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# Sialyl Lewis$^x$: A "Pre-Organized Water Oligomer"?**

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*In memory of Daniel Bellûs*

In recent years, lectins, such as selectins,[1] galectins,[2] or siglecs[3] have received increasing attention as drug targets. Among them, selectins are the most extensively studied, since they are key players in the early stages of inflammation and therefore promising targets for the treatment of diseases with an inflammatory component, such as stroke, asthma, psoriasis, or rheumatoid arthritis.[4] The key role of selectins is to promote the initial step of the inflammatory cascade, by allowing leukocytes to roll along the vascular endothelial surface. This step is followed by the integrin-mediated firm adhesion and the final extravasation to the site of the inflammatory stimulus.[5]

The specific interaction between E-selectin and its physiological ligand ESL-1 (E-selectin ligand-1) is mediated by the tetrasaccharide sialyl Lewis$^x$ (sLe$^x$, **1**).[6] Consequently, sLe$^x$ (**1**) became the lead structure for the search of drug-like, high-affinity selectin antagonists.[1,7] Elucidation of the structure activity relationship (SAR),[8] mutation studies,[9] transferred nuclear overhauser enhancement NMR spectroscopy (trNOE-NMR),[10] saturation transfer difference NMR spectroscopy (STD-NMR),[11] molecular modeling,[12] and finally X-ray crystallography[13] yielded a precise picture of the interactions of sLe$^x$ and E-selectin on an atomic level (Figure 1). Because docking studies[7] and STD-NMR experiments[11] revealed that the *N*-acetyl-D-glucosamine (D-Glc*N*Ac) and *N*-acetyl-D-neuraminic acid (D-Neu5Ac) moieties have only weak interactions with the protein,[12] they were replaced with structurally simplified mimics, resulting in E-selectin antagonists with significantly improved binding affinities. However, the affinity of these antagonists,

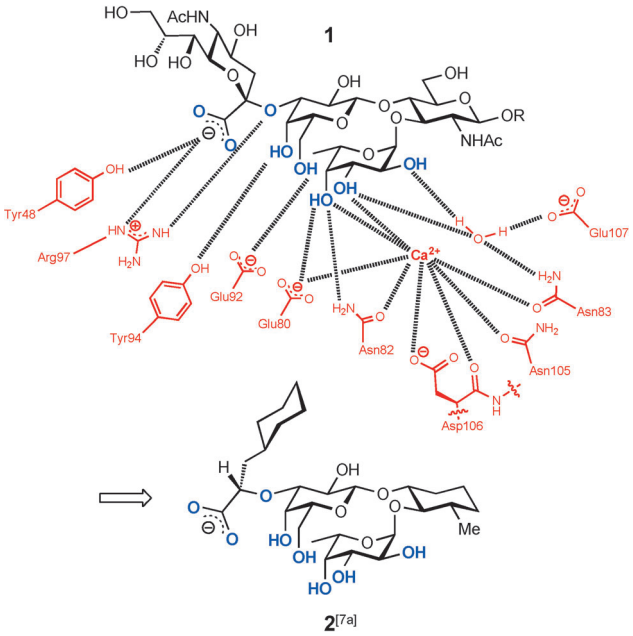


***Figure 1.*** Top: Detailed representation of the interactions between sLe$^x$ (**1**) and E-selectin as observed in the crystal structure;[13] the pharmacophores of **1** are highlighted in blue. Bottom: The glycomimetic **2** exhibits a 13 μM affinity in a cell-free ligand-based competitive binding assay.[7a]

for example, **2** in Figure 1,[7a] is still only in the low micromolar range.

Despite the progress made, the driving force of the interaction of E-selectin with its ligands has not been fully characterized to date, neither for sLe$^x$ (**1**), nor for any low molecular weight selectin antagonist. However, Wild et al. estimated the enthaplic contribution of the E-selectin/ESL-1 interaction by van't Hoff analysis, which involves the correlation of the binding affinity measured at different temperatures. The results indicated that enthalpic changes contribute only 10 to 25 % of the binding free energy $\Delta G$ and that the interaction is primarily driven by favorable entropy changes.[14]

Recently, the thermodynamic aspects of protein–ligand interactions have gained increasing interest in drug discovery.[15] Particularly enthalpy and entropy changes provide valuable information for lead optimization. Having access to these individual components of binding affinity rather than the overall value facilitates the successful design of high-affinity ligands. Herein, we report a comprehensive study on the thermodynamic fingerprint of a series of E-selectin antagonists.

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Supporting information for this article (details of the synthesis of antagonist **4**, the expression and purification of E-selectin/IgG, the competitive binding assay, and the isothermal calorimetry experiments) is available on the WWW under http://dx.doi.org/10.1002/anie.201202555.

The binding free energy ($\Delta G$) associated with a protein–ligand interaction is composed of enthalpic ($\Delta H$) and entropic ($-T\Delta S$) contributions ($\Delta G = \Delta H - T\Delta S$). The binding energy under standard conditions ($\Delta G°$), where all reactants and products are at a concentration of 1 mol $L^{-1}$, is calculated from the dissociation constant $K_D$ using the equation $\Delta G = RT\ln K_D$. With isothermal titration calorimetry (ITC),[16] $K_D$ and the enthalpy $\Delta H$ are measured directly if no changes in the protonation states occur during the interaction. The enthalpic term ($\Delta H$) represents the contribution of non-covalent interactions upon binding,[15b] that is, hydrogen bonds, electrostatic, and dipole–dipole interactions between ligand and receptor.[17] The entropy term can be dissected into translational and rigid-body rotational entropy,[18] solvation entropy,[19] and conformational entropy.[20]

For our study, an E-selectin/IgG construct consisting of the lectin domain, the EGF-like domain, and six short consensus repeats fused to the Fc part of human IgG1 was used.[21] The 148 kDa protein was expressed in Chinese Hamster Ovarian cells and purified from the conditioned culture medium by affinity chromatography, first with protein A-Sepharose, followed by a second functional purification with the monoclonal anti-hE-selectin antibody 7A9 (see the Supporting Information). The high degree of purity and functionality of the protein is reflected by the stoichiometry ($N$) of the calorimetric experiments (Table 1). Batches of up to 50 mg E-selectin/IgG were necessary for ITC measurement to reach c values close to 1.

Our calorimetric investigation had two goals: first, the determination of $K_D$ values of a series of E-selectin ligands and their comparison with data collected by a competitive binding assay (Table 1)[22] and second, the elucidation of the thermodynamic fingerprints of these ligands. The $K_D$ value for sLe$^x$ (**1**) binding to E-selectin determined by ITC is (878 ± 93) μM and is thereby in good agreement with previously reported data (e.g. 1.1 to 2.0 mM,[23] (0.7 ± 0.4) mM[10b]). In addition, the relative $K_D$ values ($rK_D$) for the antagonists **2** to **6** also nicely correlate with their relative $IC_{50}$ values ($rIC_{50}$). In analogy to earlier findings,[7a] replacement of D-Glc*N*Ac with carbocyclic mimics enhanced binding affinity up to 25-fold (**1**→**4**), whereas the replacement of D-Neu5Ac by (*S*)-cyclohexyllactic acid improved binding 2- to 5-fold (**1**→**5**; **3**→**6**; **4**→**2**).

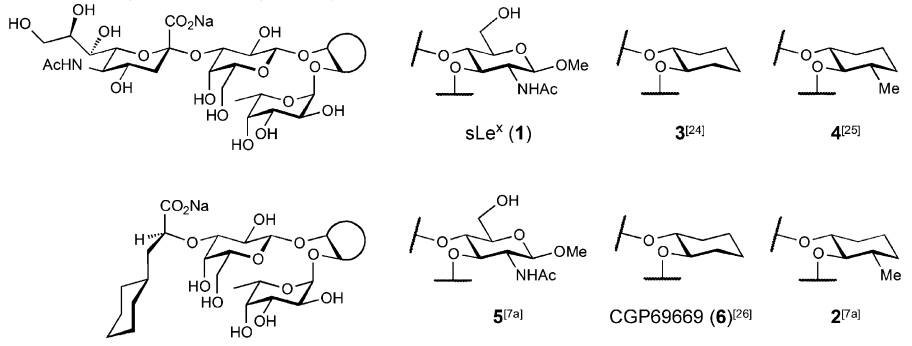
Except for some isolated cases,[14,27] lectin–oligosaccharide interactions are typically enthalpy driven with mostly unfavorable entropies.[28] In contrast, the binding of sLe$^x$ to E-selectin is driven by a large entropy term ($-T\Delta S = -23$ kJ mol$^{-1}$). Clearly, the entropy costs caused by the loss of translational and rotational degrees of freedom and conformational changes of ligand and protein upon binding are overcompensated by the beneficial entropy arising from the release of bound water molecules.[29] This argumentation is supported by two experimental observations. First, the bound conformation was identified as one of two low-energy solution conformations of sLe$^x$,[30] demanding only minor conformational adjustments upon binding. Second, the comparison of the crystal structure of apo-E-selectin and E-selectin bound to sLe$^x$ revealed only minor conformational differences.[13]

The beneficial entropy term, however, is partially compensated by an unfavorable change in enthalpy. To enable the pharmacophoric groups of sLe$^x$ (**1**) to interact with their target, a predominantly polar surface area of approximately 275 Å$^2$[13a] on both interacting moieties has to be desolvated. Because the newly formed polar interactions between the pharmacophores of sLe$^x$ and E-selectin do not fully compensate for the desolvation penalty of the polar binding interface (Figure 2),[31] a net loss of enthalpy ($\Delta H° = +5.4$ kJ mol$^{-1}$) is observed. Thus, the directed polar interactions of the pharmacophores contribute to specificity rather than affinity.

Thus, sLe$^x$ (**1**) represents a surrogate of clustered water molecules attached to a scaffold. As a "pre-organized water oligomer" it offers an array of directed hydrogen bonds for the highly specific binding to E-selectin. The clear entropic benefit of the release of water molecules from the large binding interface to bulk water and the high degree of pre-organization of sLe$^x$ result in the observed large entropy gain which provides the impetus for the binding process.

The concept of conformational pre-organization was also exploited for the development of selectin antagonists (Figure 3). More precisely, in sLe$^x$ (**1**), D-Glc*N*Ac acts as

***Table 1:*** Affinity and thermodynamic parameters for the interaction of **1**–**6** with E-selectin.[a]




| Ligand | $rIC_{50}$ | $rK_D$ | $K_D$ [μM] | $\Delta G$ [kJ mol$^{-1}$] | $\Delta H$ [kJ mol$^{-1}$] | $-T\Delta S$ [kJ mol$^{-1}$] | $N$ |
|---|---|---|---|---|---|---|---|
| **1** | 1 | 1 | 878 ± 93 | −17.5 ± 0.2 | +5.4 ± 0.7 | −23 ± 1 | 1 |
| **3** | 0.3 | 0.36 | 317 | −20.0 | −0.5 | −19.5 | 1 |
| **4** | 0.05 | 0.04 | 38 | −25.3 | +0.9 | −26.2 | 0.94 |
| **5** | 0.27 | 0.30 | 260 | −20.5 | −2.2 | −18.3 | 1 |
| **6** | 0.08 | 0.07 | 59 ± 4 | −24.2 ± 0.2 | −5.3 ± 0.4 | −18.9 ± 0.6 | 0.93 ± 0.08 |
| **2** | 0.014 | 0.02 | 19 ± 2 | −27.1 ± 0.2 | −5.8 ± 0.1 | −21.3 ± 0.4 | 0.97 ± 0.01 |

[a] Relative $IC_{50}$ values ($rIC_{50}$) and relative $K_D$ values ($rK_D$) are reported relative to the reference compound sLe$^x$ (**1**). $IC_{50}$ values were determined in a competitive binding assay.[22] $K_D$ and $\Delta H$ were measured in ITC experiments, $\Delta G$, and $T\Delta S$ were calculated according the equations $\Delta G = \Delta H - T\Delta S$ and $\Delta G = RT\ln K_D$. $N$ = stoichiometric ratio of ligand and protein.

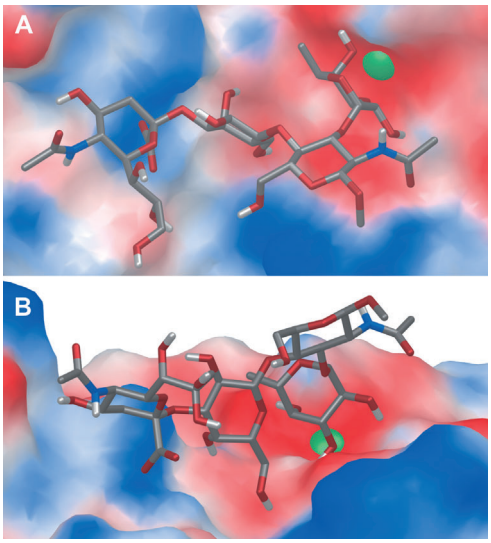



***Figure 2.*** sLe$^x$ (**1**) bound to E-selectin as observed in the crystal structure (protein data bank (PDB) code: 1G1T).[13a] A) top view: the binding epitope on E-selectin is dominated by polar residues [polar residues in red (positively charged) and blue (negatively charged), nonpolar in white]. The contact area is 275 Å$^2$. B) side view: Only a small part of sLe$^x$ directly contributes to binding (graphics generated by Maestro[32]).

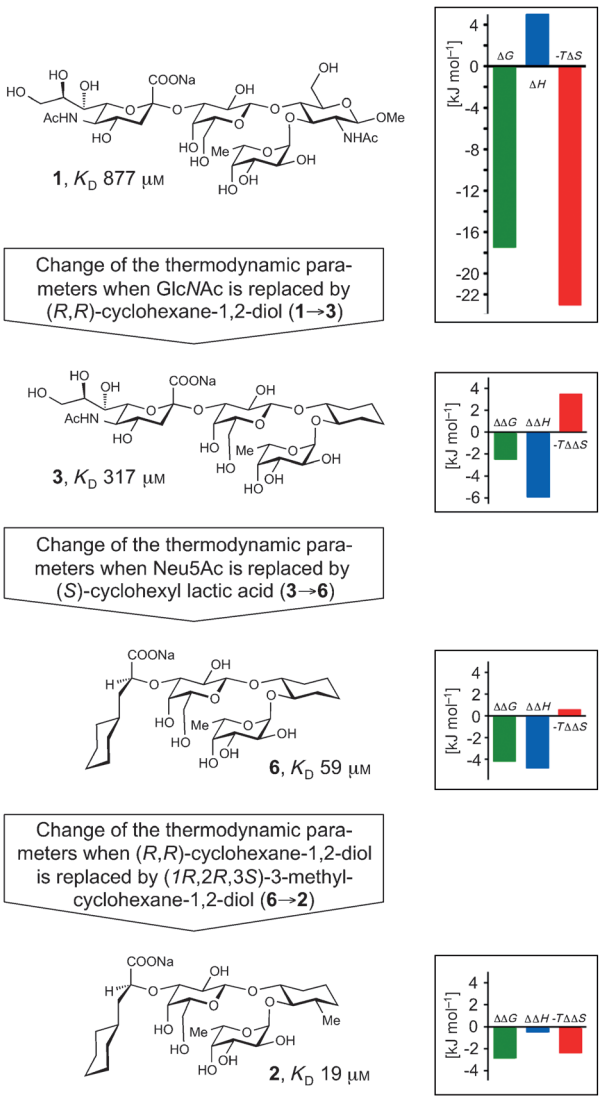



***Figure 3.*** Thermodynamic signature ($\Delta G$, $\Delta H$, $-T\Delta S$) of sLe$^x$ (**1**) (the corresponding data for the antagonists **2**–**6** are summarized in Table 1) and the changes of the thermodynamic parameters ($\Delta\Delta G$, $\Delta\Delta H$, and $-T\Delta\Delta S$) when D-GlcNAc in **1** is replaced by (*R*,*R*)-cyclohexane-1,2-diol (**1**→**3**), D-Neu5Ac in antagonist **3** by (*S*)-cyclohexyl lactic acid (**3**→**6**), and (*R*,*R*)-cyclohexane-1,2-diol in antagonist **6** by (1*R*,2*R*,3*S*)-3-methylcyclohexane-1,2-diol (**6**→**2**).

a scaffold to ensure the correct spatial orientation of L-Fuc and D-Gal in the bioactive conformation, that is, its role is to pre-organize the Le$^x$ core. D-GlcNAc itself has only weak contacts with the target protein (Figure 2B).[13] A comparable role is attributed to D-Neu5Ac, which only contributes to binding through a salt bridge involving its carboxylate group.[13] Consequently, mimics of D-GlcNAc and D-Neu5Ac were designed to stabilize the bioactive conformation and to keep the entropic costs for binding low or virtually the same as for the highly pre-organized sLe$^x$ (**1**). With (*R*,*R*)-cyclohexane-1,2-diol (**1**→**3**) which was demonstrated to be a moderate mimic of D-GlcNAc,[7a] substantial entropy costs arose ($-T\Delta\Delta S$: 3.5 kJ mol$^{-1}$, Figure 3). When D-Neu5Ac in **3** was replaced by (*S*)-cyclohexyl lactic acid (**3**→**6**, $-T\Delta\Delta S$: 0.6 kJ mol$^{-1}$), only a small entropy penalty emerged. Finally, (1*R*,2*R*,3*S*)-3-methylcyclohexane-1,2-diol (**6**→**2**) proved to be an optimal replacement of D-GlcNAc (Figure 3),[7a] resulting in an entropy term similar to that of sLe$^x$ (**1**; Table 1). Compared to antagonist **6**, the improved pre-organization of the core conformation in **2** led to a substantial reduction of the entropy costs ($-T\Delta\Delta S$: −2.4 kJ mol$^{-1}$). Because the investigated mimics are significantly less polar than D-GlcNAc and D-Neu5Ac, an additional effect needs to be taken into account, namely a substantial alteration of the solvation properties. Although only partial desolvation is necessary, the desolvation of the carbocyclic mimics is enthalpically less unfavorable compared to that of the more polar D-GlcNAc or D-Neu5Ac moieties.[31]

Strikingly, the introduction of (*R*,*R*)-cyclohexane-1,2-diol (**1**→**3**) has the same relative effect on enthalpy and entropy as the exchange of D-Neu5Ac for (*S*)-cyclohexyllactic acid (**1**→**5**), which is reflected in the same slope in the entropy–enthalpy plot in Figure 4. In both cases, a significant gain in enthalpy is partially compensated by a loss in entropy. Furthermore, when both mimics are combined in one molecule (→**6**), the effect is not additive, that is, less entropy is lost but also less enthalpy is gained than expected (**6** vs **6**$_{expected}$, Figure 4). Clearly, the exchange of the carbohydrate moieties does not only change local conformational and solvation properties, but rather the properties of the entire ligand.

In summary, thermodynamic binding parameters for the interaction of E-selectin with sLe$^x$ (**1**) and the glycomimetics **2**–**6** were investigated by ITC. The interaction of sLe$^x$ with E-selectin is driven by a large favorable entropy term which is partially compensated by an unfavorable enthalpy contribution. The exchange of residues acting as scaffolds with less

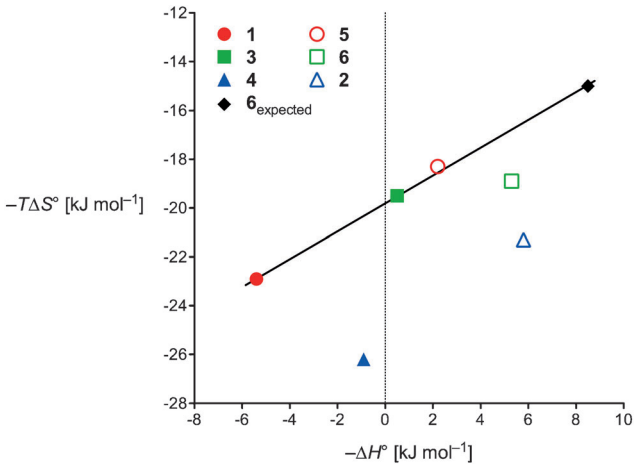



***Figure 4.*** Entropy–enthalpy plot for ligands **1** to **6** and the values expected for **6** ($\mathbf{6}_{expected}$) in case the effects caused by the mimetic replacements of D-Glc*N*Ac by cyclohexane-1,2-diol and D-Neu5Ac by (*S*)-cyclohexyllactic acid were additive.

polar mimics, that is, D-Neu5Ac for (*S*)-cyclohexyllactic acid and D-Glc*N*Ac for (*R*,*R*)-cyclohexane-1,2-diol, resulted in improved binding enthalpy, however accompanied by a loss of binding entropy. Only for mimetic structures that maintain the pre-organization of $sLe^x$ (**1**) in its bioactive conformation, as it is the case for the replacement of D-Glc*N*Ac by (1*R*,2*R*,3*S*)-3-methylcyclohexane-1,2-diol (**1**→**4** or **5**→**2**), a similar entropy term was found. Overall, the almost 50-fold improved affinity of **2** compared to **1** results from a gain in binding enthalpy, whereas the binding entropy is not significantly changed.

The development of glycomimetics with improved binding properties is intrinsically difficult because of the similarity of the ligand (carbohydrate) and the solvent (water). The results of this thermodynamic study suggest, that for a successful development of glycomimetics, carbohydrate moieties with predominantly structural tasks and no or only weak contacts with the target protein should be replaced by hydrophobic mimics, resulting in reduced desolvation penalties and therefore improved enthalpic contributions to binding. In addition, the mimetic replacement should contribute to an improved pre-organization of the binding conformation to optimize the entropy term as well. When the carbohydrate ligand is already almost optimally pre-organized in solution, as it is the case for $sLe^x$ (**1**), the identification of such mimics is a most challenging task.[7a]

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