# Nanomolar E-Selectin Inhibitors: 700-Fold Potentiation of Affinity by Multivalent Ligand Presentation

Gebhard Thoma,*[,†] Rudolf O. Duthaler,[†]
John L. Magnani,[‡] and John T. Patton[‡]

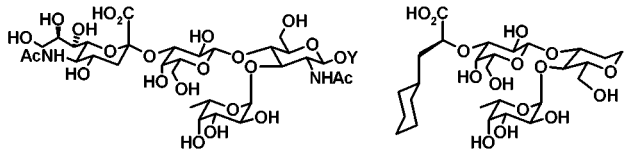
*Novartis Pharma AG*
*CH-4002 Basel, Switzerland*
*GlycoTech Corporation, 14915 Broschart Road*
*Rockville, Maryland 20850*

*Received June 19, 2001*

E-Selectin is a vascular endothelial cell surface protein which mediates leukocyte rolling on the blood vessel wall—an early stage of the inflammatory response.[1] Excessive leukocyte recruitment can cause acute and chronic inflammatory disorders such as reperfusion injuries, psoriasis, rheumatoid arthritis, or respiratory diseases.[2] A possible therapy is to inhibit the interactions of E-selectin with its physiological glycoprotein ligand, the structure of which has not been fully elucidated.[3] The tetrasaccharide sialyl Lewis X (sLe$^x$, Chart 1) is the minimum epitope recognized by E-selectin.[4] The concentration of sLe$^x$ to achieve 50% inhibition ($IC_{50}$) was determined to be 1100 $\mu$M in a static, cell-free E-selectin binding assay using immobilized E-selectin and a biotinylated sLe$^a$-polylysine conjugate as multivalent ligand.[5] By modifying sLe$^x$ we discovered the simplified analogue **1** (Chart 1) which showed 30-fold improved potency ($IC_{50}$ = 36$\mu$M) compared with sLe$^x$.[6]

In some cases high affinity inhibitors can be obtained by multivalent presentation of low-affinity ligands.[7] Polymers, liposomes, and protein conjugates containing sLe$^x$ or similar carbohydrates have been described.[8] We have reported on multivalent sLe$^x$ polyaspartic acid conjugates[9] and polylysine−sLe$^x$ conjugates such as **2** (Scheme 1).[10] Generally, the evaluation of such compounds in static, cell-free assays can be misleading because, compared to cell surfaces, plastic wells coated with either selectin ligands or recombinant selectins exhibit completely different properties with respect to both density and mobility. These factors are essential, especially for multivalent interactions. To better assess the properties of our compounds we developed a dynamic in vitro assay which mimics the nonequilibrium conditions in vivo.[11] We employed a parallel-plate flow chamber coated with human umbilical vein endothelial cells (HUVECs) to study the rolling of polymorphonuclear neutrophils (PMNs) in contact with selectins under hydrodynamic flow. Unfortunately, sLe$^x$-conjugate **2** did not show any significant E-selectin inhibition at 100 $\mu$M in the flow assay (Table 1).[10] Monovalent sLe$^x$ also did not reduce leukocyte rolling at concentrations up to 1000 $\mu$M, but inhibitor **1** showed an $IC_{50}$-value of 30−40 $\mu$M.[6]

**Chart 1**



Sialyl Lewis$^x$: Y= H

$R_1$-SH: Y= $(CH_2)_3$-NH-C(O)-$(CH_2)_3$-SH

**1**

Here we report that multivalent polylysine conjugates of antagonist **1**, such as **3** and **4** (Scheme 1), are highly active E-selectin inhibitors which reduce neutrophil rolling on activated endothelial cells with $IC_{50}$ values as low as 50 nM. Compared to monovalent sLe$^x$ these glycopolymers exhibit at least a $5 \times 10^4$-fold improved potency. We show that both the size of the polymeric carrier molecule and the ligand loading affect E-selectin inhibition.

The conjugates **3** and **4** were prepared from chloroacetylated polylysine **5** and thiol derivative **$R_2$-SH** (Scheme 1) of **1** which was obtained from the corresponding amine **6**[11] in 60−70% yield by reacting it with thiobutyrolactone in the presence of triethylamine (Scheme 2).[12] Previously, we have shown that modifications of the C-6 substituent of the 1,2-deoxyglucopyranose portion of **1** do not affect the bioactivity.[11] Therefore, this position was selected as a handle to achieve linkage to the polymer backbone. Polymer **5** and substoichiometric quantities (in case of **3**, **4a**−**e**)

† Novartis Pharma AG.
‡ GlycoTech Corp.

(1) (a) Kansas, G. S. *Blood* **1996**, *88*, 3259. (b) Varki, A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *91*, 7390. (b) Welply, J. K.; Keene, J. L.; Schmuke, J. L.; Howard, S. C.; *Biochim. Biophys. Acta* **1994**, *1197*, 215. (c) Springer, T. A. *Cell* **1994**, *76*, 301.

(2) (a) Cines, D. B.; Pollak, E. S.; Buck, C. A.; Loscalzo, J.; Zimmermann, G. A.; McEver, R. P.; Pober, J. S.; Wick, T. M.; Konkle, B. A.; Schwartz, B. S.; Barnathan, E. S.; McCrae, K. R.; Hug, B. A.; Schmidt, A.-M.; Stern, D. M. *Blood* **1998**, *91*, 3527. (b) Mousa, S. A. *Drugs Future* **1996**, *21*, 283. (c) Mousa, S. A.; Cheresh, D. A. *Drug Discovery Today* **1997**, *2*, 187.

(3) (a) Levinovitz, A.; Muehlhoff, J.; Isenman, S.; Vestweber, D. *J. Cell Biol.* **1993**, *121*, 449. (b) Lenter, M.; Levinovitz, A.; Isenman, S.; Vestweber, D. *J. Cell Biol.* **1994**, *125*, 471. (c) Steegmaier, M.; Levinovitz, A.; Isenman, S.; Borges, E.; Lenter, M.; Kocher, H. P.; Kleuser, B.; Vestweber, D. *Nature* **1995**, *373*, 615. (d) Patel, T. P.; Goelz, S. E.; Lobb, R. R.; Parekh, Raj. B. *Biochemistry* **1994**, *33*, 14815.

(4) (a) Springer, T. A.; Lasky, L. *Nature* **1999**, *349*, 196. (b) Drickamer, K. *Glycobiology* **1994**, *4*, 245. (c) Ng, K. K.-S.; Weis, W. I. *Biochemistry* **1997**, *36*, 965. (d) Phillips, M. L.; Nudelman, E.; Gaeta, F. C. A.; Perez, M.; Singhal, A. K.; Hakomori, S.; Paulson, J. C. *Science* **1990**, *250*, 1130. (e) Walz, G.; Aruffo, A.; Kolanus, W.; Bevilacqua, M.; Seed, B. *Science* **1990**, *250*, 1132. (f) Lowe, J. B.; Stoolman, L. M.; Nair, R. P.; Larsen, R. D.; Berhend, T. L.; Marks, R. M. *Cell* **1990**, *63*, 475. (g) Tiemyer, M.; Swiedler, S. J.; Ishihara, M.; Moreland, M.; Schweingruber, H.; Hirtzer, P.; Brandley, B. K. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1138.

(5) Thoma, G.; Magnani, J. L.; Oehrlein, R.; Ernst, B.; Schwarzenbach, F.; Duthaler, R. O. *J. Am. Chem. Soc.* **1997**, *119*, 7414.

(6) Thoma, G.; Kinzy, W.; Bruns, C.; Patton, J. T.; Magnani, J. L., Bänteli, R. *J. Med. Chem.* **1999**, *42*, 4909.

(7) (a) Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 321. (b) Jacob, G. S., Kirmaier, C., Abbas, S. Z., Howard, S. C., Steininger, C. N., Welpley, J. K., Sudder, P. *Biochemistry* **1995**, *34*, 1210. (c) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754. (d) Gardiner, J. M. *Exp. Opin. Invest. Drugs* **1998**, *7*, 405. (e) Roy, R. *Cur. Opin. Struct. Biol.* **1996**, *6*, 692. (f) Bovin, N. V.; Gabius, H.-J. *Chem. Soc. Rev.* **1995**, 413. (g) Miyata, T.; Nakamae, K. *Trends Polym. Sci.* **1997**, *5*, 198. (h) Kiessling, L. L.; Pohl, N. L. *Chem. Biol.* **1996**, *3*, 71. (i) Drickamer, K. *Struct. Biol.* **1995**, *2*, 437.

(8) (a) Manning, D. D.; Hu, X.; Beck, P.; Kiessling, L. L. *J. Am. Chem. Soc.* **1997**, *119*, 3161. (b) Sanders, W. J.; Gordon, E. J.; Oren, D.; Beck, P. J.; Ronen A.; Kiessling, L. L. *J. Biol. Chem.* **1999**, *274*, 5271. (c) Miyauchi, H.; Tanaka, M.; Koike, H.; Kawamura, N.; Hayashi, M. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 985. (d) Palcic, M. M.; Li, H.; Zanini, D.; Bhella, R. S.; Roy, R. *Carbohydr. Res.* **1998**, *305*, 433. (e) Stahn, R.; Schäfer, H.; Kernchen, F.; Schreiber, J. *Glycobiology* **1998**, *8*, 311. (f) Lin, C.-C.; Kimura, T.; Wu, S.-H.; Weitz-Schmidt, G. Wong, C−H. *Biorg. Med. Chem. Lett.* **1996**, *6*, 2755. (g) Roy, R.; Park, W. K. C.; Srivastava, O. P.; Foxall, C. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1399. (h) DeFrees, S. A.; Phillips, L.; Guo, L.; Zalipsky, S. *J. Am. Chem. Soc.* **1996**, *118*, 6101. (i) Spevak, W.; Foxall, C.; Charycj, D. H.; Dasgupta, F.; Nagy, J. O. *J. Med. Chem.* **1996**, *39*, 1018. (j) Welply J. K.; Abbas, S. Z.; Sudder, P.; Keene, J. L.; Broschat, K.; Casnocha, S.; Gorka, C.; Steininger, C.; Howard, S. C.; Schmuke, J. J.; Graneto, M.; Rotsaert, J. M.; Manger, I. D.; Jacob, G. S. *Glycobiology* **1994**, *4*, 259. (k) Berg, E. L.; Robinson, M. K.; Mansson, O.; Butcher, E. C.; Magnani, J. L. *J. Biol. Chem.* **1991**, *266*, 14869.

(9) Thoma, G.; Ernst, B.; Schwarzenbach, F.; Duthaler, R. O. *Bioorg. Chem. Med. Lett.* **1997**, *7*, 1705.

(10) (a) Thoma, G.; Duthaler, R. O.; Ernst, B.; Magnani, J. L.; Patton, J. T. PCT Int. Appl. WO 97/19105, 1997. (b) Thoma, G.; Patton, J. T.; Magnani, J. L.; Ernst, B.; Oehrlein, R.; Duthaler, R. O. *J. Am. Chem. Soc.* **1999**, *121*, 5919.

(11) Bänteli, R.; Herold, P.; Bruns, C.; Patton, J. T.; Magnani, J. L.; Thoma, G. *Helv. Chim. Acta.* **2000**, *83*, 2893.

(12) Poly-L-lysines with $n \approx 250$ ($M_w/M_n = 1.2$) and $n \approx 1200$ ($M_w/M_n = 1.4$) were purchased from Sigma. For the synthesis of **5** and further experimantal details see ref 10b.

10.1021/ja0164430 CCC: $20.00 
Published on Web 09/22/2001

**Scheme 1**

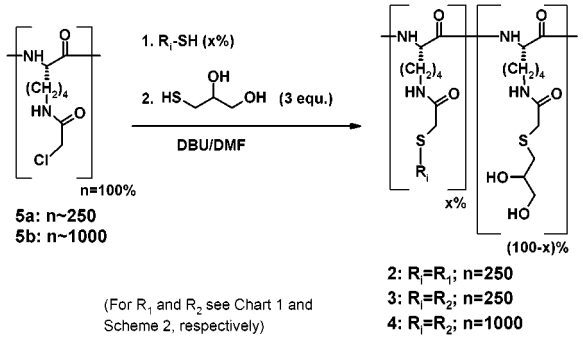
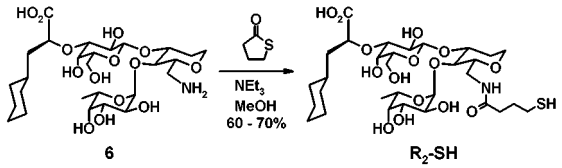



**Table 1.** E-Selectin Inhibition

| cmpd | $R_i$ (% cont.) | $n$ | flow assay $IC_{50}$ [$\mu$M][a] |
|---|---|---|---|
| $sLe^x$ | monovalent | | no inhib. at 1000 $\mu$M |
| **1** | monovalent | | 30−40[b] |
| **2** | $R_1$ (20) | 250 | no inhib. at 100 $\mu$M [10] |
| **3** | $R_2$ (20) | 250 | 2[c] |
| **4a** | $R_2$ (5) | 1200 | no inhib. at 10 $\mu$M |
| **4b** | $R_2$ (10) | 1200 | 4[a] |
| **4c** | $R_2$ (20) | 1200 | 0.2[a] |
| **4d** | $R_2$ (35) | 1200 | 0.05[a] |
| **4e** | $R_2$ (50) | 1200 | 0.05[a] |
| **4f** | $R_2$ (100) | 1200 | 0.2[a] |

[a] For multivalent compounds concentrations refer to carbohydrate ligand concentrations but not to macromolecule concentration. [b] Estimated from measurements at 200, 50, and 10 $\mu$M. [6] [c] Estimated from measurements at 10, 2, 0.2, and 0.02 $\mu$M.

**Scheme 2**




or 1.5 eq (in case of **4f**) of **$R_2$-SH** were dissolved in degassed DMF. DBU (2−3 eq) was added and the mixture stirred for 15 min. Then 3 eq of thioglycerol and 3 eq of $NEt_3$ were added to transform remaining chloroacetamide functionalities into dihydroxy side chains.[10]

All products were purified by ultrafiltration. The yields were generally higher than 90%. To investigate the influence of the molecule size we used chloroacetylated polylysine **5a** and **5b** with degrees of polymerization ($n$) of 250 and 1200, respectively. To study the effects of the ligand loading, conjugates ($n$ = 1200) with 5, 10, 20, 35, 50, and 100% carbohydrate side chains were prepared. The composition of the glycopolymers was verified by integrating several baseline-separated $^1$H NMR signals of ligand and thioglycerol as exemplified by the 80 °C $^1$H NMR spectra of **3** ($n$ = 250) and **4c** ($n$ = 1200) containing 20% carbohydrate side chains and 80% thioglycerol side chains (Figure 1). The signals of **3** ($M_n \approx 100\,000$) are slightly sharper and better resolved than the signals of **4c** ($M_n \approx 400\,000$), but even the composition of compounds as big as **4c** can be accurately analyzed. In glycoconjugate **4f** no thioglycerol side chains could be detected, indicating complete carbohydrate functionalization.

The polymers were tested for E-selectin inhibition in the flow assay at 10, 2, 0.2, and 0.02 $\mu$M to allow an estimate of their

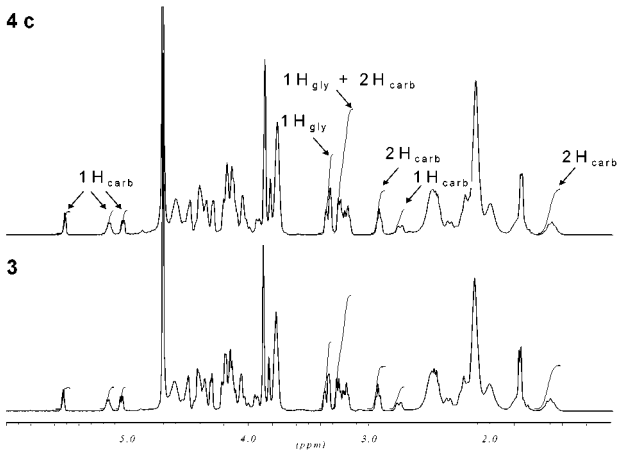



**Figure 1.** $^1$H NMR (400 MHz) spectra of glycoconjugates **3** and **4c** in $D_2O$ at 80 °C. Several baseline-separated signals can be assigned to either the carbohydrate ligand (carb) or thioglycerol (gly). Integration allows the determination of the composition within NMR accuracy.

$IC_{50}$ values. The data are based on molar concentrations of saccharide residues but not on molar concentrations of macromolecules (Table 1). All compounds (with the exception of **4a**) are by far more active than monovalent $sLe^x$, multivalent $sLe^x$-conjugate **2**, and monovalent inhibitor **1**. The potency clearly depends on the size of the multivalent molecule. Compound **3** with $n$ = 250 and 20% ligand content shows an $IC_{50}$ of 2 $\mu$M, whereas the considerably bigger conjugate **4c** with identical composition but $n$ = 1200 was 10-fold more potent. For conjugates **4a**−**f** ($n$ = 1200) we observed a strong dependence of the potency on the ligand loading. While compound **4a** with only 5% carbohydrate side chains gave no inhibition at 10 $\mu$M, glycopolymers **4b** and **4c** containing 10 and 20% of the ligand showed $IC_{50}$ values of 4 and 0.2 $\mu$M, respectively. The conjugates **4d** and **4e** with 35 and 50% carbohydrate side chains, respectively, are highly potent E-selectin inhibitors with $IC_{50}$ values of 50 nM. Interestingly, a significantly reduced affinity ($IC_{50}$ = 0.2 $\mu$M) was observed for compound **5f** with complete ligand functionalization.

Assuming a statistical ligand distribution in glycoconjugates **3** and **4**, the mean distance between two ligands in a conjugate in solution decreases with the ligand loading. A minimum ligand loading (in our case >20%) is required to achieve a ligand density which is optimal for multiple receptor recognition and, thus, allows for high affinity binding. In case of a too high ligand density most ligand residues will not bind to a receptor for steric reasons. Thus, the affinity of such conjugates is expected to drop. This is observed for compound **4f** with 100% ligand loading.

In conclusion we have demonstrated that nanomolar E-selectin inhibitors can be obtained by multivalent presentation of the micromolar small molecule inhibitor **1** (700-fold improved potency), whereas multivalent presentation of the millimolar E-selectin antagonist $sLe^x$ gave no significant effect. Our data suggest that the affinity of a small molecule antagonist has to exceed a certain threshold value to achieve multivalent amplification.

**Supporting Information Available:** Procedures for the syntheses of compounds **$R_2$-SH**, **3**, and **4a**−**f** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0164430