Articles

Studies on Selectin Blocker. 3. Investigation of the Carbohydrate Ligand Sialyl Lewis X Recognition Site of P-Selectin

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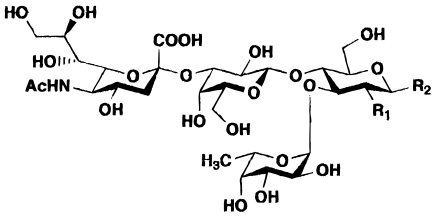
We have previously found that a 1-deoxy sialyl Lewis X (**3**), which lacks only the C-1 hydroxyl group of sialyl Lewis X (sLe^X), exhibited up to 20 times more potency than the sLe^X toward P-selectin binding. In order to explain the structure−activity relationship, we constructed structural models of the complexes of P-selectin and compounds **1**−**3** and sLe^X. From the modeling analysis, we found that the carbonyl oxygen of the *N*-acetyl group of GlcNAc in **3** formed a hydrogen bond with the amide group of Asn 82 in P-selectin. We also supposed that there was a hydrophobic interaction between the pyranose of GlcNAc in compound **3** and the imidazole ring of His 108 in P-selectin. However, it is considered that those interactions would not be appreciable in the case of sLe^X or other 1-deoxy sLe^X analogs (**1**,**2**). Accordingly, our results could be helpful in obtaining a new concept to design a potent inhibitor toward P-selectin binding.

Introduction

Selectins are one of the families of cell adhesion molecules. They have been proven to play an important role in the initial cell−cell interactions involved in leukocyte homing, platelet binding, and neutrophile extravation.[1] There are three types of selectins: E-selectin (ELAM-1), P-selectin (GMP-140), and L-selectin (LECAM-1). E-selectin is expressed on vascular endothelial cells during inflammation.[2] P-selectin appears on platelets or vascular endothelial cells,[3] and L-selectin is expressed on leukocytes.[4] The selectins consist of a cytoplasmic region, a varying number of consensus repeat sequences, an EGF-like region, and an amino-terminal calcium-dependent lectin domain. Through this lectin domain, selectins recognize carbohydrate ligands on the surfaces of their target cells.

A tetrasaccharide sialyl Lewis X (NeuAcα2,3Galβ1,4-(Fucα1,3)GlcNAc, sLe^X) is a well-known ligand for selectins.[5] In a series of studies on the structure−activity relationship of the sLe^X, we have recently reported inhibitory activities of the sLe^X and its 1-deoxy sLe^X analogs **1**−**3** (Figure 1) toward E-, P-, and L-selectin binding.[6] The most exciting result was found with the 1-deoxy sLe^X **3** with regard to its IC_{50} in a P-selectin assay. Compound **3** differs from sLe^X only by the omission of the C-1 hydroxyl group and yet has approximately up to 20-fold better activity. It is, therefore, of interest to investigate these differences and their interpretation of why **3** in particular was so active. So we have planned a model building of 1-deoxy sLe^X **3** to P-selectin and an investigation of the mode of interaction.

The crystal structure of the lectin and EGF-like domains of E-selectin have been recently resolved.[7] Though the detailed mechanism of a ligand recognition by E-selectin is not yet known, many groups have tried



Compd.	R_1	R_2	IC_{50},(mM)
1	H	H	> 1.0
2	OH	H	> 1.0
3	NHAc	H	0.044
sLe^X	NHAc	OH	> 1.0

Figure 1. Blocking activities of 1-deoxy sLe^X analogs **1**−**3** and sLe^X on P-selectin ligand binding.[6]

to solve this point in experimental and theoretical ways. Conformational analyses of the sLe^X have been studied by several groups. Ichikawa et al.[8] proposed four conformations of the sLe^X (designated GESA-A,B,C,D) based on NMR analysis and GESA molecular mechanics calculations. Cooke et al.[9] and Scheffler et al.[10] proposed that a conformation of sLe^X bound to E-selectin was almost the same as Ichikawa's GESA-C structure based on NMR analysis of the mixture of sLe^X and soluble E-selectin. Cooke et al. also proposed that 3- and 4-hydroxyl groups of fucose were coordinated to the calcium on the lectin domain of E-selectin, and the sialic acid was located near Tyr 48, Lys 111, and Lys 113, which have been considered to be essential for ligand binding toward E-selectin.[11] Additionally, Bajorath et al.[12] proposed a model of the sLe^X and P-selectin complex, which was very similar to the model of the sLe^X and E-selectin complex reported by Cooke et al. In this model, the GlcNAc residue of sLe^X seems to not be essential for sLe^X and selectin binding because the GlcNAc residue was directed toward the outer solvent and could not interact with the protein. Uchiyama et al.[13] reported that sLe^X mimics in which GlcNAc was

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replaced with a simple linker were similar to sLe^X in their activity with E-selectin. The GlcNAc residue of sLe^X might not be essential for sLe^X and E-selectin binding. However, we cannot explain the difference between the activities of sLe^X and the 1-deoxy sLe^X **3** with P-selectin by this model.

On the other hand, Kogan et al.[14] have recently reported a new binding model of the sLe^X and E-selectin complex, based on their site-directed mutagenesis experiments. The most characteristic point of their model involves the fact that a basic amino acid residue, Lys 113, does not participate in a charge-paired interaction with the sialyl carboxylate and Arg 97 in sLe^X interacts with the sialyl carboxylate in E-selectin instead. In the previous models, it was believed that the Lys 113 played a crucial role to interact with a sialic acid carboxylate of the sLe^X. However, Kogan et al. indicated that mutations on Lys 113 to neutral glutamine or acidic glutamate were still acceptable for the sLe^X recognition to the protein. In addition, the GlcNAc residue of the sLe^X was located near Glu 107 in E-selectin. Therefore, we may be able to construct the **3** and P-selectin complex model whose GlcNAc would interact with the protein, based on their model. However, it still has some problems because the residue at position 97 in P-selectin is replaced by a serine, which is not able to form the ion pair with the sialic acid carboxylate. Thus, the binding mode of sLe^X toward P-selectin would be different from those in Cooke's or Kogan's models of a selectin−ligand complex.

There are also some evidences for the differences in the binding modes of the ligands to E- and P-selectin.[15] E-selectin requires all three hydroxyl groups on the fucose of the sLe^X. It is also known that the recognition of the sLe^X to E-selectin is completely calcium-dependent. On the other hand, P-selectin requires only the 3-position hydroxyl group on the fucose of sLe^X. Removal of the 2- or 4-hydroxyl group of the fucose does not markedly affect the recognition. Furthermore, the binding of sLe^X toward P-selectin is less dependent on calcium because weak binding activity was maintained even with EDTA. Thus, it would be considered that only the 3-position among the 2-, 3-, and 4-hydroxyl groups on fucose might be critical and interact with Ca^{2+} and/or some amino acids in P-selectin.

Summarizing the above findings, it can be concluded that the mode of interactions of sLe^X and 1-deoxy sLe^X analogs **1**−**3** with P-selectin should be different from those proposed so far. In this paper, we have constructed new structural models for the ligands and P-selectin complexes to evaluate the binding modes of sLe^X and 1-deoxy sLe^X analogs **1**−**3** and to discuss their interpretation of why **3** in particular was so active to P-selectin binding.

Thus, the three-dimensional structure of P-selectin has not been reported yet. Though several groups[16] have reported its structural models based on the structure of rat mannose binding protein (MBP),[17] we have derived the P-selectin model from the crystal structure of E-selectin because P-selectin exhibits better structural homology with E-selectin than with MBP.[18] To this end, our model would be expected to be more accurate than the previously reported models, and our results could be helpful in obtaining a new concept to design a potent inhibitor toward P-selectin binding.

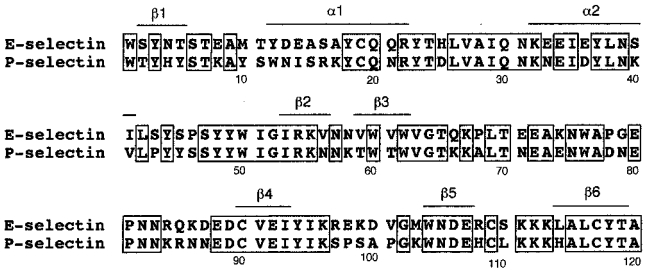


Figure 2. Alignment of the amino acid sequences of the lectin domains of human E- and P-selectins.[11] Residues conserved among E- and P-selectins are enclosed in rectangles. The secondary structures are shown above each column. Residue numbering is shown every 10 residues below each column.

Using this constructed model, we will discuss the reason for the high inhibitory activities of 1-deoxy sLe^X **3**.

Methods

Computer graphics manipulation and all calculations in this paper were carried out using MidasPlus 2.0[19] and AMBER version 4.0.1[20] with the recently published Cornel et al. all-atom force field,[21] respectively, on a SGI Indigo Elan 4000 workstation. The parameters for calcium ion were taken from Hamaguchi et al.[22] and Maynard et al.[23] The force field parameters for the carbohydrate portion of **1**−**3** and sLe^X were derived from GLYCAM_93,[24] a general force field for carbohydrates suitable to AMBER, which takes account of the exo-anomeric effect.

Modeling of the Three-Dimensional Structure of P-Selectin. We generated the model of the P-selectin lectin domain based on the crystal structure of the E-selectin lectin/EGF domain. The amino acid sequence homology between E- and P-selectin lectin domains was about 63%, and there are no insertion or deletion sequences between them (Figure 2). Nonconservative amino acids were replaced using interactive computer graphics; then replaced side chains were located at similar positions on the residues in E-selectin. A few steric clashes were removed by manual adjustment of torsion angles of the side chain. No adjustment of the polypeptide backbone was necessary. Next, the model structure was subjected to energy minimization. During the calculation, the side chains of the replaced residues and the main chains of those which were replaced with proline or glycine from the other amino acids or vice versa were free to move. This is because the conformational energy profiles of the backbone torsion angles of glycine or proline are different from those of the other amino acid residues. This calculation was carried out until the rms of the potential gradient was below 0.05 kcal mol^{-1} $Å^{-1}$, using a distance-dependent dielectric constant, $\epsilon = 80r$. The obtained model was then subjected to 100 ps of molecular dynamics calculation using an integration time step, $\Delta t = 2$ fs. The same atoms as in the previous minimization were allowed to move during the calculation. The initial velocities were chosen from a Maxwellian distribution at 50 K, and the temperature of the system was gradually increased to 298 K in the first 10 ps. The bond lengths were fixed by SHAKE algorithm.[25] A cutoff distance of 9 Å was used for nonbonded interactions. The averaged equilibrated structure was solvated by TIP3P water molecules, which were added within 10 Å from the surface of the solute.[26] This model structure was subjected to

Table 1. Torsion Angles of Glycosidyl Linkages in Sialyl Lewis X in Solution Calculated by Ichikawa et al.[8] Using the NMR Data[a]

	torsion angles of glycosidyl linkages (deg)					
	NeuAc-Gal		Gal-GlcNAc		Fuc-GlcNAc	
conformers	ϕ	ψ	ϕ	ψ	ϕ	ψ
GESA-A	160	−57	54	8	48	25
GESA-B	−170	−8	54	9	48	24
GESA-C	−79	7	55	7	48	25
GESA-D	68	−20	54	9	48	24

[a] The torsion angle ϕ is defined by the four atoms H−C1−O1−C*x* (in the case of NeuAc, by the four atoms C1−C2−O2−C*x*′) and ψ by C1−O1−C*x*′−H*x*′. The conformer adopted in our model in the GESA-conformer.

energy minimization and 100 ps of MD calculation with a dielectric constant of $\epsilon = 1$. All atoms in the system were now allowed to move during these calculations, except the atoms coordinated to the calcium ion. These atoms were fixed by harmonic position constraints with a force constant of 50 kcal mol^{-1} Å^{-2}. The averaged equilibrated structure from the MD calculations was used in the following modeling study.

Model Building of 1−3 and sLe^X Binding to P-Selectin. With the P-selectin lectin domain model described above, plausible hypothetical models for the complexes of P-selectin and **1**−**3** and sLe^X were generated using interactive computer graphics. We have constructed the models based on ideas as follows.

The conformations of **1**−**3** and sLe^X in the complexes were selected from one of four conformations of the sLe^X (GESA-A,B,C,D) reported by Ichikawa et al.[8] The torsion angles of glycosidic linkages in each conformer are listed in Table 1. In the GESA-A and -D, the carboxyl group of the sialic acid was directed to the opposite side in fucose. In the GESA-B, the direction of the carboxyl group of the sialic acid was perpendicular to the hydroxyl groups of fucose. Therefore, those three conformers (GESA-A,B,D) would be unsuitable for the P-selectin binding because it was further proposed[27] that the binding domain of sLe^X is located on the hydrophilic surface composed of fucose, galactose, and the carboxyl group of the sialic acid residues. In contrast, the GESA-C conformation would be suitable for the P-selectin binding because fucose, galactose, GlcNAc, and the carboxyl group of the sialic acid simultaneously faced the protein. So, we constructed the models of the **1**−**3** and sLe^X with P-selectin complexes based on the GESA-C conformer, which has also been adopted for the investigation of the modeling of the sLe^X and E-selectin.[9]

In our models, we supposed that only the 3-position among the hydroxyl groups on fucose would be critical and that it interacts with Ca^{2+} or amino acids of P-selectin. This is because the experimental study[15] suggested that P-selectin requires the 3-position hydroxyl group on the fucose of sLe^X; the 2- or 4-hydroxyl group of the fucose does not markedly affect the recognition. The experimental data of Lys113Ala mutagenesis[12] indicated that Lys 113 is one of the crucial residues for ligand binding toward P-selectin. So, we considered that the carboxylate of the sialic acid residue would interact with Lys 113 of P-selectin and placed the ligands on P-selectin in such a manner. Moreover, the experimental results (Figure 1) suggested that the *N*-acetyl group of 1-deoxy GlcNAc in **3** would be important for the recognition by P-selectin. So we constructed the models so that the *N*-acetyl group could interact with the protein.

The constructed hypothetical complexes of **1**−**3** and sLe^X with P-selectin were refined by energy minimization with explicit water. These models were solvated by TIP3P[28] water molecules which were added within 27 Å from the center of each ligand. All atoms of the protein and the ligand were free to move during the calculation. A cutoff distance of 9 Å was used for nonbonded interactions, and a dielectric constant of $\epsilon = 1$ was used. The minimization was carried until the rms of the potential gradient was below 0.05 kcal mol^{-1} Å^{-1}.

Results and Discussion

Model of P-Selectin Lectin Domain. The structural model of the P-selectin lectin domain constructed is illustrated in Figure 3. The crystal structure of the lectin domain of E-selectin, which was used as a template in modeling, is also shown. The rms deviation of backbone atoms between the P-selectin model and the E-selectin crystal structure was 1.26 Å. The E-selectin lectin domain is composed of two α-helixes and six β-strands (Figure 3). Those secondary structures were almost conserved in the P-selectin model.

The Ca^{2+} coordination sphere in E-selectin is formed by Glu 80, Asn 82, Asn 105, and Asp 106. These residues are conserved in P-selectin. In our model, the structure around Ca^{2+} did not alter very much from that in E-selectin because the atoms coordinated to Ca^{2+} were harmonically constrained during the modeling. Several amino acid residues in P-selectin (Tyr 48, Glu 92, Tyr 94, Lys 111, Lys 113) are known to be essential for the sLe^X binding. These residues are also conserved between both E- and P-selectins. Figure 4 shows the location of these residues on our model. Tyr 48 was located on the loop between α2 and β2. Glu 92 and Tyr 94 were located on β4 and the edge of β4, respectively. Lys 111 and Lys 113 were located on the loop between β5 and β6. Consequently, as shown in Figure 4, these residues were located on the same side of the protein surface, and it was considered to form the binding site for the ligands.

Figure 5 shows the comparison of the structure of these critical residues in our P-selectin model and the E-selectin crystal structure. In the case of E-selectin, Arg 97 formed a stacking interaction with Tyr 94, which would stabilize the structure of the loop between β4 and β5. In spite of the fact that Arg 97 in E-selectin was replaced by a serine residue in P-selectin, the structure of this loop in the P-selectin model did not change from those in E-selectin (Figures 3 and 5). This may be due to two prolines at positions 99 and 101 in the loop of P-selectin. In the P-selectin model, Pro 99 and Pro 101 would fix and stabilize the structure of the β4−β5 loop because the backbone of proline is more rigid than those of the other amino acid residues. In addition, a remarkable point was a significant difference of the spatial positions of the 108th residues in each E- and P-selectin, Arg 108 for E-selectin and His 108 for P-selectin, respectively. Particularly, the imidazole ring of His 108 in P-selectin was coming close to the center of the ligand binding site, and consequently it interacted with Glu 107 in P-selectin. In contrast, the position of Arg 108

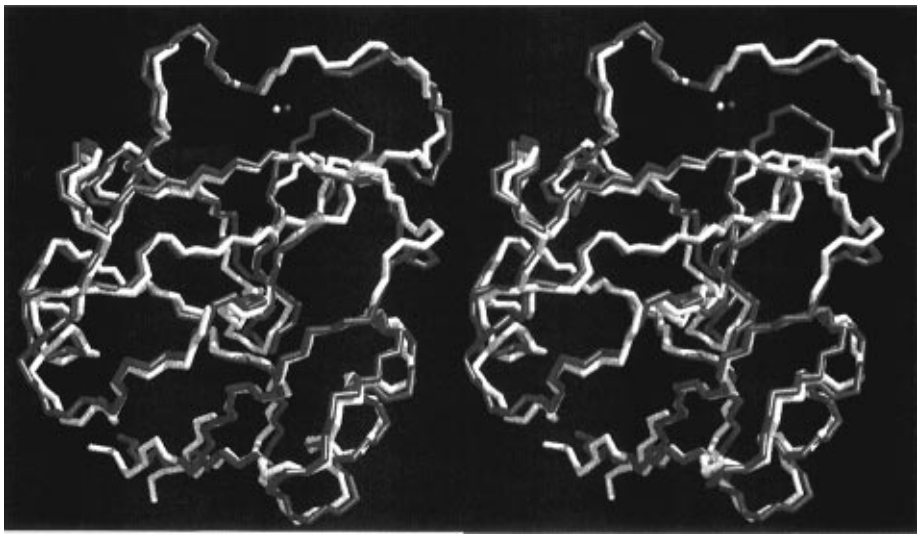


Figure 3. Stereorepresentation of superposition of the main chain of the P-selectin model lectin domain (yellow) and the crystal structure of E-selectin lectin domain (green). Only the backbone atoms are shown; rms deviation of Cα atoms between P- and E-selectins is 1.26 Å.

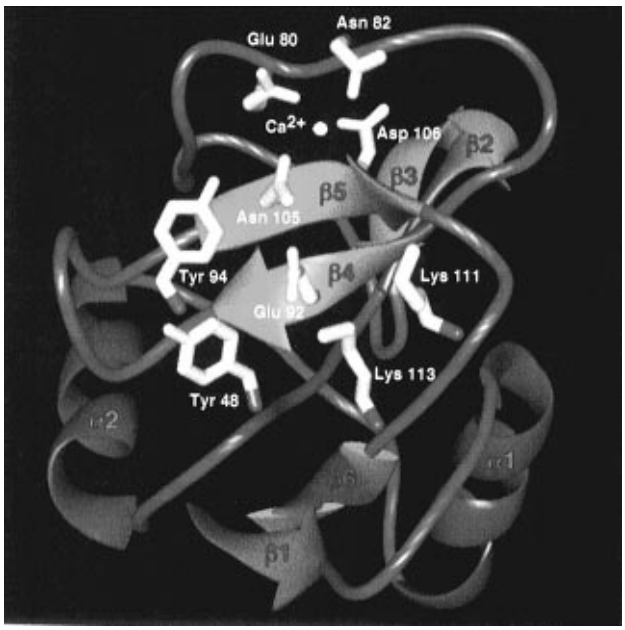


Figure 4. Ribbon diagram of the P-selectin lectin domain model. The side chains of the residues that are critical for ligand binding are also presented as the cylinder model. Residues are colored as follows: helix, blue; strand, purple; loop, green; calcium, white; critical residues, yellow.

in E-selectin was far apart from the center of the binding site because Nϵ of Arg 108 in E-selectin formed a hydrogen bond with the hydroxyl group of Ser 110 and the position of Arg 108 was fixed. In contrast, Nδ of His 108 in P-selectin could interact with the carboxylate of Glu 107 because Ser 110 in E-selectin was replaced with leucine in P-selectin. Those structural properties might correspond to the difference between the specificities of E- and P-selectins toward the ligands.

Model of 1-Deoxy sLe^X 3 Binding to P-Selectin. Figure 6 shows our final model of the 1-deoxy sLe^X **3** and P-selectin complex. Figure 7 is a schematic representation of the model. Our model involved the following features. (i) The critical 3-hydroxyl group of fucose trigonally interacted with Ca^{2+} and the carboxyl group of Glu 80 in P-selectin. (ii) No amino acid residues interacted with the 2- and 4-hydroxyl groups of fucose. (iii) The 6-hydroxyl group of the GlcNAc interacted with the amino group of Lys 111. (iv) The carbonyl oxygen of the *N*-acetyl group of GlcNAc formed a hydrogen bond with the amide group of Asn 82. (v) The pyranose ring of the GlcNAc was near the imidazole ring of His 108. Especially, the most characteristic points involved a direct interaction between the GlcNAc and P-selectin, and it was different from the models reported so far. Additionally, the 6-hydroxyl group of galactose simultaneously interacted with the hydroxyl group of Tyr 94 and the amide group of Asn 105. It seems to support that both Tyr 94 and Asn 105 are critical residues for the ligand and P-selectin binding.[12] The counter amino acid residue to the carboxyl group of the ligand was Lys 113, which was different from the model of the sLe^X and E-selectin complex reported by Kogan et al.[13]

Differences between the Interactions among the Ligands with P-Selectin. Table 2 shows the distances of the interactions among the **1**−**3** and sLe^X with P-selectin after the minimization. This table shows that each interaction is almost conserved among the ligands. The structural difference among the ligands are only C-1 and/or C-2 groups of GlcNAc (or glucose) parts. Thus, the difference of the inhibitory activities of the ligands would be based on the interactions between C-1 and C-2 groups of GlcNAc (or glucose) portions in the ligands and P-selectin. In our experimental study, the competitive P-selectin binding activity of **3**, which lacks only the C-1 hydroxyl group of the sLe^X, exhibited up to 20-fold more potency than the sLe^X toward P-selectin binding. However, the similar 1-deoxy analogs **1** and **2** were less potent, and their activity was comparable to that of sLe^X.

Table 3 shows the interaction energy between each ligand residue and P-selectin. Namely, the potential energy of interaction between **3** and P-selectin was the most stable in all of the ligands. Especially, each energy involved in the GlcNAc (or glucose) parts (B) of **3** (−37.5 kcal/mol) and sLe^X (−34.6 kcal/mol) was lower than that of **1** (−18.2 kcal/mol) and **2** (−11.1 kcal/mol). This would reflect the fact that the *N*-acetyl groups of GlcNAc in **3** and sLe^X interacted with Asn 82 in P-selectin. In contrast, **1** and **2** cannot form this kind of interaction

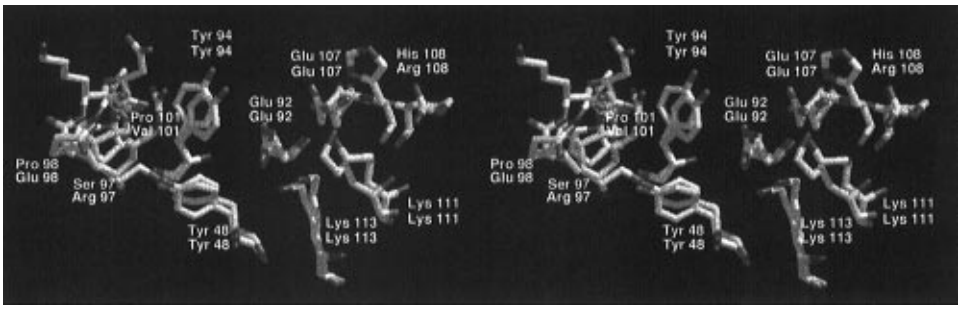


Figure 5. Stereorepresentation of comparison of the residues of the carbohydrate ligand recognition site in P-selectin model (carbon; yellow) and E-selectin crystal structure (carbon; gray). The residues which are critical for selectin function are shown. Residues are colored as follows: oxygen, red; nitrogen, blue; P-selectin carbon, yellow; E-selectin carbon, gray.

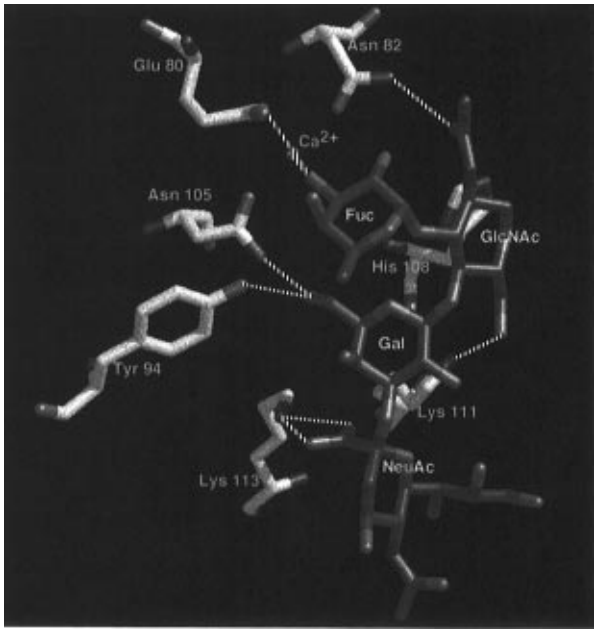


Figure 6. Detailed view of interactions between **3** and P-selectin (**3**: carbon, green; oxygen, red; nitrogen, cyan) (P-selectin: carbon, gray; oxygen, red; nitrogen, cyan; calcium, purple). Only **3** and the important residues of P-selectin are shown. The yellow dotted lines show the hydrogen bonds.

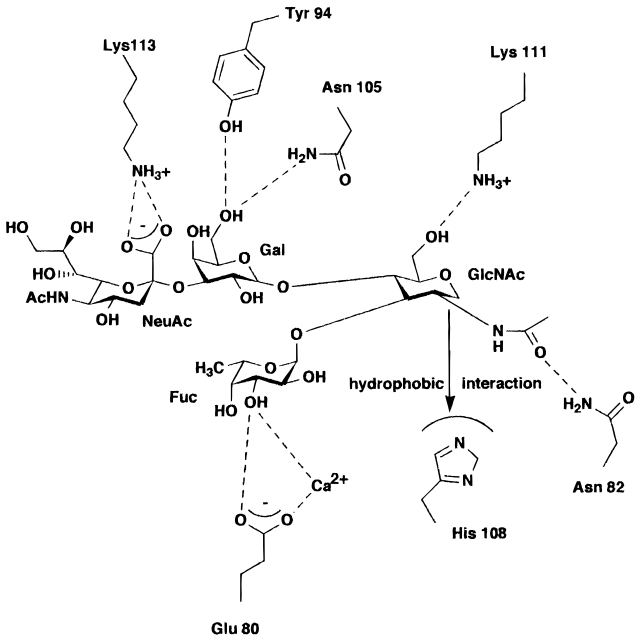


Figure 7. Schematic representation of the binding mode of P-selectin and compound **3**. The 3-hydroxyl group on fucose interacts with calcium and Glu 80. The 6-hydroxyl group on GlcNAc interacts with Lys 111. The carbonyl oxygen of *N*-acetyl group on GlcNAc interacts with Asn 82. The pyranose ring of GlcNAc forms stacking interactions with His 108. The 6-hydroxyl group on galactose interacts with Tyr 94 and Asn 105. The carboxylate of the ligand interacts with Lys 113. The dotted lines show each interaction.

because they lack 2-*N*-acetyl groups. Thus, this interaction would contribute to the binding affinity of **3** and sLe^X with P-selectin.

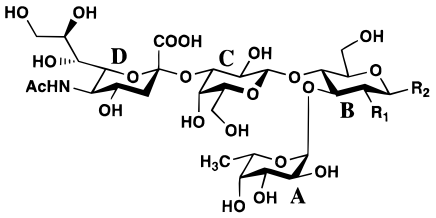
In our complex model of **3** and P-selectin, the pyranose ring of the GlcNAc in **3** was near the imidazole ring of His 108. The difference between **3** and sLe^X was only the C-1 group of the GlcNAc part. Thus, the difference between the inhibitory activities of **3** and sLe^X would correspond to the difference between the interactions between the C-1 group of GlcNAc and His 108. The lack of the hydroxyl group at the C-1 position in **3** makes the hydrophobic surface on its pyranose ring. Therefore, we speculated that the C-1 carbon of the GlcNAc in **3** would form a hydrophobic interaction with the imidazole ring of His 108. On the other hand, the C-1 hydroxyl group of the sLe^X would disrupt the important hydrophobic interaction with the imidazole ring of His 108. Accordingly, we considered that this difference would be due to the lower activity of sLe^X compared with that of **3**. In the comparison of the binding energy of the ligands (Table 3), the interaction energy between the GlcNAc part of **3** and P-selectin is lower by only 3 kcal/mol than that of sLe^X. We are not sure if this difference in the interaction energy could correspond to the difference of the inhibitory activities of **3** and sLe^X. We should also note that the values in Table 3 were just the potential energy and did not include any entropic terms which are involved in hydrophobic interaction. For the accurate evaluation of the difference of the inhibitory activities of these compounds, we might need to do more detailed calculations, for example, free

Table 2. Distances of Interactions between P-Selectin and Each Ligand (**1**−**3**, sLe^X) in the Obtained Complex Models after Minimization

	distance (Å)			
ligand/P-selectin	sLe^X	**1**	**2**	**3**
fucose O3/calcium	2.46	2.39	2.45	2.42
fucose O3/Glu 80 Oϵ2	3.02	2.84	2.82	3.08
GlcNAc NHAc carbonyl/ Asn 82 Nδ2	3.10			3.15
GlcNAc O6/Lys 111 Nζ	2.85	2.89	2.87	2.84
galactose O6/Tyr 94 Oη	2.85	3.16	2.93	2.98
galactose O6/ Asn 105 Nδ2	3.52	2.97	3.00	
sialic acid carboxylate/ Lys 113 Nζ[a]	2.71/2.82	2.75/2.78	2.75/2.76	2.77/2.73

[a] Two distances from each of two carboxylate oxygens are shown.

Table 3. Potential Energy of the Interactions between Each Residue of the Ligands (**1**−**3**, sLe^X) and P-Selectin in the Obtained Models after Minimization (kcal/mol)



1: R_1 = H, R_2 = H
2: R_1 = OH, R_2 = H
3: R_1 = NHAc, R_2 = H
4: R_1 = NHAc, R_2 = OH(α,β)

	potential energy (kcal/mol)			
residue	sLe^X	**1**	**2**	**3**
A	−39.95	−46.08	−41.80	−39.41
B	−34.56	−18.23	−11.05	−37.47
C	−13.88	−8.58	−13.28	−11.07
D	−172.43	−148.86	−173.90	−177.18
entire ligand	−260.82	−221.25	−240.03	−265.13

energy perturbation method. Furthermore, in order to confirm the role of His 108, we might need to make a mutant of residue 108 and measure the activity of **3** with that mutant. These works are now under consideration. Although our hypothesis is not completely confirmed here, our models of complexes of P-selectinand the ligands would give some ideas for designing a potent inhibitor toward P-selectin.

In conclusion, we constructed structural models of the complexes of P-selectin and **1**−**3** and sLe^X. From the modeling analysis, we found that the carbonyl oxygen of the *N*-acetyl group of GlcNAc in **3** formed a hydrogen bond with the amino group of Asn 82 in P-selectin. We also supposed that there was a hydrophobic interaction between the hydrophobic face on the pyranose of GlcNAc in **3** and the imidazole ring of His 108 in P-selectin. Both of the above would be important driving forces for the recognition to P-selectin by compound **3**. Accordingly, those findings could be helpful in obtaining a new concept for designing a potent inhibitor toward P-selectin binding.

Experimental Section

Inhibition Assay of P-Selectin−sLe^X Binding. The construction of the selectin-immunoglobulin was carried out according to a previous paper.[29] A solution of sLe^X-pentaceramide in a 1:1 mixture of methanol and distilled water was pipetted into microtiter plate wells (96 wells; Falcon Pro-Bind) at 100 pmol/50 μL/well and adsorbed by evaporating the solvent. The wells were washed twice with distilled water, blocked with 5% BSA (bovine serum albumin)−PBS (phosphate-buffered saline) for 1 h at room temperature, and washed with PBS three times.

Separately, a 1:1 volumetric mixture of a 1:500 dilution in 1% BSA−PBS of biotin-anti-human IgG (Fc) (BioSource International Inc., Lot 1201)/streptavidin alkaline phosphatase (Zymed Lab Inc., Lot 50424702) and a P-selectin-immunoglobulin fusion protein (P-selectin-Ig) was incubated at room temperature for 30 min to form a complex. The test compounds were dissolved in distilled water at 10 mM and finally diluted to final concentrations of 100, 25, 6.25, and 1.56 μM, respectively. Reactant solutions were prepared by incubating 30 μL of this solution at each concentration with 30 μL of the above complex solution for 30 min at room temperature. This reactant solution was then added to the above microtiter wells at 50 μL/well and incubated at 37 °C for 45 min. The wells were washed three times with PBS and distilled water, respectively, followed by addition of *p*-nitrophenyl phosphate (1 mg/mL) and 0.01% $MgCl_2$ in 1 M diethanolamine (pH 9.8) at 50 μL/well. The reactant mixture was developed for 120 min at 23 °C, and absorbance at 405 nm was measured. Percent binding was calculated by the following equation:

$$\% \text{ binding} = (X - C/A - C) \times 100$$

wherein X is the absorbance of wells containing the test compounds at each concentration, C is the absorbance of wells not containing the selectin-Ig and test compounds, and A is the absorbance of control wells not containing the test compounds. The results of inhibitory activities are presented in Figure 1 as IC_{50} values. The number of replicates is two.

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