Design, synthesis and activity evaluation of mannose-based DC-SIGN antagonists

Nataša Obermajer · Sara Sattin · Cinzia Colombo · Michela Bruno · Urban Švajger · Marko Anderluh · Anna Bernardi

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Abstract In this article, we describe the design, synthesis and activity evaluation of glycomimetic DC-SIGN antagonists, that use a mannose residue to anchor to the protein carbohydrate recognition domain (CRD). The molecules were designed from the structure of the known pseudo-mannobioside antagonist 1, by including additional hydrophobic groups, which were expected to engage lipophilic areas of DC-SIGN CRD. The results demonstrate that the synthesized compounds potently inhibit DC-SIGN-mediated adhesion to mannan coated plates. Additionally, in silico docking studies were performed to rationalize the results and to suggest further optimization.

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Keywords Anti-infectives · Carbohydrates · DC-SIGN · Dendritic cell-based assay · Glycoconjugates · Glycomimetics

Abbreviations BSA Bovine serum albumin 
CFSE Carboxyfluorescein succinimidyl ester 
CRD Carbohydrate- recognition domain 
DC Dendritic cell 
DCC Dicyclohexyl carbodiimide 
DC-SIGN Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin 
DMA N,N-Dimethylacetamide 
DMAP 4-Dimethylaminopyridine 
DMSO Dimethyl sulfoxide 
EDC 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide 
FBS Fetal bovine serum 
HATU O-(7-Azabenzotriazole)- N,N,N′,N′-tetramethyluronium hexafluorophosphate 
LPS Lipopolysaccharide 
MCPBA m-Chloroperoxybenzoic acid 
PAMPS Pathogen-associated molecular patterns 
PBS Phosphate buffered saline 
PFP Pentafluorophenyl 
rhGM-CSF Recombinant human granulocyte-macrophage colony-stimulating factor 
rhIL-4 Recombinant human interleukin-4 
RMSD Root mean square deviation 
SAR Structure–activity relationship 
SDS Sodium dodecyl sulfate 
SPR Surface-plasmon resonance 
THF Tetrahydrofuran 
TLC Thin-layer chromatography
Introduction

Immature dendritic cells (DC) are the first-line guard against various pathogens that invade peripheral tissues. On binding the pathogen, they differentiate to mature DC that present antigen particles to T cells [1]. DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule 3-Grabbing Non-integrin), a specific C-type lectin recognizing pathogen-cell surface glycoproteins, is probably the first transmembrane receptor on immature DCs that encounters invading pathogens and binds a vast number of diverse pathogen-associated molecular patterns (PAMPs). Normally, this binding event triggers internalization of the DC-SIGN-pathogen complex followed by pathogen lysosomal degradation and conjugation of the resulting fragments with MHC-II to initiate an adaptive immune response from T cells. Additionally, DC-SIGN serves as a signalling receptor that mediates pathogen-induced Toll-like receptor (TLR) signalling, which in turn modifies DC maturation and, therefore, the intensity of the adaptive immune response [2–5].

Some pathogens, however, have been reported to take advantage of this mechanism as they appear to deter DC maturation through DC-SIGN-mediated signalling and inhibit antigen presentation to T cells [6]. The van Kooyk group has shown that HIV-1 enters DC via DC-SIGN avoiding lytic degradation [7–9]. By doing so, HIV-1 not only escapes the host immune system, but also is presented directly to T cells which enables fully disseminated HIV-1 infection. Other pathogens are also believed to use the same principle for host invasion [10]. Thus, inhibition of pathogen interaction with DC-SIGN specific antagonists is considered as a plausible concept for new anti-infective agents. Several groups have recently demonstrated that inhibition of DC-SIGN, either by designed glycoconjugates or by antibodies, prevents pathogen attachment to DC and inhibits the infection of other immune cells at its earliest steps [11–23].

DC-SIGN specifically binds mannose and fucose-glycosylated endogenous proteins (ICAM-2 and -3) as well as mannosylated PAMPs (HIV-1 gp120, M. tuberculosis ManLAM and others) [7,9]. Moreover, mannose- and fucose-containing oligosaccharides (such as Lewis-x) bind to DC-SIGN with moderate to high affinity, while both Man and Fuc themselves have only weak affinity [24]. As a consequence, glycomimetic structures designed to inhibit DC-SIGN have been based on oligomannosides or on Lewis-x. In our previous study, we concentrated on the design and synthesis of monovalent glycoconjugates as glycomimetic DC-SIGN antagonists [12,15,21]. These molecules (Fig. 1, 1–3) have been conceived to be metabolically more stable than native oligosaccharides and/or equipped with reactive moieties to enable their multimeric presentation on multivalent supports. Ligands 1–3 had moderate to weak affinities for DC-SIGN, comparable or slightly superior to those of the starting oligosaccharide templates [12,15,21]. Thereby they displayed only limited therapeutic potential in their monomeric form. However, when presented in multimeric form, they were effective inhibitors of DC-SIGN-mediated viral infection. Indeed, the pseudo-trisaccharide 2, mimicking a linear Man-α-1,2-Man-α-1,6-trimannoside was found to inhibit DC-SIGN binding to mannosylated BSA with an IC50 of 130 µM (by surface-plasmon resonance—SPR) and, when presented on a tetravalent dendron, was also found to inhibit the trans infection of CD4+T lymphocytes by HIV at low micromolar concentrations [21].

To improve binding affinities of monovalent glycoconjugates, we have now designed mannose-based glycoconjugates derived from 1 which could bind into new binding areas of DC-SIGN carbohydrate recognition domain (CRD), that are not occupied by the native ligands. Ligand candidates were synthesized and their affinity to DC-SIGN was evaluated by an in vitro assay that measures inhibition of DC-SIGN-mediated immature dendritic cell adhesion to mannan-coated plates [25]. Additionally, docking studies were performed to rationalize the results and to suggest further optimization. Assay data demonstrate that our effort to design and synthesize mannose-based DC-SIGN antagonists has resulted in compounds which potently inhibit DC-SIGN-mediated adhesion to mannan-coated plates and have the potential to prevent DC-SIGN-mediated pathogen recognition by DC.
Table 1  Structure of compounds 16–50

![Diagram of compounds 16-50]

|   | R' = Bz | R' = H | R =     | R' = Bz | R' = H | R =     |
|---|---------|--------|---------|---------|--------|---------|
| 16|         | 26     |         | 24      | 35     | HOOC    |
| 17|         | 27     |         | 25      | 36     | H2N     |
| 18|         | 28     |         | 37      | 44     |         |
| 19|         | 29     |         | 38      | 45     |         |
| 20|         | 30     |         | 39      | 46     | Me      |
| 21|         | 31     |         | 40      | 47     | Me      |
| 22|         | 32     |         | 41      | 48     | Me      |
| 23|         | 33     |         | 42      | 49     | S       |
| 24|         | 34     | MeO     | 43      | 50     | Me      |

**Experimental**

**In Silico Molecular docking studies**

**Ligand preparation**

The molecules (Table 1) were built with ChemBioDraw Ultra 12.0 [26]. The ligand geometries were optimized with Chem-Bio 3D Ultra 12.0 using MM2 force field until a minimum 0.100 RMS gradient was reached [26]. The optimized structure was refined with GAMESS interface using the semi-empirical AM1 method, QA optimization algorithm and Gasteiger Hückel charges for all atoms for 100 steps [27]. The carboxylate (35) and non-aromatic amine (28) were kept in their ionized state at to pH 7.4.

**Receptor preparation and docking protocol**

The crystal structure of DC-SIGN CRD (chain A; 131 amino-acid sequence) in complex with Man4 (PDB code 1SL4) [28] was taken as a starting-point. The ligand Man4 was taken as a reference structure and an area of 14 Å around it was considered as the active site. The crystal structure was cleaned with FlexX 3.1.2 [29] by deleting the ligand (Man4) and crystallographic water molecules. Hydrogens were automatically added to all heavy atoms. Two water molecules (HOH residues 512 and 519; 152 and 105 in FlexX) were preserved as ‘freely rotatable water’ in docking studies since one (519 – FlexX ID: 105) stabilizes interactions between Man1 residue and the receptor while the other one (512—FlexX ID: 152) stabilizes the Man4 binding conformation.
Additionally, PharmMetal pharmacophore with spherical coordination around Ca$^{2+}$ was defined to correctly account for complex interactions between Ca$^{2+}$ (406) and Man1 residue. The side-chains of aspartate, glutamate, lysine and arginine residues were kept in their ionized state at pH 7.4.

The FlexX 3.1.2 molecular docking program for structure-based design was used for ligand docking using the standard triangle algorithm to place the ‘base fragment’. The parameters in FlexX modified/optimized for the docking studies were: (a) clash handling, (b) maximum number of solutions per iteration and (c) maximum number of solutions per fragmentation. The proposed 10 binding modes with the highest rank of the docked antagonists were evaluated using final score and RMSD as a tool to explore relative structural differences between proposed binding modes. The graphical representations of the proposed binding positions of molecules 26–36 and 44–50 were obtained using Accelrys Discovery Studio 2.5. [30].

Validation of the docking protocol

The docking procedure should be able to correctly predict the binding orientation of the molecules in the PDB database, i.e. the crystal structures of ligands in complex with proteins. We have attempted to reproduce the pose of Man$_4$ as seen in its X-ray complex with the target DC-SIGN CRD (1SL4) [28]. The top 10 docking poses were within 1.5 Å root mean square deviation (RMSD) of the crystal structure for Man1 and Man2 residues of Man$_4$. The predicted top score pose was the one that best fitted the crystal structure of Man$_4$ overlapping reasonably well (Fig. 2).

![Fig. 2](image)

Crystal structure of DC-SIGN CRD (shown as solvent-accessible surface; Ca$^{2+}$ coordinated by Man1 residue is presented as a sphere) in complex with Man$_4$ (shown as lines) and top-docked pose (shown as tubes).

Synthesis

**General**

Dichloromethane, methanol, N,N-diisopropylethylamine and triethylamine were dried over calcium hydride; THF was distilled over sodium, N,N-dimethylacetamide (DMA) was dried over activated molecular sieves. Reactions requiring anhydrous conditions were performed under nitrogen. $^1$H, $^{13}$C and $^{19}$F-NMR spectra were recorded at 400 MHz on a Bruker AVANCE-400 instrument. Chemical shifts (δ) for $^1$H and $^{13}$C spectra are expressed in ppm relative to internal Me$_4$Si as standard. Signals were abbreviated as s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Sugar signals were numbered as customary; cyclohexane protons are indicated with the letter D followed by numbers. Numbering of the cyclohexane ring in compounds 10–50 is unconventional (see Figure SI-1 in the Supplementary Information file) and it was adopted to facilitate comparison with the native disaccharide. Mass spectra were obtained with a ThermoFisher LCQ apparatus (ESI ionization), or ion-trap ESI Esquire 6000 from Bruker, or a Microflex apparatus (MALDI ionization) from Bruker, or Apex II ICR FTMS (ESI ionization—HR-MS). Specific optical rotation values were measured using a Perkin-Elmer 241, at 589 nm, in a 1 dm cell. Thin layer chromatography (TLC) was carried out with pre-coated Merck F$_{254}$ silica gel plates. Flash chromatography (FC) was carried out with Macherey-Nagel silica gel 60 (230–400 mesh). Compounds 5 [32] and 12 [31] have been described. The synthesis of 6–13 and the general procedures for the synthesis of amides 16–36 and anilides 37–50 are described. The characterization of compounds 16–50 is reported in Supplementary Information.

$$\text{(1S,2S)-4-Cyclohexen-1,2-dicarboxylic acid bis-pentafluorophenylester 6}$$

EDC-HCl (743 mg, 3.877 mmol, 3.3 eq.) was added to a solution of the diacid 5 [32] (200 mg, 1.175 mmol, 1 eq.) in dry THF (11 mL) under stirring and under a nitrogen atmosphere. After 10 min pentafluorophenolen (649 mg, 3.525 mmol, 3 eq.) was added. The solution was stirred at room temperature for 2h. After completion of the reaction the solvent was evaporated under reduced pressure, the residue was dissolved in Et$_2$O, the organic phase washed with 1M HCl and saturated Na$_2$CO$_3$, then dried over sodium sulphate. Solvent was evaporated under reduced pressure yielding 519 mg (yield = 88%) of pure 6 as a colourless oil.
[CH$_2$Cl$_2$ (4.4 mL) under stirring. The reaction was stirred by flash chromatography (8:2 Hex:EtOAc) affording 533 mg sodium sulphate and the solvent evaporated under reduced pressure. The mixture was diluted with EtOAc and washed with saturated aq. Na$_2$SO$_4$ and the solvent evaporated under reduced pressure, the reaction mixture was kept at room temperature. After completion (ca. 4 h; TLC 9:1 toluene:EtOAc) the reaction mixture was directly purified by flash chromatography (95:5 toluene:EtOAc) yielding 479 mg (yield = 95%) of pure product 10 as a yellow oil.

77% MCPBA was added (385 mg, 1.8 mmol, 1.4 eq.) to a solution of the PFP ester 6 (660 mg, 1.3 mmol, 1 eq.) in dry CH$_2$Cl$_2$ (4.4 mL) under stirring. The reaction was stirred under nitrogen at room temperature until TLC (8:2 Hexane:EtOAc) showed disappearance of the starting material. The solvent was evaporated at reduced pressure, the reaction mixture was diluted with EtOAc and washed with saturated NaHCO$_3$ and with water. The organic phase was dried over sodium sulphate and the solvent evaporated under reduced pressure, yielding 636 mg of crude product that was purified by flash chromatography (8:2 Hexane:EtOAc) affording 533 mg (78%) of pure 8.

[$\alpha$]$_D^{20}$ = +37.7 (c = 0.5; CHCl$_3$). MS (FAB) calculated for [C$_{20}$H$_9$F$_{10}$O$_5$]$^+$: 519; found: 519. 1H-NMR (400 MHz, CDC$_3$): 3.43–3.34 (m, 2H, H$_4$ or H$_5$, H$_2$ or H$_1$), 3.32 (dd, 1H, H$_3$ or H$_4$, J$_{3-4}$ = J$_{4-3}$ = 4 Hz), 3.14 (dt, 1H, H$_2$ or H$_1$, J$_{2-3}$ or J$_{1-2}$ = 6.8 Hz, J$_{2-3}$ or J$_{1-2}$ = 10.0 Hz), 2.76 (dd, 1H, H$_3$ or H$_4$, J$_{3-4}$ or J$_{4-3}$ = 1.6 Hz, J$_{3-4}$ = J$_{4-3}$ = 5.2 Hz, J$_{gem}$ = 14.8 Hz). MS (ESI) calculated for [C$_{25}$H$_{14}$F$_{10}$O$_6$Na]$^+$: 599.1; found: 599.6. 1H-NMR (400 MHz, CDC$_3$): 6.00–5.90 (m, 1H, H$_8$), 5.34 (dd, 1H, H$_9$trans, J$_{9-8}$trans = 1.3 Hz, J$_{9-8cis}$trans = 17.2 Hz), 5.25 (dd, 1H, H$_9$cis, J$_{9-8cis}$cis = 10.4 Hz), 4.12 (dd, 1H, H$_7$, J$_{7-6}$ = 5.2 Hz, J$_{7-6}$ = 12.8 Hz), 4.07 (dd, 1H, H$_7$, J$_{7-6}$ = 4.5 Hz), 3.64 (dd, 1H, H$_1$, J$_{1-2}$ = 4.0 Hz, J$_{1-2}$ = 6.8 Hz), 3.62–3.46 (m, 2H, H$_4$, H$_5$), 2.31–2.18 (m, 2H, H$_6$), 2.28–2.22 (m, 2H, H$_3$), 1.82 (d, 1H, OH, J = 3.0 Hz). 13C-NMR (100 MHz, CDC$_3$): 170.4, 170.4 (COOPFP); 142.3 (m, CF); 140.9 (m, CF); 139.8 (m, CF); 139.1 (m, CF); 138.4 (m, CF); 136.6 (m, CF); 134.4 (C$_3$); 117.4 (C$_3$); 74.9 (C$_1$); 70.3 (C$_7$); 66.5 (C$_2$); 38.9 (C$_4$); 38.4 (C$_5$); 30.5 (C$_3$); 27.2 (C$_6$).

A mixture of the acceptor 10 (418 mg, 0.725 mmol, 1 eq.) and the donor 12 [31] (644 mg, 0.870 mmol, 1.2 eq.) was co-evaporated with toluene three times. Powdered acid washed 4Å molecular sieves were added; the mixture was kept under vacuum for a few hours and then dissolved with dry CH$_2$Cl$_2$ (7.25 mL). After cooling at -20°C, TMSOTf (26 μL, 0.145 mmol, 0.2 eq.) was added to the reaction mixture under stirring. The reaction was monitored by TLC (85:15 Toluene:EtOAc, 7:3 Hexane:EtOAc) and was finished after 20 min. The reaction was quenched with Et$_3$N and the mixture was warmed to room temperature and filtered over a celite pad. The filtrate was evaporated at reduced pressure and the crude product purified by flash chromatography (75:25 Hexane:EtOAc) to yield 470 mg of 13 (56%).

MS (ESI): calculated for [C$_{57}$H$_{40}$F$_{10}$O$_{15}$Na]$^+$: 1177.2; found: 1177.3. 1H-NMR (400 MHz, CDC$_3$): 8.08 (d, 4H, H$_{Bz}$, J = 7.4 Hz), 8.00 (d, 2H, H$_{Bz}$, J = 7.2 Hz), 7.84 (d, 2H, H$_{Bz}$, J = 7.2 Hz), 7.66–7.50 (m, 3H, H$_{Bz}$), 7.49–7.34 (m, 6H, H$_{Bz}$), 7.34–7.23 (m, 3H, H$_{Bz}$). 6.11 (t, 1H, OH).
General procedure for the synthesis of amides 26–36

The amine (2.4 eq.) was added to a 0.4 M PFP-scaffold 13 (1 eq.) in dry THF under stirring and under nitrogen atmosphere at room temperature. After completion (TLC 7:3 Hexane:EtOAc, 98:2 CHCl₃:MeOH) the solvent was evaporated under reduced pressure. The crude product was purified using three consecutive 3 mL Isolute H-MN columns conditioned with 1M HCl, 1M NaOH and water. Finally, it was dissolved in 5 mL of EtOAc and eluted gravimetrically. The organic phase was collected; the solvent was evaporated at reduced pressure, obtaining the pure products 16–25. These were directly deprotected using the following general procedure: to a 0.05 M solution of the bis-amide (1 eq.) in dry methanol, under nitrogen at room temperature, a 1M solution of sodium methoxide in MeOH (2 eq.) was added. After reaction completion (TLC 96:4 and 8:2 CHCl₃:MeOH) the reaction mixture was diluted with methanol and neutralized with pre-washed Amberlite IRA 120-H⁺. The resin was filtered off and the solvent evaporated under reduced pressure. The crude was purified by flash chromatography (95:5 CHCl₃:MeOH).

(1S,2S)-4-cyclohexen-1,2-dicarboxylic acid bis-(2-trimethylsilyl)-ethylester 7

Trimethylsilylethanol (2.1 mL, 14.63 mmol, 2.2 eq.) and DMAP (56.5 mg, 0.4624 mmol, 0.05 eq.) were added to a solution of the diacid 5 [32] (1.3 g, 6.65 mmol, 1 eq.) in dry CH₂Cl₂ (9 mL) under stirring. Separately DCC (3.0 g, 14.63 mmol, 2.2 eq.) was dissolved in CH₂Cl₂ (9 mL) and the solution added dropwise to the previous one cooled at 0 °C under stirring. After 15 min the reaction mixture was warmed to room temperature and stirred for 24 h. After reaction completion (TLC 99:1 hexane:EtOAc) the reaction mixture was diluted with CH₂Cl₂ and filtered on a celite pad. The solvent was evaporated at reduced pressure. The product was purified by flash chromatography (95:5 hexane:EtOAc) obtaining 2.12 g (yield = 86%) of pure 7.

$$[\alpha]_D^{20} = +66.5 (c = 1.2; \text{CHCl}_3). \text{LC-MS (ESI)}$$

77% MCPBA was added (1.8 g, 8.00 mmol, 1.2 eq.) to a solution of the trimethylsilyl ester 7 (2.5 g, 6.66 mmol, 1 eq.) in dry CH₂Cl₂ (22 mL) under stirring. The reaction was stirred under nitrogen at room temperature. After completion of the reaction (2 h; TLC 95:5 toluene:EtOAc), the reaction mixture was diluted with CH₂Cl₂ and washed with saturated NaHCO₃ and with water. The organic phase was dried over sodium sulphate and the solvent evaporated under reduced pressure, obtaining 2.66 g of crude product that was purified by flash chromatography (9:1 hexane:EtOAc + 0.5% AcOH) yielding to 1.43 g (yield= 71%) of pure product 9.

$$[\alpha]_D^{20} = +34.5 (c = 1.15; \text{CHCl}_3). \text{H-NMR (400MHz, CDCl}_3): 1.44-4.09 \text{ (m, 4H, H}_2), 3.23-3.19 \text{ (m, 1H, H}_4), 3.21 \text{ (t, 1H, H}_5, J=5 J=5 \text{-psdax} = 4 \text{ Hz}, 2.84 \text{ (dt, 1H, H}_2, J=2 J=2 \text{-psdax} = 10.8 \text{ Hz, J=2-3psdax} = 4.8 \text{ Hz}), 2.61 \text{ (dt, 1H, H}_1, J=1 J=1 \text{-psdax} = 10.4 \text{ Hz, J=1-6psdax} = 6.4 \text{ Hz}), 2.49 \text{ (ddd, 1H, H}_3, J=3 J=3 \text{-psdax} = 14.8 \text{ Hz, J=3-4psdax} = 2 \text{ Hz}, 2.33 \text{ (ddd, 1H, H}_6, J=6 J=6 \text{-psdax} = 15.2 \text{ Hz), 2.09 (ddd, 1H, H}_7\text{-psdax}, 1.92 \text{ (ddd, 1H, H}_8, J=8 J=8 \text{-psdax} = 2 \text{ Hz}, 1.03-0.95 \text{ (m, 4H, H}_8), 0.07 \text{ (s, 18H, H}_9). \text{13C-NMR (100MHz, CDCl}_3): 174.8 \text{ (CO), 173.9 (CO), 63.1 (C}_7), 51.9 (C}_4), 50.4 (C}_5), 40.1 (C}_1), 37.8 (C}_2), 27.2 (C}_3), 26.4 (C}_6), 17.3 (C}_8), -1.5 (C}_9).$$

Epoxide of the (1S,2S)-4-cyclohexen-1,2-dicarboxylic acid bis-(2-trimethylsilyl)-ethylester 9

(1S,2S,4S,5S)-4-allyloxy-5-hydroxy-cyclohexan-1,2-bis-(2-trimethylsilyl)-ethylester 11

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Cu(OTf)$_2$ (111.2 mg, 0.3078 mmol, 0.1 eq.) was added to a solution of the epoxide 9 (1.2 g, 3.78 mmol, 1 eq.) in allylic alcohol (6.3 mL, 92.34 mmol, 30 eq.) under stirring and under nitrogen atmosphere. The solution was stirred at room temperature. After reaction completion (5 h; TLC 95:5 toluene:EtOAc) the reaction mixture was diluted with Et$_2$O, washed with 1:1 NH$_3$; saturated NH$_4$Cl, then with saturated NH$_4$Cl. The organic phase was dried over sodium sulphate and the solvent evaporated under reduced pressure. The crude product was purified by flash chromatography (85:15 toluene:EtOAc) yielding 1.26 g (yield = 91%) of pure product 11.

$[\alpha]_D^{20} = +11.3$ (c = 1.04; CHCl$_3$). $^1$H-NMR (400 MHz, CDCl$_3$): 5.95–5.80 (m, 1H, H$_8$), 5.25 (dq, 1H, H$_9$H$_{trans}$, $J_{trans} = 17.2$ Hz, $J_{gem} = J_{trans-7A} = J_{trans-7B} = 1.6$ Hz), 5.15 (dq, 1H, H$_9$A, $J_{cis} = 12.8$ Hz, $J_{cis-7A} = J_{cis-7B} = 1.6$ Hz), 4.18–4.06 (m, 5H, H$_{7B}$, H$_{10}$), 3.95 (ddt, 1H, H$_7$A, $J_{gem} = 12.8$ Hz, $J_{7A-8} = 5.6$ Hz), 3.85–3.78 (m, 1H, H$_2$), 3.43–3.37 (m, 1H, H$_1$), 3.10–2.95 (m, 2H, H$_4$, H$_5$), 2.15–1.97 (m, 2H, H$_{3eq}$, H$_{5eq}$), 1.93 (d, 1H, H$_{OH-H2} = 2.4$ Hz), 1.90–1.75 (m, 2H, H$_{3ax}$, H$_{6ax}$), 1.00–0.90 (m, 4H, H$_{11}$), 0.02 (s, 9H, H$_{12}$), 0.01 (s, 9H, H$_{12}$). $^{13}$C-NMR (100 MHz, CDCl$_3$): 174.7, 174.6 (CO), 134.5 (C$_6$), 117.1 (C$_9$); 76.8 (C$_1$); 70.1 (C$_7$); 68.3 (C$_2$); 63.2, 63.2 (C$_{10}$); 40.0, 39.6 (C$_4$, C$_5$); 30.8 (C$_3$); 27.3 (C$_6$); 17.6, 17.6 (C$_{11}$); $-1.3$ (C$_{12}$).

**Tetra-O-benzoyl α-(1,2)-pseudomannobioside bis-(2-trimethylsilyl)-ethyl ester 14**

A mixture of the acceptor 11 (140 mg, 0.313 mmol, 1 eq.) and the donor 12 [31] (278 mg, 0.375 mmol, 1.2 eq.) was co-evaporated with toluene three times. Powdered acid washed CD$_6$; 1.07–0.97 (m, 4H, H$_{11}$), 0.04 (s, 9H, (CH$_3$)$_3$Si), 0.02 (s, 9H, (CH$_3$)$_3$Si). $^{13}$C-NMR (100 MHz, CDCl$_3$): 174.7, 174.6 (CO); 134.5 (C$_6$), 117.1 (C$_9$); 76.8 (C$_1$); 70.1 (C$_7$); 68.3 (C$_2$); 63.2, 63.2 (C$_{10}$); 40.0, 39.6 (C$_4$, C$_5$); 30.8 (C$_3$); 27.3 (C$_6$); 17.6, 17.6 (C$_{11}$); $-1.3$ (C$_{12}$).

A mixture of the acceptor 11 (140 mg, 0.313 mmol, 1 eq.) and the donor 12 [31] (278 mg, 0.375 mmol, 1.2 eq.) was co-evaporated with toluene three times. Powdered acid washed 4Å molecular sieves were added. The mixture was kept under vacuum for few hours and then dissolved in dry CH$_2$Cl$_2$ (3 mL). After cooling to $-20^\circ$C, TMSOTf (11 µL, 0.0625 mmol, 0.2 eq.) was added to the reaction mixture under stirring. The reaction was stirred at that temperature and was monitored by TLC (9:1 toluene:EtOAc, 95:5 CHCl$_3$:acetone): after 30 min the reaction was quenched by adding NEt$_3$. The mixture was then warmed to room temperature and filtered on a celite pad. The filtrate was evaporated under reduced pressure and the product purified by flash chromatography (95:5 toluene:EtOAc) yielding 154 mg (48%) of 14.

$[\alpha]_D^{20} = -31.0$ (c = 1.01; CHCl$_3$). $^1$H-NMR (400 MHz, CDCl$_3$): 8.05 (d, H-Ph, $J = 5.2$ Hz), 8.00–7.90 (m, H-Ph), 7.72 (d, H-Ph, $J = 7.6$ Hz), 7.64–7.52 (m, H-Ph), 7.52–7.46 (m, H-Ph), 7.46–7.30 (m, H-Ph), 7.24 (d, H-Ph, $J = 5.2$ Hz), 6.06 (t, 1H, H$_4$, $J = 10$ Hz), 5.95–5.80 (m, 2H, H$_3$, H$_8$), 5.70 (bs, 1H, H$_2$), 5.28 (dd, 1H, H$_{7B}$, $J_{trans} = 18$ Hz), 5.23 (3H, 1H, H$_1$), 5.14 (dd, 1H, H$_{9A}$, $J_{9A-9B} = 1.2$ Hz, $J_{cis} = 10.4$ Hz), 4.63 (d, 1H, H$_{6b}$, $J_{gem} = 13.6$ Hz), 4.42 (2H, H$_{6A}$, H$_{5A}$), 4.26–4.10 (m, 4H, H$_{10}$), 4.08 (dd, 1H, H$_{7D}$, $J_{gem} = 12$ Hz, $J_{7B-8} = 5.2$ Hz), 4.02–3.97 (m, 1H, D$_2$), 3.94 (dd, 1H, H$_{7A}$, $J_{7A-8} = 5.6$ Hz), 3.72–3.68 (m, 1H, D$_3$), 3.07–2.93 (m, 2H, D$_4$, D$_5$), 2.13–1.94 (m, 4H, D$_3$, D$_5$), 1.07–0.97 (m, 4H, H$_{11}$), 0.04 (s, 9H, (CH$_3$)$_3$Si), 0.02 (s, 9H, (CH$_3$)$_3$Si).

**Tetra-O-benzoyl α-(1,2)-pseudomannobioside diacid 15**

![Tetra-O-benzoyl α-(1,2)-pseudomannobioside diacid 15](image)
69.7 (C₃); 67.0 (C₄); 63.1 (C₆); 39.0, 39.2 (C_D₄, C_D₃); 27.4 (C_D₃); 26.9 (C_D₆).

**General procedure for the synthesis of anilides 44–50**

To a 0.6 M solution of the diacid 15 (1 eq.) in dry DMA, HATU (1.1 eq.) and the appropriate aniline (3 eq.) were added under stirring and under nitrogen atmosphere at room temperature. The reaction mixture was warmed to 50 °C. After disappearance of the starting material (TLC 9:1 CHCl₃:MeOH and 98:2 CHCl₃:acetone) another aliquot of HATU (1.1 eq.) and of aniline (3 eq.) was added under stirring. The reaction was stirred at 50 °C for 3 days. After reaction completion the solvent was evaporated at reduced pressure. The product was purified by membrane filtration using Isolute Phase Separator columns. Therefore, the crude reaction product was dissolved in CH₂Cl₂ (1 mL) and charged onto a column with 1M HCl (1 mL). The organic phase was eluted and charged onto another column containing 1M NaOH. The organic phase was dried over sodium sulphate and the solvent evaporated under reduced pressure. The crude was purified by flash chromatography (98:2 CHCl₃:MeOH) to yield anilides 37–43, that were deprotected using the following procedure.

A 1 M solution of sodium methoxide (2 eq.) in MeOH was added to a 0.05 M solution of the bis-anilide (1 eq.) in dry methanol under stirring and under nitrogen atmosphere at room temperature. After reaction completion (TLC 88:12 toluene:acetone) the reaction mixture was diluted with methanol and neutralized with pre-washed Amberlite IRA 120-H⁺. The resin was filtered off and the solvent evaporated under reduced pressure. Crude anilides 44–50 were purified by flash chromatography (95:5 → 9:1 CHCl₃:MeOH).

**Biological assay**

**Preparation and culture of dendritic cells**

Buffy coats from the venous blood of normal healthy volunteers were obtained by the Blood Transfusion Centre of Slovenia, according to institutional guidelines. The study was approved by the National Medical Ethics Committee of the Ministry of Health, Republic of Slovenia, and written consent was obtained before collection of specimens.

Peripheral blood mononuclear cells were isolated using Lympholyte®-H (Cedarlane laboratories, Ontario, Canada). The cells were washed twice with Dulbecco’s phosphate-buffered saline (DPBS), counted, and used as the source for immunomagnetic isolation of CD14-positive cells (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). These were cultured in RPMI 1640 (Cambrex) medium supplemented with 10% fetal bovine serum (FBS), gentamicin (50 μg/mL; Gibco, Paisley, UK), 500 U/mL of rhGM-CSF and 400 U/mL of rhIL-4 (both Gentaur, Paris, France). On day 2, half of the medium was exchanged with starting quantities of rhGM-CSF (500 U/mL) and rhIL-4 (400 U/mL). After 5 days, non-adherent, immature DCs were harvested and characterized by flow cytometry as CD1a⁺, CD83⁻, CD86low and HLA−DRlow (data not shown). Cells were counted and re-suspended in the medium containing 500 U/mL of rhGM-CSF and 20 ng/mL LPS, and cultured for a further 2 days [33].

**Measurement of dendritic cell adhesion with CFSE fluorescence assay**

Wells of a 96-well culture plate (Nunc Nunclon Δ surface or Nunc MaxiSorp) were precoated with 50 μL of mannan (1.0 mg/mL) (Sigma) in carbonate buffer, pH 9.6, overnight at 4°C. Wells were then washed once with PBS and incubated with 1% BSA in PBS for 30 min at room temperature. Various volumes of 50 mM of stock solution of ligands were added to the wells to obtain the required final concentrations in the assay. PBS or DMSO was used in controls. Immature DCs were harvested, washed with PBS and labelled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer’s protocol (Molecular Probes, Invitrogen, Eugene, USA) [34], and re-suspended in the medium containing 500 U/mL of rhGM-CSF and either rhIL-4 (1000 U/mL) or 20 ng/mL LPS at concentration 5 × 10⁵/mL. 50 μL of this immature DC suspension was then added to wells and the cells were allowed to attach for 90 min. Wells were then washed twice with PBS, and 50 μL of lysis buffer (25 mM Tris, 0.1% SDS) was added. Each experiment was done in quadruplicate for each concentration of antagonist used and for control cells. To estimate the total immature DCs added to wells, one quadruplicate of wells was not washed with PBS, but only lysis buffer was added directly. CFSE is colourless and non-fluorescent until the acetate groups are cleaved by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester which reacts with intracellular amines, forming fluorescent conjugates. The fluorescence was measured at 520 ± 10 nm with a Tecan Saphire microplate reader (Tecan Group, Maennedorf, Switzerland, excitation wavelength 490 nm) and is directly proportional to the number of adhered cells. Cell adherence was determined by Eq. 1, where \( A_{tx} \) and \( A_{totalDC} \) are the fluorescence of CFSE determined for cells washed after different times and for all dendritic cells. \( A_{blank} \) is the background fluorescence of the buffer.

**Cell adherence (%) = \( \frac{A_{tx} - A_{blank}}{A_{total DC} - A_{blank}} \) × 100.**

\[ (1) \]

The IC₅₀ values were calculated by fitting mean values of DC-SIGN-mediated cell adherence versus log (antagonist concentration) to the logistic (sigmoid) equation by nonlinear least-squares curve-fitting using OriginPro 7.5 and 8.
software [35]. All the experimental data are presented with standard error bars. Control experiments were performed for known DC-SIGN antagonists 1 and 2, and two DC-SIGN specific antibodies (H200 and 1B10) in order to prove DC-SIGN specific inhibition.

Results and discussion

Structure-based design of substituted 1,2-mannobioside mimics

HIV-1, M. tuberculosis [7] and a number of other pathogens including viruses (HIV-1, HCV, CMV, Dengue, Ebola, SARS-CoV, HSV, coronaviruses, H5N1, West Nile virus, measles virus) and bacteria (Helicobacter pylori and Leptospira interruga) [36–44] bind to DC-SIGN through mannosylated glycoproteins (gp120 and ManLAM). To improve the binding affinity of mannose-based glycoconjugates, we have explored the known crystal structure of DC-SIGN carbohydrate-recognition domain (CRD) in complex with tetramannoside Man4 (PDB code: 1SL4) [26] in search for binding sites on DC-SIGN CRD whose binding potential is not entirely exploited by the mimics designed so far [11–16, 21]. The primary interaction of the oligosaccharide occurs by coordination of the non-reducing end residue of the oligosaccharide to a Ca2+ binding site exposed on the surface of the protein (shown as blue sphere, Fig. 3) [24]. Examination of this crystal structure allows identification of two structural features of DC-SIGN CRD as favourable sites to increase overall free energy of binding. A hydrophobic region defined by the phenyl ring of Phe 313 (Fig. 3) interacts weakly with the Man2 residue of Man4. It has been postulated that Phe 313 plays a double role in the binding mode of branched mannosides: it increases affinity (albeit weakly) by forming part of a surface complementary to the shape of Manα1-6Man moiety of Man4, and it allows access to the α-linked outer arm branch trimannose structure while preventing binding of β-linked mannosides and N-linked glycans that have only the core branch trimannose structure [24]. Man3 residue does not interact with the receptor at all, but rather it points away from the protein and allows binding of high-mannose oligosaccharides. The hydrophobic cavity/area behind Phe313 (Fig. 3) is also a likely interaction region, which does not make any contact with the Man3 residue.

Based on these observations, we have sought to modify the structure of the pseudo-disaccharide 1 (Fig. 4) to include fragments capable of interacting with one, or possibly both, proposed binding sites. The interaction of 1 and DC-SIGN was studied by NMR spectroscopy [12], suggesting that the ligand should anchor to DC-SIGN CRD through complexation of the Ca2+ binding site by its mannose residue. Indeed, preliminary docking studies confirmed this hypothesis and suggested that the cyclohexane ring of 1 may extend towards the Phe 313 region in the protein binding site. Hence, additional lipophilic fragments could be added to the framework of 1 exploiting the carboxy groups appended to the cyclohexane ring (Fig. 4). Identification of hydrophobic contacts in the proximity of the binding site of oligosaccharides and addition of lipophilic fragments to a sugar ‘core’ have often been applied to improve the affinity of glycomimetics [11, 45–49].

Fig. 3 Crystal structure of DC-SIGN CRD (shown as solvent-accessible surface) in complex with Man4 (shown as tubes) with proposed binding regions unoccupied by Man4. Mannose residues and important amino acid residues for Man4 binding are labelled as ovals, Ca2+ coordinated by Man1 residue is presented as a sphere.

The docking model used did not have the predictivity required to allow selection of the amide residues by virtual screening. A small library of substituted aromatic and non-aromatic derivatives of general structure 4 was therefore prepared (Fig. 4 and Table 1) looking for initial Structure-Activity Relationship (SAR) data. Preliminary SPR studies [50] showed that tertiary amides had no affinity for DC-SIGN, so mostly secondary amides were synthesized and only one tertiary amide (compound 28, Table 1) was tested in the present study.

The synthetic approach used is shown in Scheme 1. Two different condensation strategies were employed to synthesize amides or anilides from the preformed pseudo-disaccharides 13 and 15, respectively. These in turn were obtained by appropriate modification of the sequence previously described for the synthesis of 1, starting from enantiomerically pure acid 5 [32]. For the synthesis of amides 26–36, pentafluorophenyl esters could be used as protecting groups of the carboxylic acids in the first four steps and then exploited as activated esters for the reaction of intermediate 13 with the appropriate amines. Thus, the di-PFPester 6 was treated with MCPBA to give epoxide 8, which was
The design of novel substituted 1,2-mannobioside mimics and general structure of the bis-amide derivatives of 1.

Scheme 1 Synthetic scheme leading to amides 26–36 and to anilides 44–50. The structure of the R groups is shown in Table 1.

Opened with allylic alcohol under copper (II) triflate catalysis in almost quantitative yields. The resulting alcohol 10 was mannosylated with trichloroacetimidate 12 to give 13. This sequence afforded the scaffold 13 in four steps starting from diacid 5 with 37% overall yield. Treatment of 13 with 2.4 mol-equiv of the required amine in THF overnight afforded amides 16–25. The crude compounds after Isolute HM-N work-up (see experimental) were directly deprotected under Zemplen’s conditions to afford amides 26–36 (Note: amide 35 was obtained by NaOH hydrolysis of the tetra-O-benzoate 24).

Under the same conditions, reaction of 13 with aniline afforded 39% of mono-anilide products, along with 30% of recovered starting material. Thus, a different strategy was implemented for the synthesis of anilides 44–50, starting from the trimethylsilyl ester 7 (Scheme 1). This was elaborated as described above to afford the corresponding pseudo-disaccharide 14. Ester deprotection with trifluoroacetic acid in CH₂Cl₂ [51] afforded acid 15 which was transformed in anilides 37–43 by reaction with an excess of the appropriate aniline and 2 mol-equiv of O-((7-azabenzotriazolyl)-N,N′,N″-tetramethyluronium hexafluorophosphate (HATU) [52,53] following a carefully devised addition protocol (see experimental). The crude compounds after membrane filtration work-up (see experimental) were chromatographed and deprotected under Zemplen’s conditions to afford anilides 44–50.

Biological assay and interpretation of the results

The activity of ligand candidates was tested using an assay we recently described [25] which measures the ability of molecules to inhibit dendritic cells adhesion to mannan-coated...
candidates for IC 50 measurement. All synthesized compounds were tested in concentration 0.5 mM to assess their inhibition on immature dendritic cell adhesion (Fig. 5). The compounds that exhibited at least 50% inhibition of cell adhesion (50% of residual adhesion) at 0.5 mM concentration were tested for dose-response and their IC 50 values were determined. Based on this cut-off value, compounds 32 and 34–36 were not chosen for IC 50 determination. In addition, compounds 48–50 precipitated in the preliminary test (apparent on visual inspection), so these compounds were excluded from further testing.

IC 50 values were determined for compounds 26–36 and 44–47 (Table 2). The original binding data are reported as Supplementary Information. The highest inhibitory potency was observed for compound 28, although maximal inhibition observed for this compound was only 54% (46% adhesion). Similar, albeit slightly less potent, inhibition was observed for compounds 29, 31, 33 and 44–46. The results strongly indicate that the majority of the substituents selected were reasonable and have increased potency as DC-SIGN antagonists when compared to starting compound 1, which inhibited DC adhesion only weakly (Fig. 5). Some ligands were also found to be more potent than the pseudo-trisaccharide 2, up to 1 order of magnitude. Since amide 29 bears simple benzyl moieties and compounds 44–46 carry phenyl or 4-tolyl lipophilic groups (Table 1), we conclude that simple aromatic rings with or without small substituents are optimal for strong interaction with DC-SIGN. This strongly suggests that additional binding affinity to DC-SIGN may be attributed primarily to hydrophobic interactions and confirms our preliminary hypothesis. The assay used in this article [25] measures the influence of the antagonists on binding of DC to mannan-coated plates. Since immature dendritic cells can express various mannose binding lectins [56] it is difficult to distinguish the contributions of the different lectins to the adhesion process. However, the ability of the DC-SIGN specific antibody 1B10 to reduce adhesion by 80% suggests that DC-SIGN is a major determinant of the binding ability to mannan-coated plates, and therefore, the assay can serve as a preliminary screening. The most interesting candidates iden-

| Compound | IC 50 \( \mu \text{g/mL} \) | \( R^2 \) |
|----------|-----------------|-------|
| H200     | 6.095 ± 1.040   | 0.966 |
| 1B10     | 1.454 ± 0.651   | 0.967 |
| 2        | 299 ± 30.0      | 0.994 |
| 26       | 344 ± 10.4      | 0.999 |
| 27       | 339 ± 7.69      | 0.982 |
| 28       | 6.86 ± 0.24     | 0.988 |
| 29       | 29.5 ± 2.28     | 0.995 |
| 30       | 90.0 ± 1.87     | 0.967 |
| 31       | 44.2 ± 13.2     | 0.986 |
| 33       | 12.8 ± 3.42     | 0.982 |
| 44       | 12.5 ± 3.90     | 0.989 |
| 45       | 26.6 ± 0.72     | 0.999 |
| 46       | 45.8 ± 9.3      | 0.980 |
| 47       | 111 ± 2.10      | 0.971 |

\( ^* \) Maximal inhibition observed for compound 28 was 54% (46% adhesion)

plates (see experimental for details). To assess whether this assay identifies inhibitors of DC-SIGN-mediated adhesion, adhesion was measured in the presence of different concentrations of DC-SIGN specific antibodies, 1B10 and H200, and their IC 50 values were determined (Table 2) [54,55]. The results demonstrate that DC adhesion to the mannan-coated plate can be abolished by DC-SIGN specific antibodies (Supplementary data) and an IC 50 of 6.1 ± 1.0 µg/mL and 1.4 ± 0.6 µg/mL was determined for the two antibodies, respectively. In the presence of 1B10, a monoclonal blocking antibody, maximum inhibition of DC adhesion was high (up to 80%), confirming that the assay detects DC-SIGN specific antagonists. H200 is a polyclonal blocking antibodies (up to 80%), confirming that the assay detects DC-SIGN specific antibody 1B10 to reduce adhesion by 80% suggests that DC-SIGN is a major determinant of the binding ability to mannan-coated plates, and therefore, the assay can serve as a preliminary screening. The most interesting candidates iden-
Fig. 6 Crystal structure of DC-SIGN CRD (shown as solvent-accessible surface; Ca$^{2+}$ coordinated by Man1 residue is presented as a sphere) in complex with Man$_4$ (shown as lines) and top docked poses of compounds 28, 29, 31 and 44 (shown as tubes)

Proposed binding modes of the synthesized mannose-based DC-SIGN antagonists

The expected binding mode of 28, 29, 31 and 44 as predicted with FlexX molecular docking tool is shown in Fig. 6. Similar poses were obtained for all the other ligands analyzed, but no strong quantitative correlation was found between the calculated scores and the experimental inhibitory concentration. The conformations proposed by the FlexX show a markedly consistent binding mode for the mannose residue of all docked compounds identical to the Man1 residue in the crystal structure of DC-SIGN CRD (1SL4). Apart from Man1 residue, FlexX predicted two principal binding modes of the diamide part of the molecules:

- The first binding mode is depicted for compounds 28 and 29 (Fig. 6), where lipophilic residues stretch towards the hydrophobic cavity behind Phe 313 and the other points to the Arg345 residue (aminoacid residue at the right bottom of the figures, Fig. 6),
- The second binding mode is depicted for compounds 31 and 44, where both lipophilic residues ‘embrace’ Phe 313.

Both predicted binding modes include interaction with at least one of the targeted lipophilic sites and could explain increased affinity to DC-SIGN. Furthermore, the predicted binding modes indicate that further improvement in binding affinity might be obtained by exploiting additional interactions, for instance with Arg345 guanidine.

Conclusion

In the search for potent monovalent DC-SIGN antagonists, we have taken advantage of the known crystal structure of DC-SIGN CRD in complex with Man$_4$ tetramannoside (1SL4). After a careful examination of the DC-SIGN binding site, we identified two regions only partially exploited by native ligands which could be used to increase the potency of glycomimetic DC-SIGN antagonists. Upon this hypothesis, we designed and synthesized a small focused library of mannose glycoconjugate derivatives of the previously published DC-SIGN antagonist 1. The compounds were tested using a DC adhesion assay to mannan-coated plates and the results demonstrate that the majority of the new derivatives inhibit DC adhesion more potently, by up to 2 orders of magnitude, than the starting compound 1. Additionally, docking studies allowed qualitative rationalization of the results and suggested that the synthesized DC-SIGN antagonists occupy one or both predicted binding sites.
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