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Uncharged P-selectin blockers

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The blocking potency of P- and L-selectin was studied for certain small molecule mannosides and their polyacrylamide (PAA, 30 kDa) conjugates in comparison to SiaLe^x and fucoidan. Two experimental systems were used: (1) solid phase static assay based on recombinant selectins, and (2) P-selectin dependent rat peritoneal inflammation. βMan-SC$_6$H$_4$NO$_2$-*p* was four times more potent P-selectin inhibitor as compared to SiaLe^x. Docking of this molecule onto the P-selectin carbohydrate-binding site demonstrated that a nitro group enabled an electrostatic interaction with residue Lys 84, while the phenyl ring and the CH$_2$ at C-6 contacted the CH$_2$ groups of the same Lys residue. *In vivo*, βMan-SC$_6$H$_4$NO$_2$-*p* blocked experimental inflammation better than SiaLe^x, but significantly lower than fucoidan. *In vitro* Man-polyacrylic acid conjugates appeared to be very potent inhibitors comparable to fucoidan, uncharged Man-PAA proved rather active, comparable to SiaLe^x-PAA both *in vitro*, and *in vivo*, whereas mannan did not display any P-selectin blocking effect.
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Abbreviations: **SiaLe^x—sialyl Lewis X, Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc; Le^a—Lewis A, Galβ1-3(Fucα1-4)GlcNAc; HSO$_3$Le^a—3′-sulfo-Lewis A, 3'-HSO$_3$Galβ1-3(Fucα1-4)GlcNAc; PAA—polyacrylamide; Man6P—mannose-6-phosphate; C$_6$H$_4$—*p*-substituted phenyl; CRD—carbohydrate recognition domain; MBP—mannose-binding protein; PSGL-1—P-selectin glycoprotein ligand.**

Introduction

Leukocyte trafficking from blood vessels into sites of inflammation is a cooperative multi-step process, the first step of which is the transient rolling along the endothelial cells of blood vessels [1]. The selectins share the ability to recognize the tetrasaccharide SiaLe^x, the binding affinities for SiaLe^x are poor, being in the millimolar range [2]. Neutrophil glycoprotein, PSGL-1 generates a high affinity and biologically relevant binding with P-selectin, the main feature of this molecule is the conjunction of SiaLe^x with a negatively charged cluster of three tyrosine sulfate (sTyr) residues [3], lending high affinity to this molecule. The amino acid sequence, as well as the spatial arrangement of the selectin carbohydrate/calcium-binding site was similar to those for the mannose-binding protein (MBP) [4]. The MBP-based chimeras (MBP constructs that contain a part of the selectin carbohydrate-binding site) are capable of SiaLe^x binding [4], whereas the mannose-carboxyl group derivatives enable the ability to bind to P-selectin [5]. The syntheses of a number of analogues and mimetics of selectin ligands have been reported; as a result, it has been shown that the key “pharmacophores” for the recognition of SiaLe^x by P-selectin are the hydroxyls of fucose, and the carboxyl group of sialic acid [2,6]. A number of rationally designed glycomimetics have incorporated a L-Fuc residue on a template with acidic peptides. Such compounds have demonstrated a moderate to good activity as selectin inhibitors [2]. Other strategies have involved the synthesis of compounds in which an α-D-mannosyl moiety (isostere to L-Fuc) is linked to a carboxy group on a biphenyl template; these compounds have similar selectin blocking activity as the parent SiaLe^x [5]. Our preliminary data [7] has also shown that monosaccharide mannose presented in multimeric form was capable of inhibiting P-selectin *in vitro*.

In this study we demonstrated that both small molecule mannosides, and the multivalent form of Man displayed P-selectin blocking activity comparable to that of SiaLe^x, even without any negatively charged group.

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Methods

Fucoidan was purchased from Sigma (USA), and 1-2 mannan from *Candida parapsilosis* was a gift from Dr. V. Scherbukhin (A.N. Bakh Institute of Biochemistry, Moscow, Russia). Tween 20, human immunoglobulin, and BSA were from Sigma (USA), the peptone was from Reakhim (Russia), and the streptavidin-peroxidase conjugate from Boehringer Mannheim (Germany). All other chemicals were analytical grade from Fluka (Switzerland). 96-well microtiter MaxiSorp immunoplates were from Nunc (Denmark).

Monomeric $SiaLe^x$ and HSO_3Le^a as 3-aminopropyl glycosides were synthesized earlier [8,9]. Polymeric 30 kDa neoglycoconjugates, containing 20% mol. carbohydrate (each fifth chain of the polymer is substituted by carbohydrate), as well as biotinylated probes were obtained according to [10,11]. Carbohydrate content of the neoglycoconjugates was confirmed by acid hydrolysis followed by HPLC [10]. All the conjugates were obtained from the same batch of initial polymer.

Recombinant proteins, ZZ-selectins (monovalent) lacking the transmembrane and cytosolic domains were produced by Bernard Allet (Glaxo Institute for Molecular Biology, UK) and Nicholas Smithers (GlaxoWellcome, Stevenage, UK) as C-terminal chimeras with a ZZ domain of protein A. The ZZ domain binds firmly to human IgG. A baculovirus/insect cell expression system was used, and the IgG-purified proteins were characterized by SDS-PAGE and in cell adhesion assays [12].

Selectin assay [13]

Plates were coated with human IgG, 10 μg per ml of 0.05 M Na-carbonate buffer, pH 9.6, for 1 h at 37°C, and blocked with 3% BSA in buffer A (20 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$) for 1 h at 37°C. Plates were washed three times with buffer A with 0.1% Tween 20, 100 μl of ZZ-selectin were added in a concentration of 30 ng per ml buffer A with 0.3% BSA. Plates were incubated for 1 h at 37°C and then overnight at 4°C, followed by washing them three times with buffer A containing 0.1% Tween 20. At the next step the plates were incubated with HSO_3Le^a-PAA-biot (1 μg per ml buffer A with 0.3% BSA) and an inhibitor for 2 h at 37°C, washed and incubated with streptavidin-HRPO conjugate (1/1000). Color was developed by 30 min incubation in 0.1 M sodium phosphate/0.1 M citric acid buffer, containing 0.04% *o*-phenylenediamine and 0.03% H_2O_2, the reaction was stopped by the addition of 50 μl 1 M H_2SO_4. Absorbance was read at 492 nm in a microtiter plate reader. Blank reaction was performed by omitting the biotinylated neoglycoconjugate. Blank reading was subtracted from the final reaction optical density to give corrected absorbance values. Percent of inhibition was calculated as $(OD_A - OD_I) \times 100/OD_A$ where OD_A is the mean value of optical density in the absence of an inhibitor and OD_I is the mean value of optical density in the presence of an inhibitor.

In vivo model of P-selectin inflammation [14]

Pepton-induced inflammation in conditions of [15] is rather P- than L-selectin dependent, E-selectin participation can be ruled out [14,15]. 10 ml of 10% peptone solution in 0.9% PBS was peritoneally injected into female Wistar rats (about 200 g) under ether anesthesia in order to induce peritoneal inflammation. Equal volume of PBS was injected into the rats as the control of inflammation development. After 3 h the animals were anesthetized by ether and decapitated. 30 ml of medium containing PBS, 60 units/ml heparin, 0.02% EDTA, and 0.03% bovine serum was injected into the abdominal cavity with vigorous massage for 1 min, exudate was collected and total number of cells was counted in a cell counting camera. To count neutrophils the cell suspension was centrifuged at 400 g for 10 min. Concentrated suspension was diluted by total bovine serum 1:1, smears were made and stained by the Pappenheim method. Neutrophil number was counted in two parallel smears, 300–600 cells in each. The number of neutrophils in exudate was calculated from the neutrophil percentage and total cell number. Inhibitors were injected into the rat femoral veins under ether anesthesia once 15 min after pepton injection in 0.3 ml sterile 0.9% NaCl.

Molecular modeling of βMan-$SC_6H_4NO_2$ in the binding site of P-selectin

The crystal structure of P-selectin extracellular domain complex with glycoprotein fragment [6] was taken from the Protein Data Bank [16]. Using Sybyl software (Tripos Inc., St Louis, USA), the structure was edited in order to contain only one monomer of P-selectin together with a fucose residue in the primary binding site. The strontium ion present in the crystal structure was replaced by a calcium ion present in the native lectin. Protein hydrogen atoms were added and the partial charges were calculated using the Pullman procedure. The positions of the hydrogen atoms were refined with the use of the Tripos force field [17]. Atom types and charges for the fucose residue were defined using the PIM parameters previously developed for carbohydrates [18]. The calcium ion was given a charge of 2. The βMan-$SC_6H_4NO_2$ molecule was built in Sybyl starting from mannose taken from the monosaccharide database and chemical groups from the Sybyl fragment database. Charges were derived from semi-empirical calculations with the use of MNDO from the MOPAC package. Energy parameters for the sulfur atom at the anomeric position were derived from previous structural work on thio-derivatives of carbohydrates ([19] and refs. herein).

Accessible surface of the protein was calculated with the MOLCAD program [20] in the Sybyl package.

Results and discussion

Here we demonstrated that simple mannosides, free of a negatively charged group, bind to P- and in some cases to L-selectin.

Table 1. Inhibition of HSO_3Le^a-sp^2-PAA-biot binding with selectins by monomeric and polymeric mannosides and related compounds

	*IC_{50}, μM**	
Inhibitor	*P-selectin*	*L-selectin*
Multimeric		
Fucoidan	0.1 (0.1 μg/ml)**	0.8 (0.8 μg/ml)**
αMan-sp^1-polyacrylic acid***	0.4	0.3
βMan-sp^1-polyacrylic acid	0.5	0.3
αMan-sp^2-polyacrylic acid	0.6	60
$SiaLe^x$-sp^2-PAA	40	40
βMan-sp^1-PAA	125	500
αMan-sp^2-PAA	150	800
Man6P-sp^2-PAA	150	n.t.
αMan-sp^1-PAA	250	500
αFuc-sp^2-PAA	>1 mM	n.t.
Le^a-sp^2-PAA	NI	NI
Mannan (*C. parapsilosis*)	NI	NI
Ovalbumin	NI	n.t.
Monomeric		
βMan-$SC_6H_4NO_2$-*p*	250	300
βMan-$OC_6H_4NO_2$-*p*	500	500
αL-Fuc-OMUF	2000	NI
$SiaLe^x$	>1000	>1000
αMan-$OC_6H_4NO_2$-*p*	30% (2 mM)	30% (2 mM)
βGlc-$SC_6H_4NO_2$-*p*	30% (2 mM)	NI
βMan-SC_6H_4Cl-*p*	15% (2 mM)	NI
αMan-$SC_6H_4NO_2$-*p*	NI (2 mM)	NI
ManαOMe	NI (0.3 M)	NI
Man	NI (0.3 M)	NI
Man6P	NI (0.3 M)	NI

*Values for inhibition were the means of at least triplicate determinations, standard deviations (not shown) were less than 10%. For all glycoconjugates molar concentration was calculated on saccharide ligand (Man, $SiaLe^x$, etc.).
**Molar concentration was calculated based on the supposition that a hexasaccharide is the active unit.
***sp^1 = —OC_6H_4— connected to amide nitrogen of PAA, sp^2 = —$OCH_2CH_2CH_2$— connected to amide nitrogen of PAA.

Monomeric and multivalent mannosides were studied as $SiaLe^x$ mimetics. Data on inhibition of P- (and L-, for comparison) selectin by mannosides, as well as related and reference compounds in a solid-phase assay are presented in Table 1, whereas selected data on the *in vivo* activity are presented in Table 2.

αMan conjugated to polyacrylic acid possessed significant P-selectin blocking activity (IC_{50} 0.6 μM) that was much better than that which was shown by $SiaLe^x$-PAA (40 μM, Table 1). We could explain such a high activity with two parallel mechanisms, firstly, by the negatively charged polymer interacting with the positively charged region that forms an ionic strength-sensitive subsite (sTyr-binding) of P-selectin and secondly, by assembling the conformational $SiaLe^x$ mimetic from the Man residue and with an adjacent carboxyl group of the same polymer [7]. Thus, the polyacrylic acid carboxyl groups played a dual role here. Sharp decrease in the activity was expectable when replacing the negatively charged polymer with neutral PAA; however, the activity of Man-PAA was quite notable being at the $SiaLe^x$-PAA level (Table 1). It is noteworthy that the monomeric mannose and methyl mannoside were not inhibitors up to the 0.3 M concentration. Impact of the spacer group in the activity observed should be excluded, that follows from a similar potency of αMan-OC_6H_4-PAA and αMan-$OCH_2CH_2CH_2$-PAA (the latter hereinafter referred to as αMan-PAA). There is a temptation to explain the pronounced anti-P-selectin potency of PAA-conjugated mannose by multivalent binding. This explanation, however, does not sound convincing. Firstly, zz-P-selectin is monomeric and its density on the plastic surface in the given test-system is not high: according to calculations, the average distance between P-selectin molecules is substantially higher than the maximum possible size of stretched 30 kDa polymer (∼200 Å). Any artifactual interaction with

Table 2. The degree of inhibition of rat peritoneal inflammation by mannosides in comparison with fucoidan and SiaLe^x

	Dose, mg/rat	*Number of rats in group*	**Number of neutrophils per rat × 10^{-6}*	*Inhibition (% to control) ($p < 0.05$)*
Control	0	9	0.4 ± 0.2	
Reference compounds				
Inflammation** (no inhibitor)		12	41.4 ± 5.4	
Fucoidan	1.0	10	2.5 ± 0.5	94.0
SiaLe^x-$OCH_2CH_2CH_2NH_2$	2.0	11	37.0 ± 5.6	10.6
SiaLe^x-PAA	1.0	9	30.0 ± 6.2	26.6
Mannosides in test				
Inflammation** (no inhibitor)		22	45.7 ± 4.5	
βMan-$SC_6H_4NO_2$-*p*	2.0	7	26.9 ± 5.9	41.1
	5.0	5	26.6 ± 6.8	41.8
αMan-OC_6H_4-polyacrylic acid	1.0	4	31.2 ± 2.0	31.7
	2.0	6	18.8 ± 3.1	58.9
αMan-PAA	2.0	7	24.5 ± 5.0	46.4
Mannan (*C. parapsilosis*)	2.5	5	42.5 ± 11.6	7.0
	5.0	3	38.9 ± 13.4	14.9

*The data are presented as mean ± SEM.
**Animals of this group were injected intravenously with 0.3 ml 0.9% NaCl (no inhibitor).

PAA backbone is also excluded; a complete absence of the Le^a-PAA and Fuc-PAA activity testifies to this. Secondly, Man-PAA was shown to block the entry of neutrophils into the P-selectin based [15] inflammation site *in vivo* (Table 2) whereas P-selectin forms no clusters on endothelial cells [4]. Monomeric mannoside β-Man-$SC_6H_4NO_2$-*p* inhibited neutrophils entry to experimental inflammation site similarly to the polymer αMan-PAA; the degree of inhibition by these compounds is at least not less than that of SiaLe^x-PAA. Thus the reason for the higher potency of PAA-conjugated mannose might not depend on the multivalent binding to CRD of P-selectin, but on some other additional effects, *e.g.*, additional calcium bridging between the mannose residue and the protein "not-CRD" region.

The β-anomer Man-$SC_6H_4NO_2$ displayed P-selectin binding of 250 μM, it was much more active *in vitro* than the natural ligand, tetrasaccharide SiaLe^x. The α-anomer of Man-$SC_6H_4NO_2$ was in this case almost inactive. A high βMan-$SC_6H_4NO_2$ potency can hardly be explained only by the stacking of its aromatic ring with an amino acid in the selectin CRD, this following from the fact that the replacement of the nitro group by chloride abolishes the binding. To understand why a formally uncharged nitro group enhanced the binding, we modeled the complex derived with the docking of this mannoside to the P-selectin CRD [6].

Optimization of the binding site, including fucose, calcium and side chains of adjacent amino acids did not yield to significant geometry change when compared to the crystal structure. From this complex, a mannose residue was substituted to the fucose residue while keeping two hydroxyl groups within close proximity from the calcium ion. This produces 4 different docking modes: O-3 and O-4 of fucose can be replaced by the mannose O-3 and O-4, O-4 and O-3, O-2 and O-3 or O-3 and O-2. Energy minimization of each complex was performed in several cycles with optimization of the sugar position and amino acid side chains orientation. As for the four docking modes of mannose in the fucose binding site of P-selectin, one appears to be impossible because of steric conflicts. The three possible complexes are displayed in Figure 1 together with the binding mode of fucose for comparison. In binding mode A, O-3 and O-2 of mannose were superimposed on O-3 and O-4 of fucose. When these two monosaccharides are superimposed with such an inversion of "rotation sense" in the ring, they display striking similarities as displayed in Figure 1. Therefore, mannose mimics the fucose bound in the crystal structure and the hydrogen bond network is conserved. It should be noted the first experimental evidence for mannose as mimetic of fucose in leukocyte-endothelial interaction in [21]. Oppositely, when O-3 and O-4 of mannose are located close to the Ca^{2+} ion, docking modes are different, as well as the hydrogen bond network. Nevertheless, the binding site is large enough to accommodate these docking modes, and the energy levels are similar. Interestingly, the two binding modes described as Man_B and Man_C correspond closely to the ones reported in the crystal structures of other C-type lectins that are specific for mannose. Docking mode Man_B is similar to what has been observed for α-Me mannoside in the binding site of rat liver mannose binding protein (MBP-C) [22] whereas Man_C is similar to the binding mode of mannose-containing oligosaccharide to the serum analog (MBP-A) [23].

For each of the proposed binding modes, the *p*-nitrophenylthio group was added to the mannose and the complex was optimized. There was no steric hindrance. The effect of a

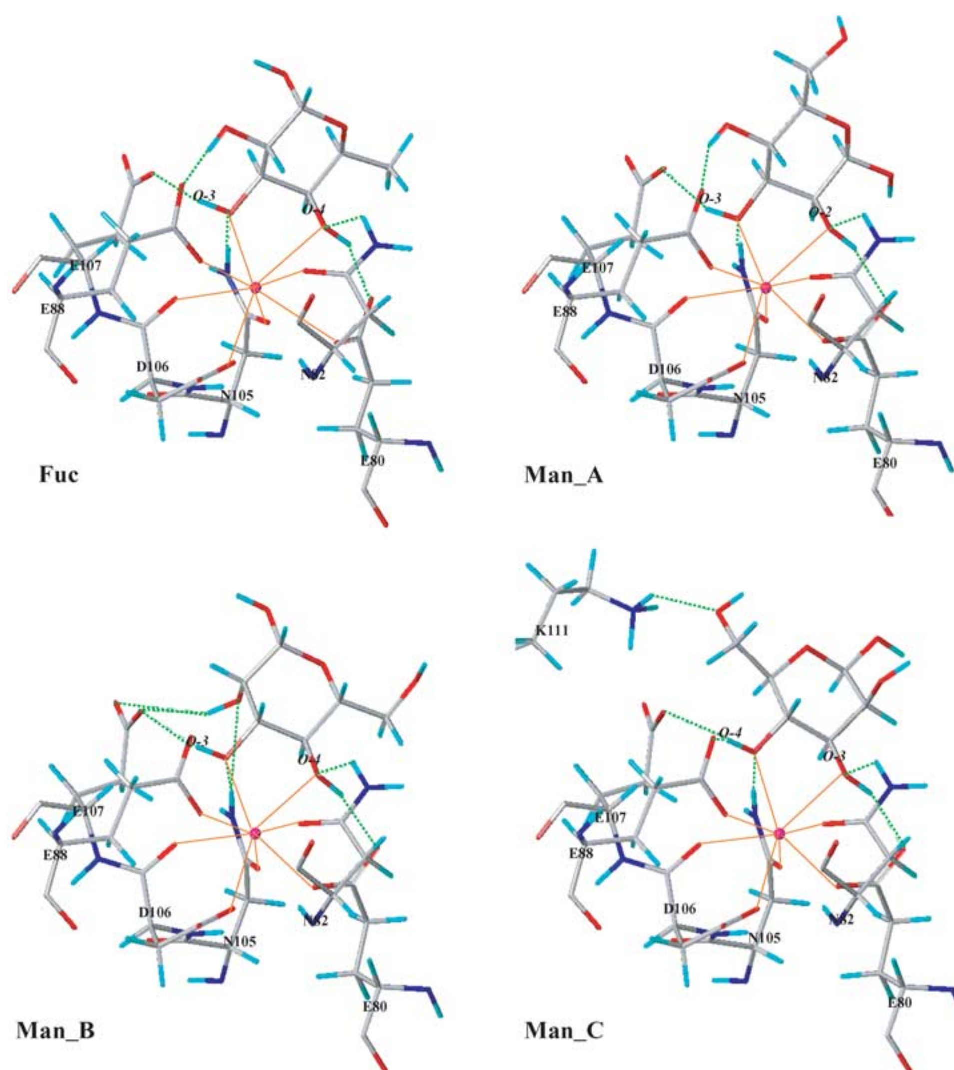

Figure 1. Three different modes of the interaction between α-Man and P-selectin and a comparison with the binding mode of fucose in the crystal structure [6]. Hydrogen bonds are drawn as green dotted lines, and the coordination of carbon as orange lines.

thio group at the anomeric position is to create a kink in the shape of the ligand (the C1_S_C angle is smaller than 100°, as seen in the crystal structures of such compounds [19]). This kinked shape helps in creating contact between the *p*-nitrophenyl group and the amino acids that surround the primary binding site. In Man_B docking mode, no contact exists between the nitrophenyl and the protein. In the two other binding modes, the complex was further stabilized by electrostatic interactions between the negatively charged oxygen atoms of the nitro group and basic amino acids. The amino acid involved are Lys 84 and Lys 111 for the binding modes Man_A and Man_C, respectively. There is no direct hydrogen bond between the nitro group of the ligand and the lysine sidechains, but the contacts are close enough to generate stabilization by electrostatic interaction, the O. . . N distance being 4.1 Å in complex Man_A and 4.8 Å in Man_B. The Man_A mode, which as aforementioned is similar to L-Fuc binding mode, also displays additional hydrophobic interaction between both the CH_2 at C-6 and the phenyl with the CH_2 groups of Lys 84 (Figure 2)—residue not essential for natural ligand binding [24]. The calculated energy differences between the three docking modes cannot be used for any sort of prediction. Nevertheless, because of its high stereochemical similarity with fucose in the crystalline complex, and because of the additional stabilization throughout the *p*-nitrophenyl group, we propose that binding mode Man_A to be the most probable one. Our modeling has contributed to the explanation of good activity of βMan-$SC_6H_4NO_2$ thus advancing new possibilities to search for low molecular weight blockers of P-selectin, different from the conventional method that requires a carboxyl group in mimetics [2]. Furthermore, βMan-$SC_6H_4NO_2$ displayed a pronounced activity not only in the static test system, but also *in vivo* on the rat model upon intravenous administration (the given studies) as well as on a mouse model during hypodermic injection [25].

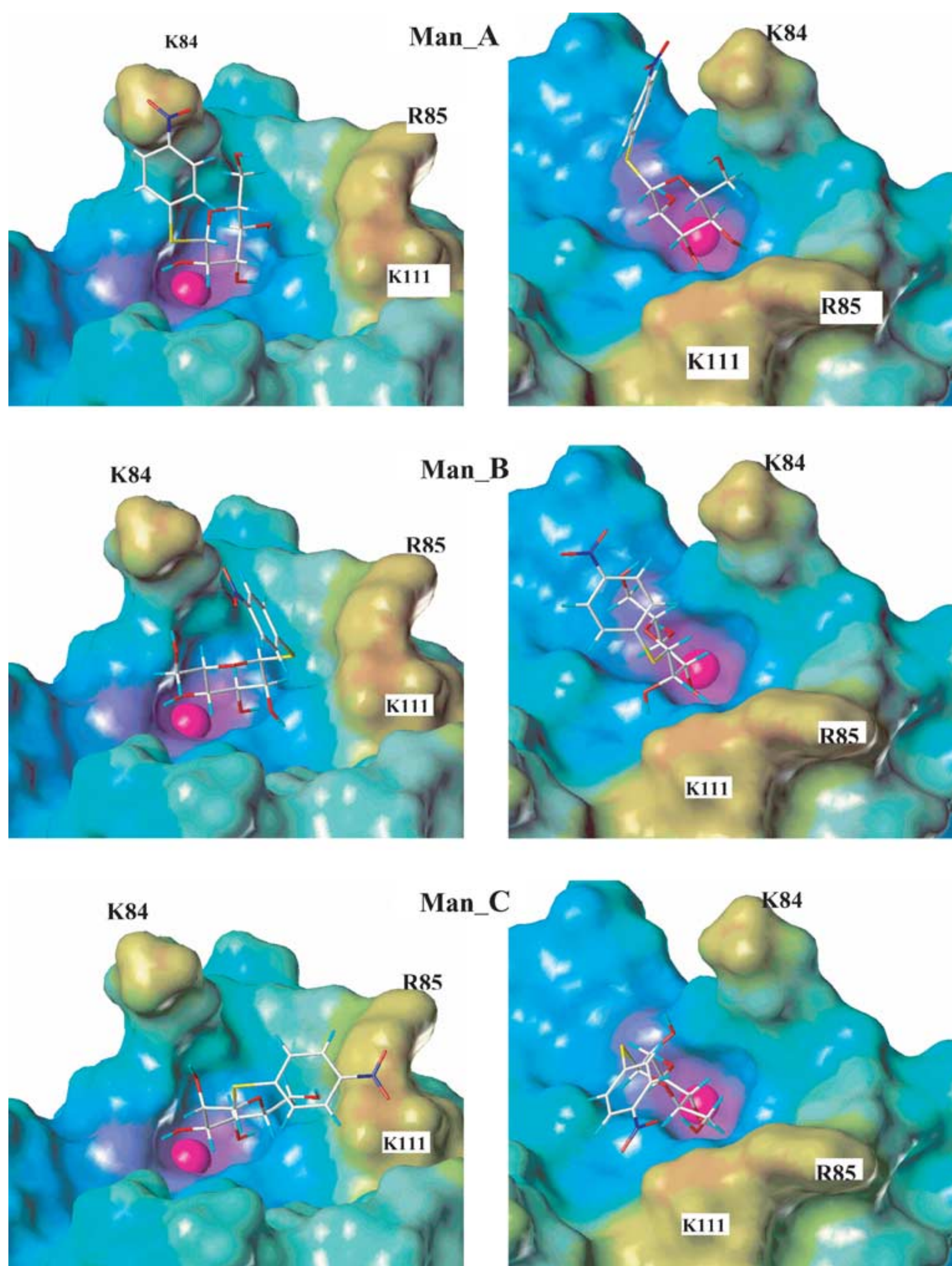

Figure 2. Three proposed binding models for the compound βMan-$SC_6H_4NO_2$-*p* in the binding site of P-selectin. The protein has been represented with its accessible surface colored according to the electrostatic potential (from blue for negatively charged area to orange-red for positively charged ones). In Man_A mode there are electrostatic interactions between oxygen atoms of $-NO_2$, and hydrophobic interactions of C_6H_4, with Lys 84 residue.

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