuc}), 68.36, 68.14 (4-C_{Nana}, 7-C_{Nana}), 67.75, 67.34, 66.71 (2-C_{Fuc}, 4-C_{Gal}, 5-C_{Fuc}), 62.62 (9-C_{Nana}), 61.51 (6-C_{Gal}), 59.70 (6-C_{GlcNAc}), 55.17 (2-C_{GlcNAc}), 51.73 (5-C_{Nana}), 39.81, 39.46 (Spacer-$\underline{C}H_2NH$, 3-C_{Nana}), 30.64, 30.18 [$C(NO_2)\underline{C}H_2\underline{C}H_2$], 28.55, 28.25, 25.75, 24.78 ($NCH_2\underline{C}H_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_2O$), 22.31, 22.07 (NAc), 15.31 (6-C_{Fuc}).

Preparation of trimeric sialyl Lewis-X (7) (method A): 98 mg (0.108 mmol) tetrasaccharide lactone **1c**[6] and 30 mg (0.033 mmol) **5** in 10 ml pyridine were stirred at 60°C until the reaction was complete (ca.10 h), as monitored by TLC. The solvent was evaporated, the residue dissolved in 10 ml water and adjusted to pH 12 with an aqueous 1 N NaOH solution. After 1 h the solution was neutralized with ion exchange resin (H^+-form). Chromatography on Biogel P4 with water as eluent delivered 17 mg **7** (15% based on **5** and 14% based on **1c**) as a colourless solid. Electrospray ionization MS (ESI-MS): m/e = 1106.5 $[M-3H]^{3-}$; $C_{139}H_{237}N_{13}O_{77}$ (3322.4). Traces of the bivalent compound (2420 g/mol) are detectable at m/e = 805.8 $[M-3H]^{3-}$. ^{1}H-NMR (500 MHz, D_2O): δ = 1.13 (d, 9H, J = 6.5 Hz, 6-H_{Fuc}), 1.22-1.33 (m, 18H, $NCH_2CH_2C\underline{H}_2C\underline{H}_2CH_2CH_2O$ and $NHCOCH_2CH_2C\underline{H}_2CH_2CH_2CO$), 1.40-1.60 (m, 24H, $NCH_2C\underline{H}_2CH_2CH_2C\underline{H}_2CH_2O$ and $NHCOCH_2C\underline{H}_2$-$CH_2C\underline{H}_2CH_2CO$), 1.75 (pseudo-t, J = 12 Hz, 3H, 3-$H_{Nana/ax}$), 1.97, 1.99 (2s, 18H, 2 NAc), 2.15-2.27 (m, 18H, $C\underline{H}_2C\underline{H}_2CNO_2$ and $CH_2CH_2C\underline{H}_2CONH$), 2.74 (dd, 3H, J = 4.5, J = 12.5 Hz, 3-$H_{Nana/equ}$), 3.13 (2t, 12H, J = 6.5 Hz, $C\underline{H}_2NHCO$), 4.04 (dd, 3H, J = 3.0, J = 9.5 Hz, 3-H_{Gal}), 4.48 (2d, 6H, 1-H_{Gal}, 1-H_{GlcNAc}),

4.78 (m, 3H, H-5_{Fuc}), 5.07 (d, 3H, J = 4.0 Hz, 1-H_{Fuc}). ^{13}C-NMR (125.7 MHz, D_2O): δ = 176.50, 174.92, 173.97, 173.90, 173.79 (CO), 101.52 (1-C_{Gal}), 100.88 (1-C_{GlcNAc}), 99.56 (2-C_{Nana}), 98.51 (1-C_{Fuc}), 93.39 ($\underline{C}NO_2$), 75.55, 75.16, 74.79, 74.76 (3-C_{Gal}, 5-C_{GlcNAc}, 3-C_{GlcNAc}, 5-C_{Gal}), 73.25, 72.81 (4-C_{GlcNAc}, 6-C_{Nana}), 71.95, 71.80 (4-C_{Fuc}, 8-C_{Nana}), 70.36 (Spacer-$\underline{C}H_2O$), 69.42, 69.17, 68.21, 68.01, 67.62, 67.21, 66.58, (2-C_{Gal}, 3-C_{Fuc}, 4-C_{Nana}, 7-C_{Nana}, 2-C_{Fuc}, 4-C_{Gal}, 5-C_{Fuc}), 62.49 (9-C_{Nana}), 61.38, 59.56, (6-C_{Gal}, 6-C_{GlcNAc}), 55.75 (2-C_{GlcNAc}), 51.60 (5-C_{Nana}), 39.67, 39.17, 39.12 (2x Spacer-$\underline{C}H_2NH$, 3-C_{Nana}), 35.58 ($OC_6H_{12}NHCO\underline{C}H_2$), 30.55, 30.06 [$C(NO_2)\underline{C}H_2\underline{C}H_2$], 28.45, 28.26, 27.79, 25.64, 25.40, 24.99, 24.68, ($NCH_2\underline{C}H_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_2O$ and $NHCOCH_2\underline{C}H_2\underline{C}H_2\underline{C}H_2CH_2CO$), 22.17, 21.93 (NAc), 15.18 (6-C_{Fuc}).

Preparation of trimeric sialyl Lewis-X (8): 355 mg (0.283 mmol) tetrasaccharide lactone **1d**, freshly prepared from the anomeric azide by hydrogenation according to the literature[8], and 44.8 mg (0.0787 mmol) tris-succinimide **3** were dissolved in 2.5 ml THF and 2.5 ml pyridine and stirred for 12 h at ambient temperature. The residue obtained after evaporation of the solvents was subjected to flash column chromatography on Kieselgel (dichloromethane/ methanol 10:1) to give 277 mg (88%) of the protected trimeric intermediate. The partially protected compound (250 mg) was deprotected in the following reaction steps: Hydrogenation in 30 ml methanol/ 1,4-dioxane (10:1) with 200 mg of 10% Pd/C as catalyst for 16 h at ambient pressure, filtration, evaporation of the solvents gave an intermediate which was subsequently subjected to lactone hydrolysis in the following manner: Treatment with 0.8 ml 1 M aqueous sodium hydroxide solution in 40 ml water and 5 ml methanol for 1 h, neutralisation with ion exchange resin (Amberlite IR-120) and column chromatography on Biogel P2 with water as eluent gave 157 mg (93%) **8** as colourless solid (overall yield: 82%). $[\alpha]_D^{20}$ = -27.3° (c = 1; water); Electrospray ionization MS (ESI-MS): m/e = 1364.1 $(M+2Na)^{2+}$; $C_{103}H_{168}N_{10}O_{71}$ (2682.5). ^{1}H-NMR (300 MHz, D_2O, 3-TMS-propionic acid-d_4 sodium salt): δ = 1.18 (d, 9H, J = 6.5 Hz, 6-H_{Fuc}), 1.80 (pseudo-t, J = 12.1 Hz, 3H, 3-$H_{Nana/ax}$), 1.86, 1.90 (2s, 18H, 2 NAc), 2.19-2.29 (m, 12H, $C\underline{H}_2C\underline{H}_2CNO_2$), 2.77 (dd, 3H, J = 4.5 Hz, J = 12.5 Hz, 3-$H_{Nana/eq}$), 3.58 (5-H_{Gal}), 3.66 (6-H_{Nana}), 3.65, 3.86 (9-H_{Nana}), 3.68 (6-H_{Gal}), 3.79 (4-H_{Fuc}), 3.84 (5-H_{Nana}), 4.08 (dd, 3H, J = 2.7 Hz, J = 9.6 Hz, 3-H_{Gal}), 4.55 (d, 3H, J = 7.5 Hz, 1-H_{Gal}), 4.84 (m, 3H, 5-H_{Fuc}), 3.90 (8-H_{Nana}), 5.10 (d, 3H, J = 7.1 Hz, 1-H_{GlcNAc}), 5.11 (d, 3H, J = 3.4 Hz, 1-H_{Fuc}). ^{13}C-NMR (75.4 MHz, D_2O, 3-TMS-propionic acid-d_4 sodium salt): δ = 177.9, 177.8, 177.0, 176.7 (CO), 104.5 (1-C_{Gal}), 102.5 (2-C_{Nana}), 101.5 (1-C_{Fuc}), 95.7 (C-NO_2), 81.1 (1-C_{GlcNAc}), 79.9 (C_{GlcNAc}), 78.5 (3-C_{Gal}), 78.2 (C_{GlcNAc}), 77.8 (5-C_{Gal}), 75.8 (C_{GlcNAc}, 6-C_{Nana}), 74.8 (4-C_{Fuc}), 74.7 (8-C_{Nana}), 72.1 (2-C_{Gal}), 72.1, 71.1, 71.0, 70.5, 70.2 (4-C_{Nana}, 7-C_{Nana}, 3-C_{Fuc}, 2-C_{Fuc},

4-C_{Gal}), 69.6 (5-C_{Fuc}), 65.5 (9-C_{Nana}), 64.4 (6-C_{Gal}), 62.4 (6-C_{GlcNAc}), 57.5 (2-C_{GlcNAc}), 54.6 (5-C_{Nana}), 42.7 (3-C_{Nana}), 33.2, 33.0 ($\underline{C}H_2$), 25.1, 24.9 (NAc), 18.2 (6-C_{Fuc}).

Selectin cell adhesion assays: The activity of compounds *in vitro* was measured in adhesion assays as the inhibition of the binding of promyelocytic leukemia HL60 cells (ATCC CRL1964) to recombinant solid phase bound selectin-fusion proteins. Genetic constructs for expression of extracellular portions of the human selectins E and P, joined to human immunoglobulin heavy chain hinge, CH2 and CH3 regions were obtained from *B. Seed*, Massachusetts General Hospital, Boston, USA. The soluble E- and P-selectin-IgG fusion proteins contain the signal sequence, the lectin-like domain, the EGF repeat domain and six (E-selectin) and two (P-selectin) of the complement regulatory-like domains.[25,26] CD4-IgG served as a negative control.[28] Recombinant proteins were expressed as soluble proteins after DEAE/dextran facilitated plasmid DNA transfection into COS cells according to standard procedures.[27] The 96well round bottomed ELISA plates (Maxisorp, Nunc, Kamstrup, Denmark) were precoated for 1 h at room temperature (RT) with 100 µl/well of 10 µg/ml goat-anti-human-Ig (Cappel, Durham, USA) in 50 mM Tris-HCl (pH 9.5). After washing with PBS, plates were blocked for 0.5 h with 150 µl/well of 1% BSA in PBS. Next, plates were coated with fusion proteins by incubating the plates for 1.5 h with either 100 µl/well CD4-IgG, E-selectin-IgG or P-selectin-IgG fusion protein in COS-cell culture supernatants diluted to the same fusion protein content (1-2 µg/ml) as determined by an Ig specific ELISA. 100 µl/well of Fc-receptor blocking buffer (1 mg/ml γ-globulin, Sigma, Deisenhofen, FRG) in binding buffer (see below) was added to prevent HL60 cell binding to immobilised goat anti human Ig antibody *via* Fc receptors. After one PBS wash, 40 µl/well binding buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 100 µM $MgCl_2$. 100 µM $MnCl_2$, 100 µM $CaCl_2$, 1 mg/ml BSA) were added together with test compounds or antibodies (see below). Parallel to the preparation of the plates, HL60 cells, grown in DMEM medium with 20 % FCS, were washed two times with PBS before incubation for 20 min in Fc-receptor blocking buffer at RT. 10^5 cells/well in 50 µl Fc-receptor blocking buffer were left to sediment and to adhere for 10 min, before the plates were slowly immersed at a 45° angle in stop buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 100 µM $MgCl_2$, 100 µM $MnCl_2$, 100 µM $CaCl_2$) to remove all unbound cells. After inverting the plate on filter paper and one repeat of the washing step, 50 µl/well of staining solution (4 % formaldehyde, 0.5 % Triton X-100, 17 µg/ml Hoechst 33528 DNA dye) was slowly added and incubated at RT for 15 min. The amount of bound cells was quantitated in a cytofluorometer (CytoFluor 2300, Millipore, Bedford, USA). Specific cell binding was calculated by subtraction of the signals in wells coated with CD4-IgG (nonspecific binding) from the signals in wells coated with selectin-IgG. Assay performance was controlled by using adhesion blocking mouse anti-E-selectin monoclonal antibody (clone BBIG- E4, R&D systems, Abingdon, UK) and compound **1a** as inhibition controls. IC_{50} values were determined according to the four parameter logistics model.[40] Each IC_{50} determination was repeated on another day at least once.

Inhibition of leukocyte adhesion *in vivo:* The animal experiments have been performed according to the "German Animal Protection Law" from 1993 and the declaration of Helsinki and reviewed by the ethics commitee of the German Federal Government (permission No. H2-01/36 and H2-01/34).

Animal preparations: Female Sprague-Dawley rats (Mollegaard, Denmark) weighing 180-200 g were anaesthetized with an intramuscular injection of 1.25 g/kg urethane (Riedel-de-Haen, Hannover, Germany) under light ether anaesthesia. The animals breathed spontaneously through a tracheal tube and body temperature was maintained at 37°C with a regulated heating pad. Rat mesentery, exposed cautiously[41] through a hypogastric incision on a thermocontrolled (37°C) window of the microscope stage, was covered with paraffin liquid at 37°C, equilibrated with 95% N_2/ 5% CO_2. With three blunt needles attached to modelling clay, the ileo-caecal portion of the mesentery was fixed in position. The experiment lasted 2 hours. For each experiment, the rat femoral artery and vein were cannulated for monitoring blood pressure and heart rate (Statham transducer, P 23 Db), or intravenous (i.v.) injection of lipopolysaccharide (LPS) or drugs, respectively. Sixty minutes after surgery LPS (from *E.coli*, Serotype 0269B6, phenol extraction, SIGMA, St.Louis, MO; 15 mg/kg in 1 ml/kg bodyweight) was injected.

Intravital microscopy: A Zeiss inverted camera microscope ICM 401 (Oberkochen, Germany) was used to investigate the mesenteric microcirculation. The ICM was equipped with transillumination using 10x oculars, a 25/0.35x objective for leukocyte counting, and LD-Epiplan 40-0.60x long working objective.

Measurements of leukocyte adhesion: Following a 30 min postsurgical equilibration period during which the tissue was allowed to stabilize, leukocyte adhesion was measured in postcapillary venules of 10-30 μm diameter and 100 μm length. Leukocytes adhering to the endothelium were counted in 2-3 segments of postcapillary venules at 10 minutes intervals.[42] A leukocyte was considered adherent to the endothelium if it was stationary for more than 30 seconds. The mean values of the numbers of adhering cells between 10 and 30 minutes after surgery were calculated and served as control for the model. The drug was administered 30 min after surgery and the LPS-endotoxin 30 min thereafter. All data between the 70th and 120th minute after surgery were taken for calculations, whereby the untreated LPS-controls were compared with the drug treated animals. The statistical results were reported as mean and standard error of mean (SEM). Significances were determined with the nonparametric Mann Whitney U-test and expressed as $p < 0.05$.

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44. Work is in progress to prepare polyvalent ligands as well as small monovalent mimetics of **1a** with improved efficacy *in vitro* and *in vivo*.

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