Drug Discovery

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A LecA Ligand Identified from a Galactoside-Conjugate Array Inhibits Host Cell Invasion by *Pseudomonas aeruginosa***

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Abstract: *Lectin LecA is a virulence factor of Pseudomonas aeruginosa involved in lung injury, mortality, and cellular invasion. Ligands competing with human glycoconjugates for LecA binding are thus promising candidates to counteract P. aeruginosa infections. We have identified a novel divalent ligand from a focused galactoside(Gal)-conjugate array which binds to LecA with very high affinity ($K_d = 82$ nM). Crystal structures of LecA complexed with the ligand together with modeling studies confirmed its ability to chelate two binding sites of LecA. The ligand lowers cellular invasiveness of P. aeruginosa up to 90% when applied in the range of 0.05–5 μM. Hence, this ligand might lead to the development of drugs against P. aeruginosa infection.*

Pathogens generally initiate their infection by using host-specific epitopes for adhesion and penetration. Frequently, the host-specific marker is a glycoconjugate that is recognized by a lectin present on the pathogen surface.[1] In the case of *P. aeruginosa*, the structural basis for the preference of the LecA lectin towards galactose and globotriaosylceramide (Gb3) was elucidated.[2,3] It was demonstrated in a *P. aeruginosa* model of infection that the lectin is involved in lung injury and mortality[4–7] and recent data suggested that LecA promotes bacterial invasion into host cells.[7] The lectin is a tetramer and structural data demonstrated an overall rectangular shape with pairing of neighboring binding sites separated by ca. 30 Å. This structural knowledge has stimulated intense work to design templates presenting galactose residues with a spacing that matches the dimeric geometry of LecA neighboring binding sites[8–16] with the potential of acting as anti-pathogenic agents[17,18] or disrupting biofilm formation.[9,15] These studies clearly highlighted the potential benefit of oligovalent interactions since dissociation constants lower than 90 nM and 20 nM were obtained for tetravalent[11,16] and divalent[13] compounds, respectively. Nevertheless, a more systematic investigation in the linker, including more chemical diversity, had not been carried out. Furthermore, no ligand has thus far been shown to compromise host invasion.

In an independent work, we reported a technology to rapidly access heteroglycoconjugate libraries emulating the architectures of complex glycan with additional points of diversity that included aromatic substituents probing beneficial secondary interactions.[19] A profile of the lectin LecA from *P. aeruginosa* revealed specific interactions that served as the lead for a focused library (Figure 1). This library was designed based on the selectivity of LecA for galactose, on the ca. 30 Å distance between the two adjacent binding sites, and on the observed benefit of an aryl substituent in the linker. Following these considerations, the library included the following elements: four different branching geometries and lengths (R1.2–5) as well as the unbranched monovalent control (R1.1); five different spacer groups with different lengths and geometries (R2.1–5); the four permutations of 1,2- 1,3- 1,4- and 1,6-α-galactose disaccharide (R3.2–5) in addition to the β-thioarylgalactose (R3.1); five different aryl groups in the linker moiety. This afforded a library of 625 unique monovalent and divalent glycans with a connectivity ranging from 28 to 47 atoms between the anomeric carbons of the terminal galactoses and diverse conformational constrains. The library was synthesized using peptide nucleic acid (PNA)-encoded synthesis (see Figure 1 for the structure of fragments).[19] The quality of each synthetic step within the library was verified by analysis of the cleavage product from an aliquot of resin (see the Supporting Information (SI) for full details of synthesis and analysis).

The library was hybridized at 2.5 μM to a microarray containing 24 copies of each DNA sequence for statistical analysis of binding data. At this concentration, each DNA spot on the array is saturated with the ligand. Screening for the preferred ligand for LecA at concentrations from 50 to

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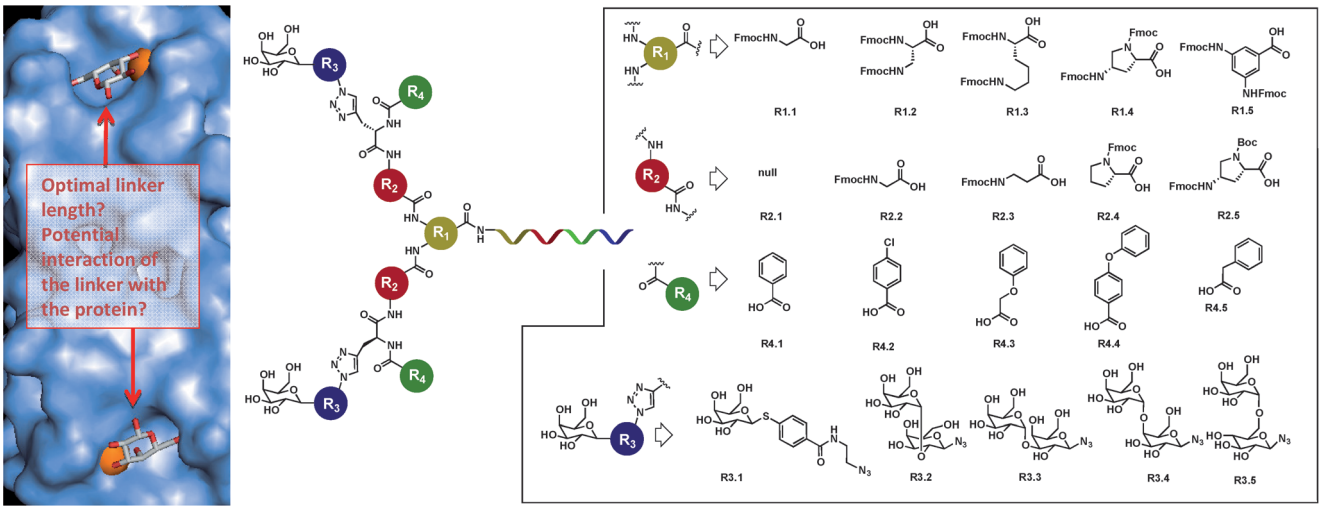

Figure 1. Design of a focused library of ligands bridging the neighboring binding sites of LecA. Left: Image generated from pdb ID: 1OKO. Right: Structure of the library component.

1.8 nM using 3-fold dilution gave a saturated signal for the best ligand down to 10 nM. At 5.6 and 1.8 nM of LecA, a single compound clearly stood out as the fittest ligand (1.3–2.5–3.1–4.3; see Figure 2 for array image and Figure 3 for titration). The signal corresponding to this compound had a standard deviation of less than 3% across the 24 spots providing reliable binding accuracy. This profile suggested a clear binding preference for a unique linker combination with the phenoxyacetate group and β-thiophenyl galactose. For comparison, the B-subunit of Shiga toxin which also binds to Gb3,[20] was profiled with this focused library. While a much higher concentration was required to obtain binding (200 nM), a clear preference was obtained for a different linker combination with 4-phenoxy benzoate and α1–4-linked digalactoside.

In order to assess the contribution of the lectin interacting with a ligand hybridized to different DNA strands rather than a unique divalent ligand (Figure 4), the concentration of ligand hybridized on the array was reduced and the relative intensity of the fittest divalent ligand (1.3–2.5–3.1–4.3) was compared to the monomeric version (1.1–2.5–3.1–4.3). As shown in in Figure 4, as the concentration of the ligand on the surface is reduced (hybridizing from 2.5 to 0.03 μM of library, 3-fold dilution), the signal ratio between the fittest divalent ligand and its monomeric version increases. This observation is consistent with the speculation that at high concentration, the ligand density is sufficiently high for the lectin to interact with multiple ligands across different hybridization sites.[21] At lower ligand concentration and hence surface density, the binding to the divalent ligand is preferred compared to the

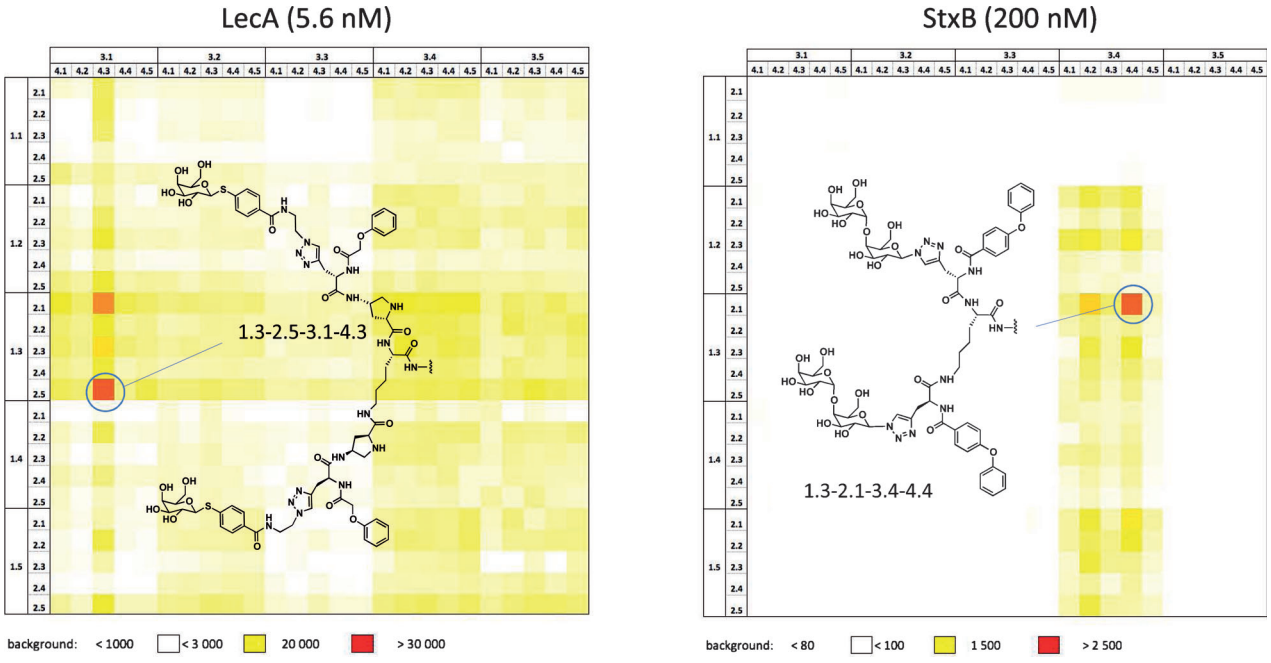

Figure 2. Binding profile of LecA (left) and the B-subunit of Shiga toxin (StxB) (right).

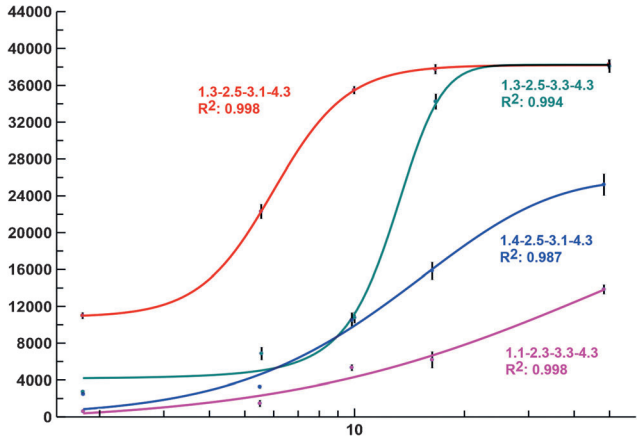

Figure 3. Plot of fluorescence intensities (*y*-axis) measured at different LecA concentrations (*x*-axis, nM, log scale) for four related compounds.

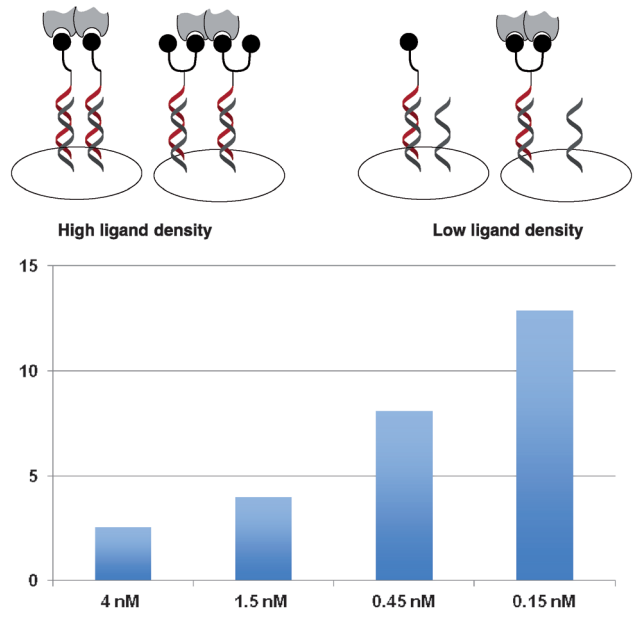

Figure 4. Top: Schematic representation of oligomeric protein interacting with monovalent versus divalent ligands on the array surface (top). LecA is tetrameric protein with two pairs of binding sites on opposite sides. For clarity, only half of the tetrameric protein with the two binding sites on the same face is depicted. Bottom: Intensity ratio between divalent ligand (1.3–2.5–3.1–4.3) and monovalent ligand (1.1–2.5–3.1–4.3) at decreasing ligand density (concentration of PNA used in the hybridization is the concentration for a single PNA conjugate, that is, 1/625 of library concentration). Experiments performed with 5.6 nM of LecA.

monomeric ligand and the ratio of observed intensity reaches a factor of 12-fold.

Next, the fittest divalent ligand (Figure 2: 1.3–2.5–3.1–4.3: **SI-1**, see Figure S1 for detailed structure) was resynthesized substituting the PNA tag for an arginine residue (**SI-2**) and its affinity to LecA was measured by isothermal calorimetry (ITC). The divalent compound displayed very strong affinity ($K_d = 82$ nM) while the monovalent reference *para*-nitrophenyl-β-galactoside (βPNPGal) had a dissociation constant of 26 μM (see Table S1). The monovalent compounds **SI-3** and **SI-4** containing the aryl-β-thiogalactoside and truncated portion of the linker had higher affinity than βPNPGal (5.4 and 7 μM) but were still much weaker ligands than the divalent **SI-2**, demonstrating the requirement of the two galactose units to obtain nanomolar affinity. These results clearly indicate a strong synergy of interaction between the two galactose units of the divalent ligand (> 100 fold increase in affinity per galactose unit).

The crystal structure of the fittest divalent ligand without the nucleic acid tag (**SI-2**) complexed with LecA was obtained by cocrystallization under two different conditions (Li_2SO_4 and $MgSO_4$ with a resolution of 1.65 and 1.57 Å, respectively, Table S2 and Figure S2–S4; PDB ID: 4CP9 and 4CPB). In both structures, the asymmetric unit containing one LecA tetramer provided clear density, however, better ligand density was observed for the cocrystalisation using Li_2SO_4. The structure obtained for the aryl-β-thiogalactoside is consistent with previous structure of LecA having the thioaryl ring establishing a T-shape interaction with His50 (Figure 5).[9,14] The triazole ring was also clearly seen and was stabilized by a water-bridged interaction involving the side chains of His50, Tyr36 and Asp47. Finally, the proline ring on one side was also stabilized through water bridge interaction with a sulfate ion. While the cocrystal structure confirmed the divalent nature of the identified ligand and provided information regarding interactions of the triazole ring, a clear diffraction for the whole linker could not be obtained (Figure S5). This may be the result of the close packing effect of neighboring tetramer in addition to the presence of sulfate ions from the crystallization buffer affecting the solvent exposed portion of the linker. In order to release the ligand from the packing forces, a molecular dynamics trajectory was calculated with Amber force-field using a dimer of LecA in water solution with manual building of the ligand starting from the crystal structure observations. Constraints on the position of the galactose residues were maintained during 50 ns and then fully released for a further 110 ns trajectory (Figure S6–S8). During the unrestrained trajectory, the two galactose residues were stable in their binding sites, maintaining the coordination contacts with the calcium ions. The adjacent thioaryl rings displayed some mobility, but stayed in close contact with His50. The rest of the linker had a flexible behavior, although a clustering analysis demonstrated the emergence of preferred conformations (Figure 5). In several clusters representing a sizable part of the trajectory, the phenoxy rings of the linker stabilized the complex through hydrophobic interactions at the protein surface. Stacking with neighboring thioaryl groups of the same ligand was also observed, which may further contribute to the stabilization.

We next investigated the potential of the identified ligand to block bacterial entry in the human lung epithelial cell line H1299 using an Amikacin protection assay. In this assay, αPNPGal (**3**) used at a concentration of 10 mM resulted in ca. 40% inhibition of bacterial uptake. However, when using compound **SI-1** (1.3–2.5–3.4–4.3) hybridized to its complementary DNA at a 2000 times lower concentration (5 μM), the

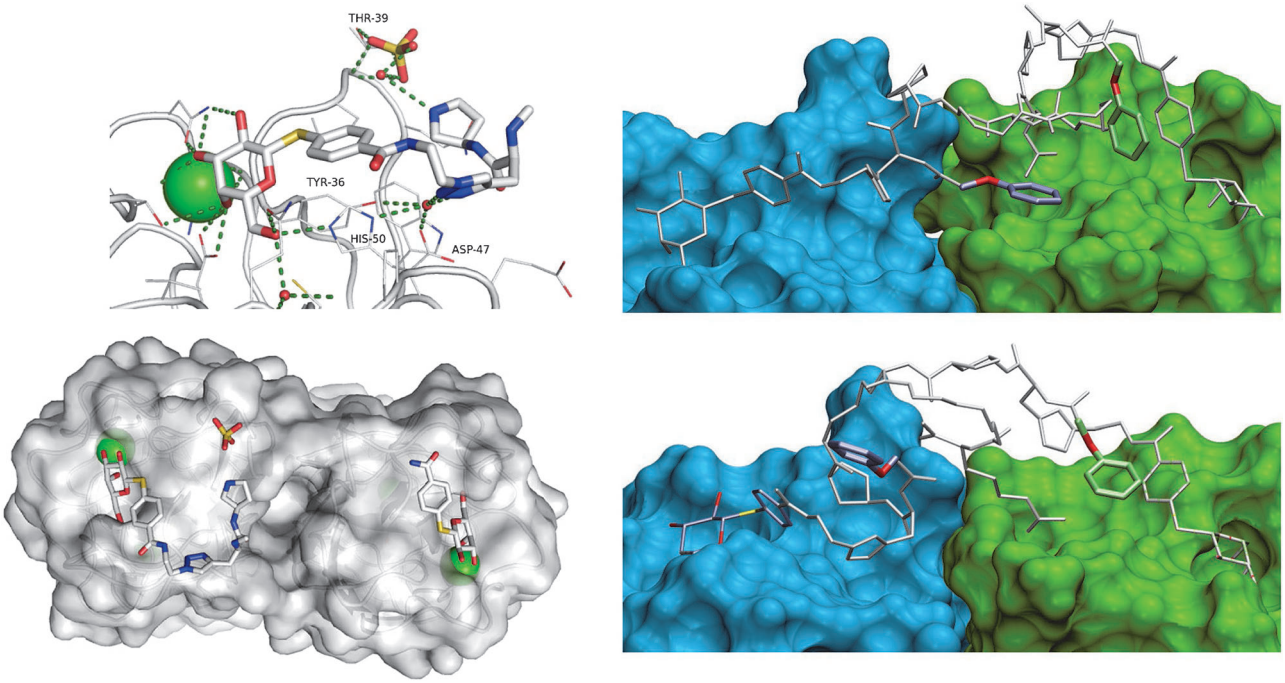

Figure 5. Left: Crystal structure of LecA complexed with divalent galactose compound (**SI-2**) and close view of the binding site with larger fragment located by X-ray analysis (PDB ID: 4CP9). Right: Two snapshots of molecular dynamics trajectory representative of two clusters of conformation populated for 10% and 36% of the trajectory for the top and bottom panels, respectively.

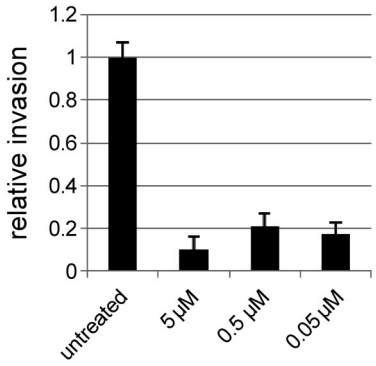

Figure 6. Inhibition of *P. aeruginosa* invasion of human lung epithelial cells. Bacterial cells were left untreated or incubated with the indicated concentrations of compound **SI-2** hybridized to its complementary DNA prior to inoculation with the human lung epithelial cell line H1299. Bacterial invasion was measured by the Amikacine protection assay. The number of intracellular, ligand-treated bacteria was normalized to untreated bacteria (values ± SD).

internalization was reduced by 90% (Figure 6). At 50 nM, the same ligand was still able to lower the uptake of *P. aeruginosa* by 83%. Higher order oligomer of the divalent ligand obtained by hybridization of **SI-1** to various DNA or PNA templates, or the ligand without the nucleic acid tag (**SI-2**) also exhibited inhibitory activity at low micromolar concentrations in a dose-dependent manner, decreasing cell entry by 30–86% (Figure S9). However, the use of higher order oligomers of the divalent ligand did not result in a significant inhibitory benefit.

Multivalent glycocompounds have been shown to interfere with lectin-mediated pathogen adhesion.[17] Glycopolymers, glycodendrimers, and glyconanoparticles bearing a large number of epitopes are efficient at binding lectins and pathogens but they induce aggregation resulting in poor performance as therapeutics. Conversely, glycans designed to chelate neighboring sites[22] on a small lectin oligomer are more promising, granted sufficient affinity can be achieved. Such an approach has been very successful against the pentameric B-subunit of Shiga toxin with a "starfish" shaped compound reaching nanomolar affinity.[23] Targeting neighboring sites of LecA has been performed using a variety of tetravalent and divalent compounds with the best divalent ligand reported reaching affinity range of 20–100 nM, which is a three order of magnitude improvement compared to monomeric galactose.[8,12–13,16] Molecular modeling suggests that such gains in affinity are the result of bridging neighboring sites 30 Å apart. The divalent ligand (**SI-2**) reported herein could be characterized by a crystal structure, confirming that the linker indeed spans the distance between the sites. This linker maintains some flexibility, as was observed in the only two other crystal structures of lectin/multivalent complexes, that is, Shiga toxin complexed with starfish inhibitor[23] and wheat germ agglutinin complexed with bivalent GlcNAc ligand.[24] Molecular dynamics based on structural data points towards a stabilizing role of phenoxy residues within the linker that can interact with hydrophobic patches on the protein surface. The combinatorial chemistry approach favored the selection of flexible ligands with optimal contact with the surface and with a gain in enthalpy, which appears complementary to the structure-based design of more rigid

spacer proposed by other groups, gaining in entropy.[8,13,16,25] The ability of this ligand to block cellular invasion might lead to the development of promising intervention strategies against *P. aeruginosa*.

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