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Target Selectivity of FimH Antagonists

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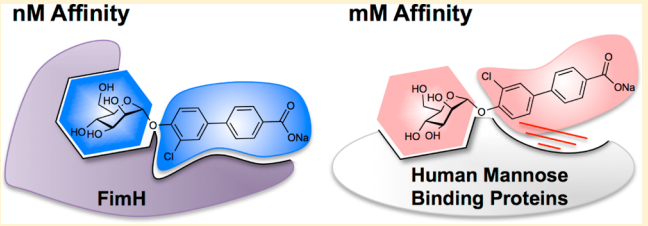
Supporting Information

ABSTRACT: Mannose-based FimH antagonists are considered new therapeutics for the treatment of urinary tract infections (UTIs). They prevent the adhesion of uropathogenic *Escherichia coli* (UPEC) to urothelial cell surfaces triggered by the lectin FimH, which is located at the tip of bacterial type 1 pili. Because all reported FimH antagonists are α-D-mannosides, they are also potential ligands of mannose receptors of the human host system. We therefore investigated the selectivity range of five FimH antagonists belonging to different compound families by comparing their affinities for FimH and eight human mannose receptors. On the basis of the detected selectivity range of approximately 5 orders of magnitude, no adverse side effects resulting from nonselective binding to the human receptors have to be expected. FimH antagonists can therefore be further considered as potential therapeutics for the treatment of UTI.



INTRODUCTION

Urinary tract infections (UTIs) are primarily caused by uropathogenic *Escherichia coli* (UPEC) (70−95% of cases) expressing type 1 pili.[1] At the tip of these pili, the lectin FimH is located. It enables the bacteria to adhere to oligomannosides of the glycoprotein uroplakin Ia (UPIa), which is located on uroepithelial cells.[2] This initial adhesion is a prerequisite for the infection to take place, because it prevents the rapid clearance of *E. coli* from the urinary tract by the bulk flow of urine and at the same time enables the invasion of the host cells.[2a,b] FimH antagonists, such as α-D-mannopyranosides, have been shown to interfere with the attachment of UPEC to their host cells, thus providing a novel therapeutic opportunity for prevention and treatment of UTIs as an alternative to antibiotics.[3] To date, several FimH antagonists have been investigated in vitro.[4] Furthermore, in vivo studies with methyl α-D-mannopyranoside,[5a] *n*-heptyl α-D-mannopyranoside (**1**, Figure 1),[5b,d] biphenyl α-D-mannopyranosides such as **2** and **3**,[5c−f] and indolinylphenyl α-D-mannopyranosides like **5**[5g] exhibited a considerable potential to reduce bacterial infections.

Target selectivity is of great concern in drug development and should be evaluated in the early stages of preclinical development.[6] Because all reported FimH antagonists are α-D-mannopyranosides and therefore also potential ligands for mannose receptors of the human host system, target selectivity of these FimH antagonists is a pivotal concern. Although various antagonists were already tested in vivo,[5] their target selectivity was not verified so far. Mammalian mannose receptors are present on many tissues throughout the whole body and are involved in numerous biological processes, such as cell−cell adhesion[7] and serum glycoprotein homeostasis.[8] They also intervene in the innate and the adaptive immune response by recognizing molecular patterns on pathogens.[7,9] Consequently, nonselective interactions of FimH antagonists with these various mannose receptors would have a profound impact on these processes and could cause severe side effects. A high selectivity of FimH antagonists is therefore of importance for a clinical application and should be evaluated in the early stages of preclinical development.

The majority of human mannose-binding lectins belong to the group of pathogen-recognition receptors (PRRs). Most PRRs are members of the C-type lectin superfamily.[10] They are either secreted as soluble plasma proteins or expressed as membrane-bound proteins on the surface of cells of the immune system such as macrophages, dendritic cells, or Langerhans cells. Secreted PRRs, such as the mannose binding protein (MBL)[11] and the lung surfactant protein D (SP-D),[12] bind to pathogens and simultaneously associate with cell surface receptors, triggering signaling pathways such as the lectin complement activation pathway, which results in enhanced phagocytosis of the pathogens as well as activation of the host defense system.[13] MBL and SP-D belong to the collectin family and share a similar collagen-like domain connected to the C-terminal C-type lectin domain, which contains the carbohydrate recognition domain (CRD). They consist of homotrimers, which oligomerize with 2−6 other trimers, forming high molecular weight complexes.[14] Transmembrane PRRs, which are classified into type I and type II C-type lectins, are also involved in the phagocytosis of pathogens, leading to their elimination or their processing for antigen presentation.[15] The type I C-type lectins such as the macrophage mannose receptor (MMR)[9] contain multiple CRDs within a single polypeptide backbone. In contrast, the type II C-type lectins such as langerin,[16] DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN),[17] DC-specific ICAM-3-

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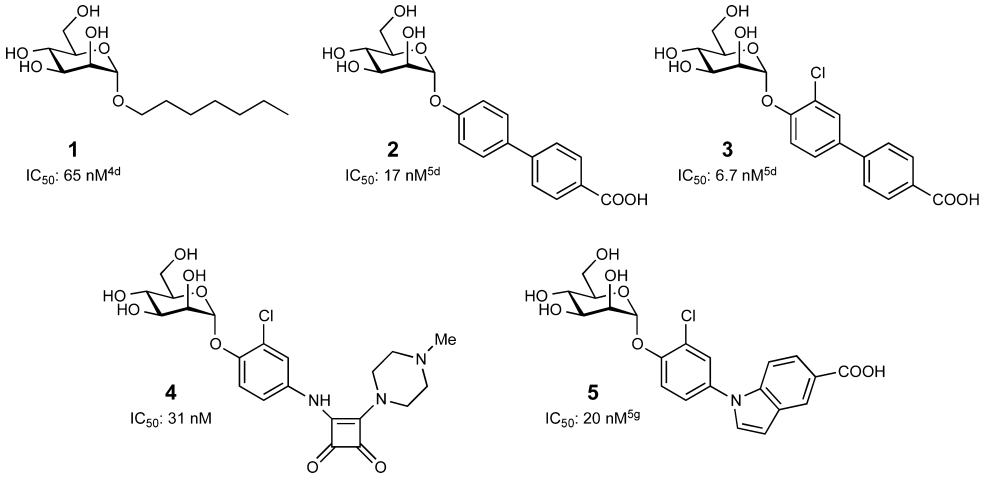


Figure 1. FimH antagonists tested for their selectivities for various human mannose-binding lectins: *n*-heptyl α-D-mannopyranoside (**1**),[4d] biphenyl α-D-mannopyranoside derivatives **2** and **3**,[5d] squaric acid derivative **4**, and indolylphenyl derivative **5**.[5g] IC_{50} values for FimH were obtained by a competitive binding assay.[4d]

grabbing nonintegrin related (DC-SIGNR),[18] dectin-2,[19] and dendritic cell lectin (DLEC)[20] exhibit only a single CRD. However, by formation of homomultimers, type II C-type lectins can greatly enhance their binding affinity. This was shown for the trimeric langerin[21] and tetrameric DC-SIGN and DC-SIGNR.[19] The multimeric arrangement of the CRDs further supports the discrimination between innate and extrinsic carbohydrate epitopes.[22]

For defense mechanisms against a broad range of microorganisms, human mannose-binding receptors require highly specific binding. Thus, MMR exhibits a preference for branched sugars with terminal D-mannose, L-fucose, or *N*-acetyl-D-glucosamine moieties that are specifically expressed on mycobacteria and fungi.[9a] Dectin-2 selectively binds to high mannose structures predominantly expressed on the surfaces of yeast and fungi,[23] whereas DC-SIGN recognizes high mannose oligosaccharides and Lewis blood group antigens such as Lewis^x or Lewis^a, found on mycobacteria, some viruses (e.g., HIV), and fungi.[18,24]

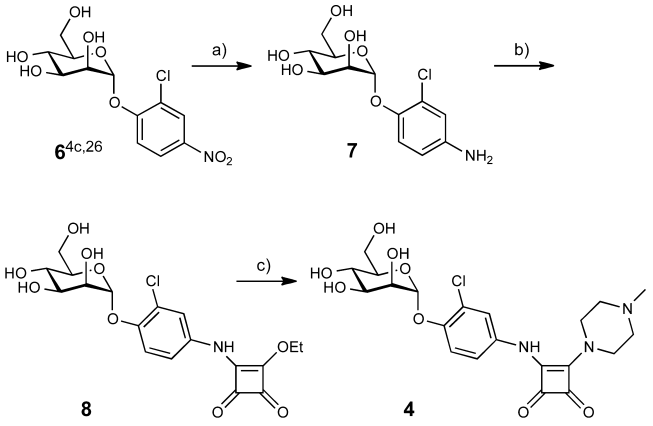
To ensure that FimH antagonists do not cause any adverse side effects due to nonselective binding to human mannose receptors, their selectivity profile to eight different PRRs was established. Nonselective binding may also have a profound impact on the serum half-life of FimH antagonists, since binding to PRRs often triggers endocytosis and would result in their elimination from circulation. To address this selectivity issue, we tested five mannose-based FimH antagonists with diverse aglycones (alkyl, biphenyl, squaric acid, and indolinylphenyl derivatives) for their binding affinity to various mannose-binding lectins (MBL, SP-D, MMR, DC-SIGN, DC-SIGNR, langerin, dectin-2, and DLEC).

RESULTS AND DISCUSSION

With a competitive binding assay,[4d] five high-affinity FimH antagonists belonging to different compound families (Figure 1, **1**−**5**[4d,5d,g]) were tested for their selectivity for eight human mannose receptors.

Synthesis of FimH Antagonists. *n*-Heptyl α-D-mannopyranoside (**1**),[25] the biphenyl α-D-mannopyranosides **2**[5d] and **3**,[5d] and the indolylphenyl α-D-mannopyranoside **5**[5g] were synthesized as previously reported. The synthesis of FimH antagonist **4** (Scheme 1) started from nitrophenyl mannoside **6**, which is

Scheme 1[a]



[a]Reagents and conditions: (a) H_2 (1 atm), cat. PtO_2, morpholine, MeOH, 45 min. (b) Diethyl squarate, MeOH, 1 day, 61% (2 steps).[4c] (c) *N*-Methylpiperazine, DIPEA, MeOH, 18 h, 90%.

easily available from peracetylated D-mannose.[4c,26] Because the reported procedure[4c] for the hydrogenation to aniline **7** using palladium on charcoal as a catalyst resulted in a substantial loss of the chloro substituent, platinum dioxide in the presence of morpholine was applied.[27] The mannosylated ethyl squarate **8** was then obtained in analogy to Sperling et al.[4c] Finally, treatment of ester **8** with *N*-methylpiperazine yielded amide **4** in 90%, which was ready for biological testing.

Binding Assays. The cell-free competitive binding assay[4d] is based on the interactions of a biotinylated polyacrylamide (PAA) glycopolymer [Manα1−3(Manα1−6)Manβ1−4Glc*N*Acβ1−4Glc*N*Acβ-PAA, TM-PAA] with the mannose receptors. Complexation of the biotinylated glycopolymer with streptavidin coupled to horseradish peroxidase allows for the quantification of the binding potencies of the tested FimH antagonists.

For our selectivity study, two parameters, the protein concentration and the TM-PAA concentration, were optimized to obtain comparable optical densities (ODs) for the different lectins in the competitive binding assay (Figure 2 and Table 1). Because of distinctive coating properties of the proteins, different protein concentrations (2.5−20 μg/mL) were

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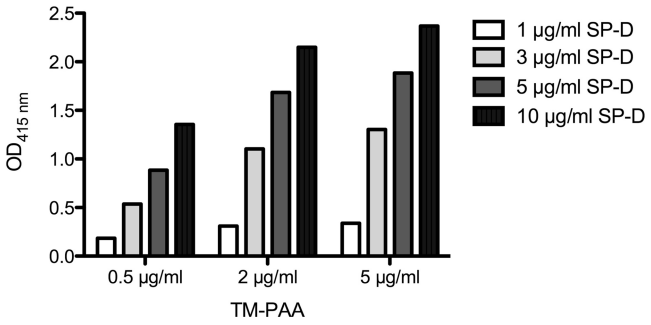

Figure 2. Assay development and optimization on the example of the human lectin SP-D. Different concentrations of coated SP-D (1−10 μg/mL) in combination with various TM-PAA concentrations (0.5−5 μg/mL) were tested.

Table 1. Optimized Protein and TM-PAA Concentrations Used in the Competition Assays for Each Individual Lectin

	μg/mL		
protein	[protein]	[TM-PAA]	$OD_{415\ nm}$
FimH	20	0.25	2.20
MBP	10	5	1.82
SP-D	5	5	1.88
MMR	5	2	2.03
langerin	10	2	2.12
dectin-2	10	5	1.92
DLEC	10	5	1.93
DC-SIGN	2.5	1	2.15
DC-SIGNR	10	5	2.10

necessary to obtain comparable levels of immobilization. The TM-PAA concentration in turn required adaptation due to different affinities of the various lectins. Because of the multivalent oligosaccharide presentation, the affinity of the polymer is expected to be higher than the affinity of the corresponding free oligosaccharide. We therefore used low TM-PAA concentrations between 0.5 and 5 μg/mL, which correspond to 16−160 μM TM-PAA assuming a molecular mass of approximately 30 kDa. As a representative example, the results of this optimization process for the lectin SP-D are summarized in Figure 2. Briefly, when 5 μg/mL SP-D was used for the immobilization step, an OD_{415nm} of approximately 2 was obtained with 5 μg/mL TM-PAA. The protein and polymer concentrations leading to comparable ODs for the other investigated lectins are summarized in Table 1.

For the competitive binding assays, concentrations of 1 mM antagonists and 50 mM D-mannose (positive control) were used. The results are summarized in Figure 3. The ODs obtained in the absence of an antagonist were set to 100% TM-PAA binding, the background in the absence of the polymer to 0% TM-PAA binding. D-Mannose showed a strong inhibition of binding for all proteins at a concentration of 50 mM (more than 90% inhibition). As expected, at a concentration of 1 mM, the antagonists **1**−**5** strongly inhibited binding of the polymer to FimH, whereas none of the antagonists showed relevant inhibition potencies for the tested human lectins. The highest inhibition of TM-PAA binding was observed for the antagonists **3** (54%) and **5** (58%) to MMR, for compound **3** (63%) to langerin, and for compound **2** (50%) to DLEC (indicated by asterisks, Figure 3). On the basis of their ODs, the IC_{50} value (concentration at 50% inhibition) of these antagonists can be estimated to be in the order of 1 mM. As compared to the low nanomolar FimH affinities of the five investigated antagonists (Figure 1), the affinities for the human lectins are at least 5 orders of magnitude lower, indicating an excellent selectivity margin for a therapeutic application of these FimH antagonists.

Infection studies in a mouse disease model, using compounds **1**−**3**, were previously reported.[5d] In these studies, a high dose of 50 mg/kg was applied, resulting in a substantial reduction of the bacterial infection caused by UPEC UTI89 [reduction of the colony-forming units (CFU) in the urine by 2 orders of magnitude and in the bladder by 4 orders of magnitude]. Furthermore, the in vivo pharmacokinetic parameters were determined, including the maximal plasma concentrations (C_{max}) of the FimH antagonists after iv application. Maximal concentrations of 39 μg/mL for **1**, 35 μg/mL for **2**, and 39 μg/mL for **3** were detected in blood samples, which correspond to C_{max} values of 144, 80, and 97 μM, respectively. Low binding to mammalian mannose receptors is expected even at these concentrations, since the IC_{50} values of the antagonists **1**−**3** for the eight tested human mannose receptors are approximately 10-fold higher than the detected maximal blood concentrations in treated mice. Furthermore, with improved antagonists like indolylphenyl derivative **5**,[5g] the dose of 50 mg/kg could be reduced to 1 mg/kg, thus additionally increasing the selectivity margin.

Binding affinities of various human mannose receptors to monosaccharides, such as D-mannose, L-fucose, and D-galactose, have already been characterized in previous studies. Monovalent sugars showed only weak binding affinities in the millimolar range toward DC-SIGN,[18] DC-SIGNR,[28] dectin-2,[23] langerin,[21] or MMR.[29] The functional affinity to carbohydrates necessary for pathogen capturing is predominantly an effect of avidity, caused by the combined strength of multiple interactions with ligands. The presentation of multivalent carbohydrates on the pathogen surface and the multimerization and/or clustering of the receptors on the host cells greatly support binding between the interaction partners. Therefore, multivalent presentations of α-D-mannosidic antagonists[4a,30] might be prone to cause severe side effects due to strong binding to human mannose receptors.

CONCLUSION

On the basis of the presented data, adverse side effects resulting from nonselective binding of monovalent FimH antagonists to the investigated mannose-binding lectins are not considered to be a critical issue for their potential therapeutic application to treat UTI. Although this selection does not cover the entire mammalian mannose-binding proteins, it represents the most abundant and best-characterized receptors expressed in various tissues. The 10^5-fold lower affinity for the tested human receptors as compared to the bacterial FimH lectin confirms a high selectivity safety range. This primarily results from the fact that the investigated FimH antagonists were optimized by introducing hydrophobic substituents at their reducing end, enabling the interaction with the tyrosine gate, the entrance to the ligand-binding site, which is a unique feature of FimH.[31] Furthermore, because of the importance of multivalent ligand presentation in nature, monovalent α-D-mannopyranosides in general can be considered to exhibit only low affinities to human mannose receptors.

EXPERIMENTAL SECTION

General Methods. Commercially available reagents were purchased from Sigma-Aldrich or Acros. Methanol (MeOH) was

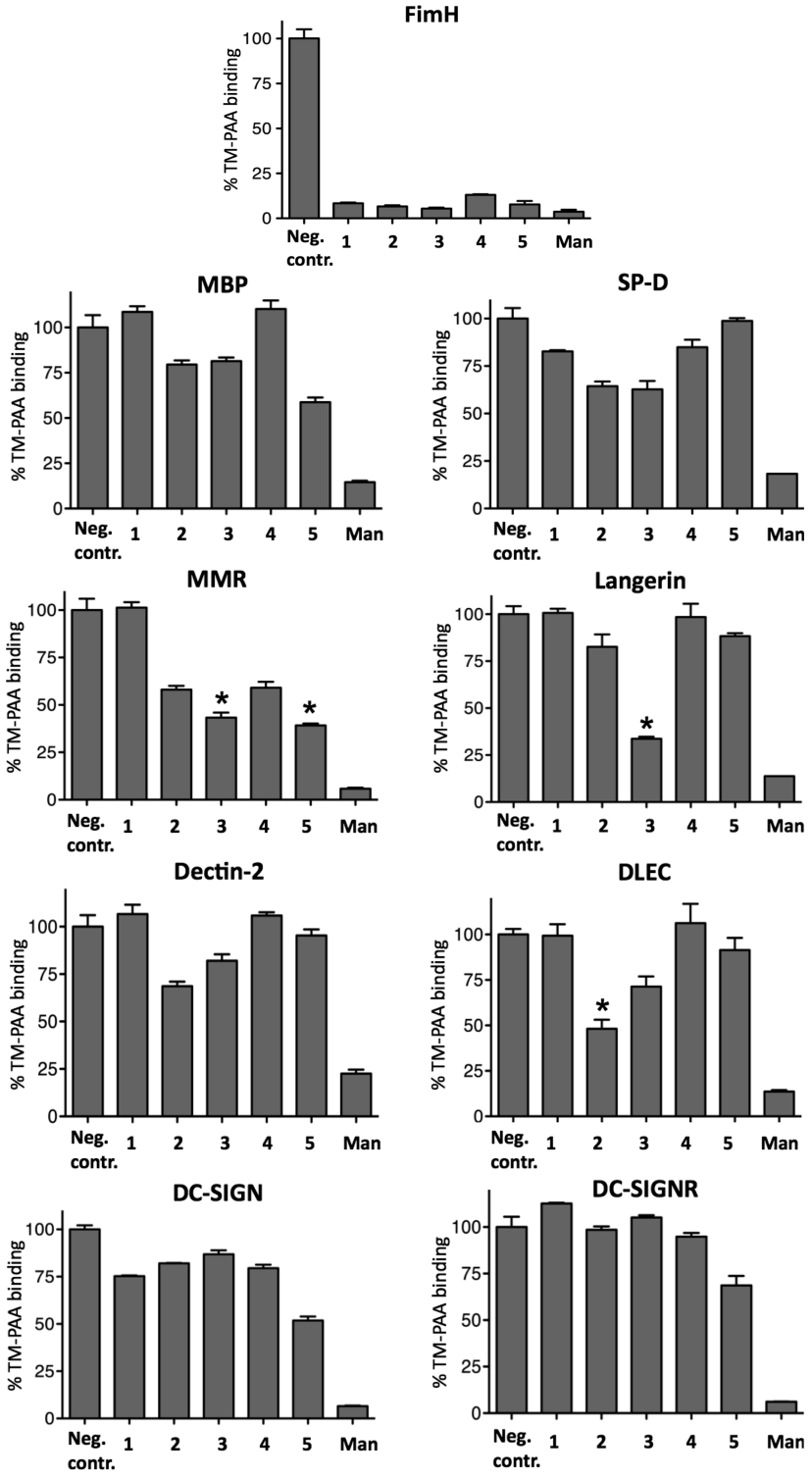

Figure 3. Selectivity profile of FimH antagonists **1**−**5**. Competitive binding assays with FimH, MBP, SP-D, MMR, langerin, dectin-2, DLEC, DC-SIGN, and DC-SIGNR to evaluate the selectivity of compounds **1**−**5**. Inhibitory capacities of the compounds were tested at a concentration of 1 mM. D-Mannose (Man) served as a positive control (50 mM). The binding signals of TM-PAA to the proteins in absence of antagonists were set to 100%, and background signals were set to 0%. Asterisks indicate inhibition of TM-PAA binding by 50% or more. The assays were performed in triplicate.

dried by distillation from sodium methoxide. Optical rotation was measured at 20 °C on a Perkin-Elmer 341 polarimeter. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance 500 UltraShield spectrometer at 500.13 MHz (^{1}H) or 125.76 MHz (^{13}C). Chemical shifts are given in ppm and were calibrated on residual solvent peaks. Assignment of the ^{1}H and ^{13}C NMR spectra was achieved using 2D methods (COSY and HSQC). Electron spray ionization mass spectra (ESI-MS) were recorded on a Waters micromass ZQ instrument. High-resolution mass spectra (HR-MS) were obtained on an ESI Bruker Daltonics micrOTOF spectrometer equipped with a TOF hexapole detector. Reactions were monitored by TLC using glass plates coated with silica gel 60 F_{254} and visualized by

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using UV light and/or by charring with a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4) with heating to 150 °C for 5 min. MPLC separations were carried out on a CombiFlash Rf from Teledyne Isco equipped with RP-18 reversed-phase flash columns. LC-MS separations were done on a Waters system equipped with a Waters SunFire C_{18} OBD (5 μm, 19 mm × 150 mm) column, sample manager 2767, pump 2525, PDA 2525, and micromass ZQ mass spectrometer.

Compound Purity. The test compounds **1**−**3** and **5** were purified by reversed-phase chromatography (RP-18 column, gradient of MeOH in H_2O, compound **1**−**3**[5d,25]) or chromatography on silica (DCM/MeOH/H_2O, compound **5**[5g]) followed by Bio-Gel P2 (exclusion limit 1800 Da, Bio-Rad Laboratories) size exclusion chromatography (elution with water containing up to 20% MeOH at 0.25 mL/min) prior to HPLC, HR-MS, NMR, and activity testing. Compound **4** was purified by preparative LC-MS (Waters SunFire C_{18} OBD column, H_2O/MeCN + 0.2% HCO_2H). The purity of all test compounds was determined by NMR and HPLC to be ≥95% [method A (compounds **2**, **3**, and **5**): Beckman Coulter Gold, consisting of pump 126, DAD 168 (190−410 nm), and autosampler 508; column, Waters Atlantis T3 (3 μm, 2.1 mm × 100 mm); A, H_2O + 0.1% TFA; B, MeCN + 0.1% TFA; detection, 254 or 270 nm; gradient, 5% B → 95% B (22 min); and flow rate, 0.5 mL/min. Method B (compounds **1** and **4**): Agilent 1100/1200 with UV detector (190−410 nm) and ELSD; column, Waters Atlantis T3 (3 μm, 2.1 mm × 100 mm); A, H_2O + 0.1% TFA; B, H_2O/MeCN (90:10) + 0.1% TFA; gradient, 5% B (1 min), 5% B → 70% B (15 min), 70% B (1 min), 70% B → 5% B (3 min); flow rate, 0.5 mL/min; and detection, 254 nm or ELSD]. For the ^{1}H NMR spectrum and HPLC trace of compound **4**, see the Supporting Information.

4-Amino-2-chlorophenyl α-D-Mannopyranoside (7). A suspension of **6**[4c,26] (430 mg, 1.28 mmol), morpholine (30 μL), and PtO_2 (50 mg) in MeOH (20 mL) was hydrogenated (1 atm H_2) for 45 min. Then, the mixture was filtered and concentrated in vacuo to give crude **7** (443 mg) as a colorless oil, which contained approximately 15% morpholine and was used in the next step without further purification. ^{1}H NMR (500 MHz, CD_3OD): δ 3.71−3.81 (m, 3H, H-4, H-5, H-6a), 3.83 (dd, *J* = 1.7, 12.7 Hz, 1H, H-6b), 3.95 (dd, *J* = 3.4, 9.1 Hz, 1H, H-3), 4.11 (dd, *J* = 1.8, 3.4 Hz, 1H, H-2), 5.27 (d, *J* = 1.7 Hz, 1H, H-1), 6.61 (dd, *J* = 2.7, 8.7 Hz, 1H, C_6H_3), 6.78 (d, *J* = 2.7 Hz, 1H, C_6H_3), 7.09 (d, *J* = 8.7 Hz, 1H, C_6H_3). ^{13}C NMR (125 MHz, CD_3OD): δ 62.6 (C-6), 68.4 (C-4), 71.9 (C-2), 72.3 (C-3), 75.7 (C-5), 102.1 (C-1), 115.7, 117.4, 121.2, 153.2 (6C, C_6H_3). ESI-MS: *m*/*z* calcd for $C_{12}H_{17}ClNO_6$ $[M + H]^+$, 306.1; found, 306.0.

2-Chloro-4-[(2-ethoxy-3,4-dioxocyclobuten-1-yl)amino]-phenyl α-D-Mannopyranoside (8).[4c] To a solution of **7** (443 mg) in MeOH (15 mL) was added diethyl squarate (379 μL, 2.56 mmol) under argon, and the reaction mixture was stirred at rt for 1 d. Then, the solvent was removed in vacuo, and the residue was purified by MPLC on RP-18 (H_2O/MeOH) to yield **8** (337 mg, 61% from **6**) as a yellow solid. ^{1}H NMR (500 MHz, CD_3OD): δ 1.47 (t, *J* = 7.1 Hz, 3H, OCH_2CH_3), 3.66 (dt, *J* = 3.7, 9.9 Hz, 1H, H-5), 3.75 (d, *J* = 3.7 Hz, 2H, H-6), 3.79 (t, *J* = 9.8 Hz, 1H, H-4), 4.00 (dd, *J* = 3.4, 9.6 Hz, 1H, H-3), 4.16 (dd, *J* = 1.8, 3.3 Hz, 1H, H-2), 4.81 (q, *J* = 7.1 Hz, 2H, OCH_2CH_3), 5.56 (d, *J* = 1.5 Hz, 1H, H-1), 7.23 (m, 1H, C_6H_3), 7.32 (d, *J* = 9.0 Hz, 1H, C_6H_3), 7.47 (s, 1H, C_6H_3). ^{13}C NMR (125 MHz, CD_3OD): δ 15.7 (OCH_2CH_3), 61.4 (C-6), 67.2 (C-4), 70.8 (C-2), 71.4 (C-3), 71.6 (OCH_2CH_3), 74.9 (C-5), 100.0 (C-1), 119.2, 120.2, 122.4, 124.7, 134.6, 152.5 (C_6H_3), 168.9, 176.2 (C═C), 183.3, 186.9 (2 CO). ESI-MS: *m*/*z* calcd for $C_{18}H_{21}ClNO_9$ $[M + H]^+$, 430.1; found, 430.1.

2-Chloro-4-[(2-(4-methylpiperazin-1-yl)-3,4-dioxocyclobut-en-1-yl)amino]phenyl α-D-Mannopyranoside (4). Compound **8** (72.5 mg, 0.169 mmol) was dissolved in MeOH (7.5 mL) at 50 °C. After it was cooled to rt, *N*-methylpiperazine (28.0 μL, 0.252 mmol) and diisopropyl-ethylamine (DIPEA) (145 μL) were added, and the reaction mixture was stirred for 18 h at rt. Then, the solvent was removed in vacuo, and the residue was purified by LC-MS (RP-18, H_2O/MeCN + 0.2% HCO_2H) to give **4** (73.9 mg, 90%) as a white powder after a final lyophilization from H_2O/dioxane. $[\alpha]_D$ +74.5 (*c* 1.00, MeOH). ^{1}H NMR (500 MHz, CD_3OD): δ 2.47 (s, 3H, NCH_3), 2.76 (m, 4H, 2 CH_2), 3.66 (ddd, *J* = 2.1, 5.6, 9.8 Hz, 1H, H-5), 3.71 (dd, *J* = 5.6, 11.8 Hz, 2H, H-6a), 3.74 (t, *J* = 9.7 Hz, 1H, H-4), 3.79 (dd, *J* = 2.1, 11.8 Hz, 2H, H-6b), 3.88 (m, 4H, 2 CH_2), 3.96 (dd, *J* = 3.4, 9.4 Hz, 1H, H-3), 4.09 (dd, *J* = 1.8, 3.3 Hz, 1H, H-2), 5.48 (d, *J* = 1.4 Hz, 1H, H-1), 7.15 (dd, *J* = 2.7, 8.9 Hz, 1H, C_6H_3), 7.33 (d, *J* = 8.9 Hz, 1H, C_6H_3), 7.35 (d, *J* = 2.7 Hz, 1H, C_6H_3). ^{13}C NMR (125 MHz, CD_3OD): δ 45.4 (NCH_3), 47.4 (2C, 2 CH_2), 55.0 (2C, 2 CH_2), 62.7 (C-6), 68.2 (C-4), 71.8 (C-2), 72.3 (C-3), 76.0 (C-5), 101.2 (C-1), 119.0, 121.6, 123.8, 125.0, 135.1, 150.2 (C_6H_3), 164.9, 169.2 (C═C), 183.3, 185.9 (2 CO). HR-MS: *m*/*z* calcd for $C_{21}H_{26}ClN_2NaO_8$ $[M + Na]^+$, 506.1306; found, 506.1303.

Expression and Purification of DC-SIGN CRD-Fc and DC-SIGNR CRD-Fc. Plasmids containing the full-length cDNA of DC-SIGN and DC-SIGNR were kindly provided by Daniel A. Mitchell, Clinical Sciences Research Institute, Warwick Medical School (Coventry, United Kingdom). Standard molecular techniques[32] were used for the cloning of the CRD of DC-SIGN (DC-SIGN CRD; aa residues 250−404, GenBank accession no. M98457) and DC-SIGNR (DC-SIGNR CRD; aa residue 262−398, GenBank accession no. Q9H2x3). The DC-SIGN/DC-SIGNR CRD encoding inserts were amplified by PCR using primers containing the restriction sites *Eco*RI and *NcoI*, respectively. The insert was ligated into the corresponding cloning site of the pFUSE-hIgG2-Fc2 expression vector (Invivogen, Toulouse, France). The constructs were amplified in chemocompetent DH5α *E. coli* (Novagen, Lucerne, Switzerland), and their correctness was confirmed by DNA sequencing.

CHO-K1 cells (American Type Culture Collection No. CCL-61) were cultivated in Ham's Nutrient Mixture F-12 supplemented with 2 mM L-glutamate (Invitrogen, Paisley, United Kingdom), 10% fetal calf serum (FCS, Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (both Sigma-Aldrich, Basel, Switzerland). The CHO-K1 cells were transfected with the DC-SIGN CRD or DC-SIGNR CRD expression vector using the FuGENE HD transfection reagent (Roche Applied Science, Rotkreuz, Switzerland). Stably transfected CHO-K1 cells were selected by treatment with Zeocin (0.5 μg/mL, Invitrogen), and single clones were obtained by limiting dilution. For protein production, the cells were cultivated as described above, and the culture medium containing the secreted DC-SIGN CRD-Fc and DC-SIGN CRD-Fc chimera was harvested weekly. Purification of the recombinant protein was achieved by applying conditioned medium on a protein A-sepharose column (BioVision, Mountain View, CA) attached to a fast protein liquid chromatography apparatus [BioLogic (FPLC) system, BioRad, Reinach BL, Switzerland], with loading buffer I [20 mM Tris/HCl, pH 7.6, 150 mM NaCl, and 0.05% (v/v) Tween-20]. The protein was eluted with elution buffer I (0.5 M acetic acid/ammonium acetate, pH 3.4). The collected protein was further purified on a L-fucose-sepharose column (prepared in house) using loading buffer II (20 mM Tris/HCl, pH 7.8, 0.5 M NaCl, and 25 mM $CaCl_2$) and elution buffer II (20 mM Tris/HCl, pH 7.8, 0.5 M NaCl, and 2 mM EDTA). For long-term storage, the protein was stored at −80 °C.

FimH, Human Langerin, DLEC, SP-D, Mannose Binding Protein (MBP), Dectin-2, and MMR. The FimH CRD linked with a thrombin cleavage site to a 6His-tag (FimH-CRD-Th-6His) was expressed in *E. coli* strain HM125 and purified by affinity chromatography as described in Rabbani et al.[4d] Human langerin, DLEC, SP-D, MBP, dectin-2, and MMR were purchased from R&D systems (Minneapolis, MN).

Competitive Binding Assay. Biotinylated trimannose (TM)-PAA polymer (20 μL, 1 mg/mL, Lectinity, Moscow) was mixed with 80 μL of assay buffer (20 mM HEPES, 150 mM NaCl, and 10 mM $CaCl_2$, pH 7.4), 20 μL of FCS, and 80 μL of streptavidin−horseradish peroxidase conjugate (500 U/mL, Roche, Mannheim, Germany) and incubated for 2 h at 37 °C. The complex was stable for several weeks when stored at 4 °C.

For assay development FimH, DC-SIGN, DC-SIGNR, MBP, langerin, DLEC, SP-D, dectin-2, and MMR were each diluted in assay buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, and 10 mM

$CaCl_2$) at concentrations of 1, 3, 5, and 10 μg/mL and were coated on microtiter plates (F96 MaxiSorp, Nunc, Langenselbold, Germany) with 100 μL/well overnight at 4 °C. The coating solution was discarded, and the wells were blocked with 200 μL/well of a 3% bovine serum albumin (BSA) solution in assay buffer for 2 h at 4 °C. After three washing steps with assay buffer, the streptavidin−peroxidase-coupled TM-PAA polymer (0.5−5 μg/mL) in assay buffer (50 μL/well) was added to the wells. The plates were incubated for 3 h at 25 °C and 350 rpm and then washed four times with assay buffer. After the addition of 100 μL/well of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate (Invitrogen), the colorimetric reaction was allowed to develop for 4 min and then stopped by adding 100 μL/well of 2% aqueous oxalic acid. The OD was measured at 415 nm on a microplate reader (Spectramax 190, Molecular Devices, CA).

For measuring the binding properties of FimH antagonists to the mannose binding receptors, a mix (total volume 100 μL/well) of the test compounds **1**−**5** (final concentration 1 mM) or α-D-mannose (final concentration 50 mM) and the streptavidin−peroxidase-coupled TM-PAA polymer in assay buffer (final concentration see Table 1) was added to the protein-coated wells and developed as described above.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

HPLC trace and ^{1}H NMR spectrum for compound **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); BSA, bovine serum albumin; CFU, colony-forming units; CRD, carbohydrate recognition domain; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin; DC-SIGNR, DC-specific ICAM-3-grabbing nonintegrin related; DLEC, dendritic cell lectin; DIPEA, diisopropyl-ethylamine; FCS, fetal calf serum; MBP, mannose binding protein; MMR, macrophage mannose receptor; OD, optical density; PAA, polyacrylamide; TM-PAA, Manα1−3(Manα1−6)Manβ1−4GlcNAcβ1−4GlcNAcβ-PAA; PRR, pathogen-recognition receptors; SP-D, lung surfactant protein D; UPEC, uropathogenic *Escherichia coli*; UPIa, uroplakin Ia; UTI, urinary tract infection

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