Structural Considerations for Building Synthetic Glycoconjugates as Inhibitors for *Pseudomonas aeruginosa* Lectins

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1. Introduction

Pseudomonas aeruginosa (PA) is a ubiquitous pathogenic bacteria, which is a leading cause of chronic infections and death among immunocompromised and hospitalised patients, including those with cystic fibrosis (CF). PA has been classified as a ‘Critical’ pathogen by the World Health Organisation and is of particular concern in light of the growing global problem of antimicrobial resistance. Various approaches to treating PA, in addition to traditional antibiotics, have been reported including inhibition of quorum sensing, biofilm-formation, iron-chelation and interfering with biosynthetic pathways of the bacterium. PA produces characteristic carbohydrate-binding proteins, the soluble lectins LecA and LecB, which play a role in biofilm-formation and lung-infection, including those with cystic fibrosis (CF).

Death among immunocompromised and hospitalised patients, particularly airway epithelial cells. LecA is demonstrated to be crucial for biofilm-formation while LecB mediates interactions with exopolysaccharides. LecA also plays a key role in internalization of PA into host cells, as well as being cytotoxic, causing damage to lung- and gut-epithelial cells. By contrast, LecB is not cytotoxic, but affects ciliary beating frequency of airway epithelial cells. Both are C-type lectins dependent on the presence of Ca²⁺ and Mg²⁺ ions for their function and exist as tetramers in their natural form (Figure 1). The structural basis of their recognition of host cell surface-glycans is reviewed in detail by Imbert et al.

LecA has medium-range affinity (Ka 3.4 × 10⁴ M⁻¹) for D-galactose (D-Gal), however this is still tenfold higher than average affinities of C-type lectins for their ligands and this interaction is very selective. The protein sequence and folding are unique to PA, however its binding mode is quite typical for C-type lectins. LecA is highly conserved amongst clinical strains. High selectivity of this lectin for D-Gal derives from H-bonding interactions of the O2 and O3 atoms with the Asn107 residue, which itself is also involved in coordination of Ca²⁺ in the binding site (Figure 1b). O4 interacts with Thr104 and Asp100 in a similar fashion. Furthermore O6 is stabilised by interactions with a water molecule, His50 and Gin53. The shortest distance between Gal-binding sites in LecA is ~29 Å.

LecB is more varied in sequence and folding among clinical isolates. It has an unusually high affinity for fucoside terminated glycans: K, with L-fucose (L-Fuc) is 1.6 × 10⁶ M⁻¹, two orders of magnitude higher than LecA for Gal. This particularly high affinity comes at the cost of a loss in specificity; this lectin also recognises D-mannose and D-arabinose, among others. The Fuc O2, O3 and O4 atoms coordinate Ca²⁺ ions in the binding pocket and furthermore O5, O1, O2 and O3 form an intricate H-bonding network with neighbouring residues, particularly Asp99, Asp96, Asp104, Ser23 and structural water (Figure 1d). The shortest distance between neighbouring Fuc-binding sites is ~40 Å.

While X-ray diffraction crystallography (XRD) provides insight into ligand binding, it is not always possible to obtain this...
structural information, nor is it necessarily representative of ligand-protein binding in vivo. While no single biophysical assay tells the whole story, several methods are used in conjunction to obtain complimentary information and construct a profile to characterise lectin-inhibition.

The most commonly used assays include: Hemagglutination Inhibition Assay (HIA); Enzyme-Linked Lectin Assay (ELLA); and competitive assays based on fluorescence polarisation (FP). HIA is a qualitative turbidimetric assay\(^{[26–28]}\), while the latter two techniques provide IC\(_{50}\) numbers as a measure of the concentration of competitive ligand added.\(^{[29–33]}\) Isothermal Titrination Calorimetry (ITC) provides dissociation constants (K\(_d\)) of ligands to lectins as well as thermodynamic parameters for interactions in solution,\(^{[34,35]}\) and allows stoichiometry of interactions to be inferred. Surface Plasmon Resonance (SPR) is significantly less common, but nonetheless provides not only details on affinity but also kinetics of on-surface interactions.\(^{[36–39]}\) ITC data in the PA lectin-inhibitor field are particularly comparable as in most cases they were measured either by, or in collaboration with, Prof. Imberty’s team at University of Grenoble-Alps, leading to high degrees of methodological consistency across many classes of ligand described in this review. It is important to note that precise comparison between binding affinity values from different studies requires careful consideration, since techniques and methodologies vary. The figures in tables below are therefore provided to be illustrative of trends.

1.2. Lectin Inhibition as a therapeutic strategy

Lectin-inhibition is a promising therapeutic strategy under the anti-adhesion umbrella of infectious disease treatments. Preventing pathogen-adhesion to host tissues and biofilm-formation results in bacteria that are (a) unable to attack host cells; and (b) more exposed to drug treatments. Furthermore, anti-adhesion is a non-bactericidal strategy, which is especially attractive given the rise in antibiotic-resistant strains in common nosocomial pathogens, including PA. Non-bactericidal strategies do not introduce evolutionary pressure to select for resistant strains.\(^{[40]}\)

There are some excellent review articles detailing advances in various treatment strategies for PA,\(^{[5,6]}\) as well as bacterial biofilm-inhibition\(^{[1]}\) and antivirulence drugs and pathoblockers more generally.\(^{[41]}\) Some of these highlight people with CF specifically as an at-risk group from PA-infection. This is largely attributed to their expression of more Man- and Fuc-terminated glycans on bronchial epithelial cells and mucins, as well as higher levels of O-glycosylation, compared to the general population.\(^{[23,42,43]}\)

LecA and LecB have been shown to inhibit Ciliary Beat Frequency (CBF) in human airway cilia in vitro,\(^{[79,44]}\) which makes mechanical clearance of the airway more challenging, resulting in mucus accumulation during lung infection. Treatment of affected cell cultures with L-Fuc and D-Gal solutions restored normal CBF.
Both lectins also play a role in lung-injury and it was shown that monosaccharide lectin-inhibitors reduce injury and lung bacterial load in a murine model.\textsuperscript{[48]} A multi-carbohydrate solution (d-Man, l-Fuc and d-Gal) also inhibits adhesion of mucoid and non-mucoid PA strains to CF bronchial epithelial cells, and in a murine model of acute pneumonia, diminished lung-damage, bacterial spread, and inflammatory responses. Ex vivo experiments on dissected murine lungs and tracheas showed it induced rapid but reversible formation of bacterial clusters, which had enhanced susceptibility to antibiotics.\textsuperscript{[45]}

These findings are consistent with two examples of clinical application of sugar inhalation as a treatment for PA infections, namely, use of a l-Fuc/d-Gal solution administered by inhalation combined with Tobramycin to clear a persistent nosocomial PA infection in a 9 month old with CF;\textsuperscript{[46]} and a following clinical study, monitoring the effect of twice daily inhalations of the same solution on 11 adult CF patients over 21 days.\textsuperscript{[47]} The solution was administered as the only treatment for 4 participants and in combination with intravenous antibiotics for 7 participants. In all cases, the infection was cleared with no side effects or inflammation. Hauber and colleagues also report that, particularly when combined with antibiotics, PA counts in patients’ sputum significantly decreased, as did inflammation factor TNFα in both sputum and blood.

A recent opinion piece by Titz and co-workers,\textsuperscript{[49]} inspired by entry of GMI-1070 (a selectin antagonist) into Phase-III clinical trials, concludes that lectin-inhibition is a promising target for development of new anti-microbial strategies with drug-like properties. This is an expanding and exciting field, with potential for broader applicability, as new lectins are discovered and characterised each year, including in other pathogens.\textsuperscript{[49–52]}

2. Synthetic Monovalent and Divalent Ligands

Given the promising results of studies described in Section 1.2, it is unsurprising that improvements on the affinity of the natural carbohydrates have been sought in hopes of developing new therapies and drug-like molecules based on monosaccharides shown in Figure 2 and other natural glycans. Simple modifications, more advanced rational design, and examinations of the relationships between structure and activity have resulted in several classes of monovalent and divalent ligands which better target PA’s lectins.

2.1. Monovalent LecA and LecB inhibitors: towards drug-like molecules

The affinity of LecA for Gal has $K_d$ of 87.5 μM. As early as 1992, specificity of this binding was investigated with a range of simple galactoside and thiogalactoside derivatives, including the widely-used reagent IPTG.\textsuperscript{[54]} It became clear from these data that LecA had marked preference for galactoside-derivatives featuring aromatic aglycons at the anomic position, opening an avenue for fine-tuning structure to create more potent ligands. 5- and O-aryl-galactosides can effectively inhibit hemagglutination of erythrocytes by LecA, as well as fully inhibit its binding of labelled Gal. These compounds had $K_d$ in the $\sim 10$ μM range.\textsuperscript{[55]} Structures and summary of binding data are given in Table 1. In 2013, Roy developed monovalent LecA-inhibitors with aromatic thioglycosides, seeking higher-affinity ligands, which were stable to glycosidases.\textsuperscript{[56]} The best candidates identified in this study were 5-naphthyl galactoside.

![Figure 2: Most common monosaccharide epitopes for targeting PA lectins.](image)

| Aglycon $^{[a]}$ | Ref. | $K_d$ [μM] $^{[b]}$ | $IC_{50}$ [μM] $^{[c]}$ | r.p. $^{[c]}$ | HIA MIC [μM] |
|----------------|------|----------------|----------------|-------------|--------------|
| βGal | [53] | 32.4 | 2.7 | 0.2 |
| αFuc | [54] | 8.8 | 9.9 | 2.1 |
| βFuc | [55] | 9.9 | 8.83 | 2.1 |
| -O-(CH$_2$)$_2$-NO$_2$ | [54] | 1.41 | 6.2 | 0.55 |
| -O-Tol | [55] | 4.2 | 20.8 | 0.7 |
| -OEt | [56] | 6.3 | 3 | 11.1 | – |
| -OTol | [55] | 7.4 | 11.8 | 2.1 |
| -OH | [55] | 5.4 | 16.2 |
| -O-Hex | [55] | 6.3 | 13.8 | 2.1 |
| -OH2 | [56] | 5.4 | 5 | 16.2 |
| -K-P-L-NH$_2$ | [53] | 4.2 | 20.8 | 0.08 |
| -OAg | [57] | 5.8 | 46 | 12.1 | 250 |
| -OOPin(Au) | [58] | 6.8 | 0.7 |

[a] Peptides are represented using one letter codes for l-amino acids; [b] ITC; [c] ELLA.
and lactoside, with $K_d$ of 7.9 and 5.4 μM, respectively, representing a marked but not dramatic improvement in potency.

Nearly simultaneously, Reymond and co-workers delved into the nature of interactions responsible for observed affinity-increases for Gal derivatives with aromatic aglycons, through a Structure-Activity Relationship (SAR) study. A library of galactosides with $S$- and $O$- linked aglycons was analysed by ITC, and their MIC of hemagglutination determined. Additionally, XRD of LecA in complex with six of these compounds revealed favourable “T-shaped” CH–π interactions between aromatic aglycons and the proton of the His50 imidazole in the carbohydrate-binding pocket (Figure 3). Furthermore it was observed that electron-rich aglycons had shorter CH–π distances than their electron-poor counterparts, which is consistent with the thermodynamic data obtained in ITC, as those with electron-rich aglycons had stronger bonding enthalpies (around $-11.5 \text{kcal/mol}$). Furthermore, this study also identified five further low-micromolar monovalent LecA ligands, including O-tolyl and coumarin derivatives (Table 1).

Ligands used as monovalent “reference” compounds in order to calculate relative potencies per epitope (r.p./n) of multivalent LecA inhibitors (see Section 3) have themselves also demonstrated low-micromolar affinities. For example, GalAG0, the monovalent tripeptide “arm” of glycopeptide-dendrimers (Section 3.2) has $K_d$ of 4.2 μM, while GalOPhNAz used in developing glycoclusters (Section 3.4) had $K_d$ of 5.8 μM and was a significantly better inhibitor than analogues with non-aromatic flexible oligo(ethylene glycol) aglycons.

Aiming to enhance binding affinity using molecular dynamics simulation-aided design, ligands extended to include remote aromatic groups (e.g. GalExt, $K_d$ 6.8 μM) gave additional interactions with a central hydrophobic pocket of LecA’s tetramer, confirmed by XRD and PrOF-NMR. However no great improvement in affinity was observed because enhanced binding enthalpies were balanced by entropic penalties. Another approach to overcome modest affinities is installation of an electrophilic ‘warhead’ into ligand structures, to achieve persistent covalent LecA-inhibition. GalEpox (Figure 4) was shown by mass spectrometry analysis to bind the Cys62 residue (cf. Figure 1b). GalEpox was also co-crystallised with LecA, clearly showing proximity to Cys62. A fluorescein-derivative was also successfully used to label the protein (see Section 4).

Finally, non-carbohydrate catechol-based glycomimetics were recently reported for LecA-inhibition. XRD of CAT-LecA complex showed CAT bound in the carbohydrate-binding site in an analogous way to galactosides, a first example of this type of behaviour for C-type lectins. CAT had a $K_d$ of 1.11 mM, (by SPR). No significant biofilm inhibition results are reported for monovalent galactosides. Moreover, breaking through to nanomolar affinities for LecA has proven impossible with monovalent ligands, and even development of a competitive FP assay was challenging due to the only average avidity of the interaction. Divalent inhibitors have proven much more successful in this regard (Section 2.2).
GlCNac moiety’s O3 group with Ser23 and O6 with Asp96 in the binding pocket. FucOxaAc was designed to mimic the GlCNac moiety and is reported to match the affinity of Lewis". 

As for GalAGO above, Reymond and co-workers tested the monovalent fucosyl analogue as a LecB ligand. Fuc2G0 had Kd of 5.9 μM, just slightly below that of the non-peptidic reference FucPHNO (Kd 5.3 μM) reported in the same article. Andreini et al. reported a family of N-fucosides as relatively high potency inhibitors, of which FucHexPhOH (Kd 1.2 μM) is the highest affinity ligand. The presence of the amide at the anomeric position perturbs the highly conserved H-bonding network involving Fuc, a bound water molecule and the peptide backbone (observed in most if not all LecB-fucoside complexes, Figure 1d) and this explains the difference between FucHexPhOH and higher affinity ligands.

A downside of LecB’s high affinity for αFuc is that fucoside-glycans are ubiquitous in natural systems and can be recognised by many pathogens, so it is not surprising that LecB also has a reasonably high affinity for Me-α-D-mannoside (Kd 71 μM), a target that offers higher selectivity than can be achieved with αFuc, making it attractive for developing inhibitors with both high selectivity and high affinity, if optimised with structurally informed modifications.

This ambitious enterprise was undertaken by Titz and co-workers in hopes to identify drug-like candidates. Me-α-D-mannosides were modified at the primary alcohol with small libraries of triazoles, amines, amides and sulfonamides, and screened via FP competitive binding assays with LecB, to determine IC50 values (Table 2). While it had been anticipated that amine derivatives might form a salt bridge with Asp96, disappointing binding affinities were not supportive of this. Amine and triazole derivatives only had IC50 values in the 500-100 μM range. On the other hand, three amide derivatives displayed IC50 values <100 μM, with cinnamide ManNH CinA standing out at 37.4 μM (Table 2). Furthermore, all but one sulfonamide derivative were good inhibitors with IC50 <50 μM, e.g. ManNH SulfA. These two high-affinity inhibitors were identified as lead compounds and their Kd determined by ITC as 18.5 and 3.3 μM respectively. Both compounds feature drug-like qualities and comply with Lipinski’s rule of five, even promising oral availability according to ADMET calculations. While L-Fuc is 26 times more potent than Me-α-Man as an inhibitor, ManNH SulfA is 21.5 times more potent without being a fucoside - a significant increase comparable to the natural ligand and conducive to further lead-optimisation. XRD reveals ManNH SulfIA binds the two lectin Ca2+ ions through O4 and O5 in an identical fashion to Me-α-D-Man. It also shows that the sulfonamide forms a H-bond with Asp96 carboxylate in addition to lipophilic interactions of the aryl substituent with the protein surface, which help explain the excellent binding affinities obtained. Furthermore, it was demonstrated that both ManNH SulfA and ManNH CinA are able to inhibit adhesion of whole PA bacteria to a fucosylated surface at 200 μM.

Table 2. LecB affinity for selected monovalent ligands.

| Compound   | Ref. | Kd [μM] | IC50 [μM] | r.p./Me-α-Man |
|------------|------|---------|-----------|--------------|
| ManNH CinA | [32] | 18.5    | 37.4      | 3.8          |
| ManNH CinB | [66] | 27.4    |           |              |
| ManNH CinC | [66] | 33.6    |           |              |
| ManNH CinD | [66] | 10.9    | 19.9      | 6.5          |
| ManNH SulfA| [32] | 3.3     | 3.4       | 21.5         |
| HepNH SulfA| [67] | 101     |           |              |
| HybNH CinA | [69] | 3.09    | 4.21      | 23           |
| HybNH SulfA| [69] | 1.27    | 0.97      | 55.9         |
|              |      | (0.31)  | (0.34)    |              |
| HybNH SulfB | [69] | 0.83    | 1.80      | 85.5         |
|              |      | (0.29)  | (0.44)    |              |
| HybNH SulfC | [70] | 1.52    |           |              |
|              |      | (0.14)  | (0.44)    |              |
| HybNH SulfD | [70] | 1.20    | 1.87      | 59           |
|              |      | (0.29)  | (0.44)    |              |

[a] ITC; [b] FP assay; [c] LecB from PA14 clinical strain.

A family of mannoheptoses with cinnamide and sulfonamide substituents was also synthesised by extending the sugar in hopes of increasing affinity by freeing the O6 position to form H-bonds with Ser23, as seen interactions with natural D-Man. However, these ligands, such as HepNH SulfA, do not represent an improvement in affinity over their mannose- ANALOGUES and this strategy was less impactful than anticipated.

Although low-micromolar affinities were achieved with Man-based inhibitors, the carbohydrate-recognition domain of LecB is highly specific for fucosides. At a point where no further optimisation of Man-based compounds could be achieved, a biophysical study comparing the binding properties of various Fuc and Man derivatives identified the molecular origins of this selectivity and the most favourable interactions in the binding
pocket, evaluating individual contributions of each substituent group to the binding. From this panel of derivatives, a hybrid bespoke glycomimetic featuring the CH₃ group present in Fuc and the O6 terminus of Man was identified as “the best of both worlds”, allowing for the lipophilic interaction of the methyl group, as well as the O6-Ser23 H-bonding interaction observed for Man. This hybrid-glycomimetic is conducive to further drug design as substituents can be introduced that result in interactions with the nearby Asp96 and hydrophobic interactions, in addition to the already enhanced affinity, as discussed for sulfonamide-derivatives of Man above.⁶⁶

Sulfonamide- and cinnamide-derivatives of the hybrid-glycomimetic such as HybNHCinA, HybNHsulA, and HybNHsulB, (all analogues of previously identified Man-based lead compounds) showed improved binding affinities and anti-biofilm activity against both the common reference strain PAO1 and the more virulent PA14 strain (reduction of biofilm by >75 % with respect to the control). All were, in fact, more potent inhibitors of LecB from PA14, reaching <450 nM affinities in the case of both sulfonamide derivatives by two separate assays; their affinities for the wild-type LecB (PAO1) are also excellent (Table 2). Inhibitors showed no toxicity and good selectivity for LecB even in the presence of langerin (a human C-type lectin selective for Fuc and Man). Selectivity for LecB even in the presence of langerin (a human C-type lectin selective for Fuc and Man). In vitro ADME experiments showed good metabolic stability of the inhibitors against liver microsomes and murine and human plasma, as well as promising oral availability in a murine model. High concentrations measured in the plasma of the mice and a urinary excretion pathway presents the possibility to use these liver microsomes and murine and human plasma, as well as separate assays; their affinities for the wild-type LecB (PAO1) are potent inhibitors of LecB from PA14, reaching >75 % with respect to the control). All were, in fact, more compounds against PA urinary-tract infections. Furthermore, concentrations measured in the plasma of the mice and a urinary excretion pathway presents the possibility to use these liver microsomes and murine and human plasma, as well as separate assays; their affinities for the wild-type LecB (PAO1) are potent inhibitors of LecB from PA14, reaching >75 % with respect to the control). All were, in fact, more

2.2. Divalent inhibitors: role of linker length and rigidity

While many excellent monovalent inhibitors for LecB have been developed, similar work with LecA is not prevalent for reasons explored above. Introducing multivalency into inhibitor-design can boost the strength of interactions. Pieters and co-workers, in particular, have carried out significant work understanding and optimising the role of linker-length, rigidity and multivalency in increasing LecA binding affinity of glycoconjugates. Bridging adjacent carbohydrate-binding sites has proven an effective path to more potent inhibitors, capable of chelating the two nearest binding sites, Figure 5a.⁷¹ These sites are ca. 29 Å apart, as measured from XRD (cf. Figure 1a). Microarrays of different valency glycodendrimers were tested with LecA and observed multivalency effects correlated well with this inter-binding site distance; di- and tetravalent compounds showed strong interactions with the lectin. ELLA assays showed low-micromolar affinity (16-fold enhancement per β-Gal).⁷²

Pieters argued that the role well-defined spacers has been underestimated in LecA-inhibitor design and that candidates with flexible polyethylene glycol-based spacers, such as DivalPEG, may not be appropriate for effective binding, giving only millimolar affinities, despite having appropriate length to chelate binding sites (Table 3).⁷³ As part of a rational design strategy, a rigid spacer with alternating 1,2,3-triazole (Tz) and 1–4-linked glucose components was described, with overall linear geometry and good water solubility.⁷⁴

Compounds with three spacer-units proved most effective, with nanomolar affinities (Table 3) determined by both an ELISA-type assay and ITC, while increasing or decreasing spacer-length pushed affinities back into the millimolar range. Upon adding a triazole-glucose unit to the structure, going from U2C1 to U3C1, a relative potency enhancement per galactose

a) Chelate binding

b) Aggregative chelate binding

c) Aggregation

Figure 5. Illustration of some potential binding modes for galactosides with LecA: a) chelate binding mode with divalent inhibitor; b–c) aggregative chelate and aggregation with tetravalent inhibitors. (adapted from ⁷⁵)
Experimental binding data of C6-modified structures, however showed minimal impact of positively-charged groups at this position (U3C1-pyr and U3C1-NH₂ in Table 3), suggesting that such protein-ligand interactions are of minimal importance in solution.

XRD of U3C1-LecA complex confirms the chelate-binding mode proposed by molecular modelling and ITC. Interestingly, unlike other aromatic aglycons (see Figure 4) the triazole does not form CH···π interactions with His50. This is perhaps due to favourable H-bonding interactions between flanking glucose units and structural water molecules on the protein surface, a bonding network including His50, Tyr36 and Gln40. While replacing triazolyl aglycons with phenyl does indeed enhance enthalpy of binding for rigid divalent inhibitors of similar length, such as U2PhGal, concomitant entropic losses mean that this series of inhibitors is less potent. Entropic penalties of protein rearrangement negate any advantage of this common strategy for Kᵢ enhancement. Solubility was also an issue for some phenylene-derivatives.

An analogue of U3C1, replacing central bis(triazolyl)glucose motif of with a bis(thiourea)cyclohexyl group, yielded U3TU with equivalent LecA binding potency, and with significantly simplified core synthesis (halving the number of steps to 7). Replacing this core with a phenyl ring, U3Ph, led to increased affinity, with increased r.p./n of 258 (compared to 111 for U3C1), which remains among the highest enhancements reported, even including multivalent systems (Section 3 below). More flexible butyl-centred linkers (U3Bu) gave similarly impressive results. U3C1 and U3C3 showed weak biofilm-inhibition (50% at 150 μM), comparing very poorly with tetravalent glyoclusters GalAG2 (vide infra). This points to the importance that cross-
linking lectins typically has, in addition to chelate-binding, when it comes to anti-biofilm therapeutic activity.\textsuperscript{[87]} Indeed, a tetravalent derivative, Bis[U3Ph]\textsuperscript{[88]} could much more effectively disperse or inhibit biofilms, giving 46% inhibition at only 28 μM.\textsuperscript{[89]} Stoichiometry of interaction determined from ITC supports 1:4 interactions, aggregating two LeC tetramers, as illustrated in Figure 5b. This study gratifyingly shows the power of rational inhibitor-design, informed by lectin structure and binding-site topology.

Low-valency galactoclusters TzNA-B based on dendritic triouthiourazine cores with inter-galactoside distances less than 29 Å, unsurprisingly had modest affinities. Fucoside analogues also showed poor LeC binding; however, these clusters could inhibit PAO1 biofilm formation at 5 mM.\textsuperscript{[90]} Another class of divalent fucosides with flexible linkers were shown to have good affinity for LeC (up to 90 nM), but due to the longer distance between LeC binding sites chelate-binding was not achieved, only cross-linking of neighbouring tetramers.\textsuperscript{[91]}

A galactoside-conjugate DNA-based array was used to identify a potent LeA inhibitor from a library of 625 divalent candidates, with ligand design informed by the structural considerations already discussed. PePa was identified by this method, and ITC of its arginine-derivative PEPb showed a K\textsubscript{d} of 82 nM. PePa (when hybridized with its complementary DNA) showed the ability to block cellular invasion by PA in human lung epithelial cells by 80–90% at as low as 50 nM (compared to modest protection at 10 mM by monovalent galactoside).\textsuperscript{[92]}

Recently, Titz and co-workers reported a remarkably elegant route to low nanomolar divalent LeA-inhibitors, requiring only four synthetic steps from pentaacetylated α-Gal. Using phenyl-galactoside as a LeA targeting motif, para- and meta-hydrazides were reacted with bis-benzaldehydes of varying spacer-length. All examples had nanomolar affinity, with linker-length playing a key role in fine-tuning K\textsubscript{d}. The shortest example in the para-family, B5p proved the most potent divalent LeA ligand to date (K\textsubscript{d} 10.8 nM, Table 3); molecular modelling indicated that spacers were of appropriate length to bridge LeA’s adjacent galactose-binding sites. Selectivity of this ligand for LeA over human galectin-1 was also established. The synthetic simplicity of this design is very promising for future optimization of structures, even potentially to enhance their drug-like properties.\textsuperscript{[93]}

### 3.1. Multivalent systems immobilised on nucleotide support

Reactions and linkages widely employed in DNA-synthesiser technology were adopted as a source of inspiration for modular inhibitor design by Morvan, Vidal and co-workers. In their first article on this topic, they construct glyoclusters from building-blocks, consisting usually of glycosides (with linker), phosphor-amidite tethers, a scaffold, and nucleotides or a short strand of synthetic DNA (Figure 8).\textsuperscript{[94]} Components are assembled through well-known and reliable reactions; phosphoramidite chemistry (using a DNA-synthesiser) for the pentaerythriyl-phosphodiester core, and microwave-assisted CuAAC to conjugate glycosides giving clusters such as PeOP (4-, 6-, 8- and 10-valent ficoclusters).\textsuperscript{[95]} In a binding competition assay vs L-Fuc, all exhibit sub-micromolar IC\textsubscript{50} for LeC, Table 4. There is a clear advantage of selected phosphodiester-containing clusters.

| Compound | Ref. | Scaffold | Lectin | K\textsubscript{d} [nM] | IC\textsubscript{50} [μM] | r.p.n |
|----------|------|----------|--------|----------------|-----------------|------|
| PeOp-10  | [87] | Pe       | LeB    | 0.25\textsuperscript{[a]} | 2.2              |      |
| PeOp-4-DNA | [90] | Pe       | LeB    | 15\textsuperscript{[b]} | 18.8\textsuperscript{[b]} |      |
| MAN2     | [90] | Man      | LeB    | 14\textsuperscript{[b]} | 17.5\textsuperscript{[b]} |      |
| MAN3\textsubscript{rec} | [90] | Man      | LeC    | 11\textsuperscript{[b]} | 13.8\textsuperscript{[b]} |      |
| DMCH\textsubscript{rec} | [90] | DMCH    | LeC    | 8\textsuperscript{[b]} | 8\textsuperscript{[b]} |      |
| DMCH\textsubscript{gal} | [91] | DMCH    | LeA    | 1550\textsuperscript{[b]} | 63\textsuperscript{[b]} |      |
| MAN3\textsubscript{gal} | [91,92] | Man    | LeA   | 395\textsuperscript{[b]} | 29\textsuperscript{[b]} | 1.5 |
| MAN4     | [91,92] | Man     | LeA    | 60\textsuperscript{[b]} | 2826\textsuperscript{[b]} | 141 |
| MAN5     | [91,92] | Man     | LeA    | 39\textsuperscript{[b]} | 4218\textsuperscript{[b]} | 211 |
| MAN6     | [91] | Man     | LeA    | 6803\textsuperscript{[b]} | 170\textsuperscript{[b]} |      |
| MAN3\textsubscript{rec-α} | [92] | Man     | LeA    | 11000\textsuperscript{[b]} | 27.6\textsuperscript{[b]} | 1.6 |
| MAN4-a   | [92] | Man     | LeA    | 194\textsuperscript{[b]} | 0.26\textsuperscript{[b]} | 90\textsuperscript{[b]} |
| MAN5-a   | [92] | Man     | LeA    | 157\textsuperscript{[b]} | 0.06\textsuperscript{[b]} | 111.5\textsuperscript{[b]} |
| XyloGal  | [93] | Xylo    | LeC    | 49\textsuperscript{[b]} | 0.006\textsuperscript{[b]} |      |
| ManniGal | [93] | Mannitol | LeC     | 50\textsuperscript{[b]} | 0.006\textsuperscript{[b]} |      |
| ManniFuc | [93] | Mannitol | LeC     | 84\textsuperscript{[b]} | 0.006\textsuperscript{[b]} |      |
| RiboFuc  | [93] | Ribo    | LeC    | 56\textsuperscript{[b]} | 0.006\textsuperscript{[b]} |      |
| MAN7     | [94] | Man     | LeA    | 20\textsuperscript{[b]} | 180000\textsuperscript{[b]} |      |
| MAN8     | [95] | Man     | LeA    | 19\textsuperscript{[b]} | 0.006\textsuperscript{[b]} |      |
| MAN9     | [13] | Man     | LeA    | 395\textsuperscript{[b]} | 0.006\textsuperscript{[b]} |      |

(a) ELLA; (b) Competitive on-surface assay, where higher values indicate stronger binding;\textsuperscript{[97]} (c) K\textsubscript{d} determined on array;\textsuperscript{[98]} (d) ITC.

| Table 4. Affinity of selected phosphodiester-containing clusters. |
relationship between valency and inhibition, however the increase in r.p./n is not particularly impressive, and not strong enough to be attributed to a “cluster” effect. The best compound of this family was decavalent PeOP-10 (IC\textsubscript{50} 250 nM).

Following from this, DNA-Directed Immobilisation (DDI) technology was adopted to rapidly assess libraries of surface-bound glyoclusters as ligands for PA lectins via glycan microarray approach. An in-depth discussion of DDI technology, design and applications can be found in Morvan’s review.[88]

Mannose-centred glyoclusters were the first instance of carbohydrates as scaffolds for PA lectin-targeting. Tethered via DDI at the anomeric position, the four remaining hydroxy-groups were functionalised with EG\textsubscript{3}-linked monosaccharides to yield tetravalent clusters MAN1 with a defined topology provided by the mannose core. A heterocluster, combining both MAN1\textsubscript{Gal} and MAN1\textsubscript{Fuc} through a long flexible linker, was also synthesised. All three ligands were screened for activity with LecA and LecB through a fluorescence assay, with heterocluster having a marked increase in fluorescence in the presence of both lectins (rather than only one), and much higher than the homoclusters in all instances. However, no quantitative binding measurements were reported.[89]

A series of sixteen fucosylated ligands was synthesised, varying the topology and spatial arrangement.[90] This small library featured monovalent compounds; linear compounds of various valencies based on dimethanolcyclohexane (DMCH), and bis-pentaerythrityl (Pe) scaffolds; and finally carbohydrate-scaffolds based on Man, Gal and d-glucose (Glc). In addition to presentation topology, the influence of linker-length was also studied, with 13, 17 and 21 atoms between the alcohol groups of the hexose scaffold and the anomeric oxygen of the Fuc epitope. An on-surface assay was developed by the group in this work: the clusters immobilised on glass slides and tagged with Cy3 were incubated with Alexa 647-tagged LecB, then increasing concentrations of Fuc were added to determine IC\textsubscript{50} by measuring the decrease of the Alexa 647 fluorescence signal. Therefore, in this case higher IC\textsubscript{50} values indicate better binding of the cluster to target lectin, as a higher concentration of the natural ligand was needed to displace 50% of the interactions. Selected IC\textsubscript{50} values are shown in Table 4. Tetravalent compounds PeOP-4-DNA and MAN2, based on two different scaffolds, were the standouts from the study (IC\textsubscript{50} 15 and 14 \mu M, respectively). It was observed that among the carbohydrate-centred fucosimetics, those based on Man cores, such as MAN3\textsubscript{Gal} performed significantly better than analogues based on Gal or Glc scaffolds (IC\textsubscript{50} of 11, 1 and 2 \mu M, respectively), indicating benefits of Man-topology, however the PeOP-4-DNA resulted in the greatest enhancement in binding (r.p./n of 18.8). It was also pointed out that longer linkers seem to positively influence binding, as among the Man-centred compounds r.p. increases with linker length and across the board compounds with a 21-atom linkers (e.g. MAN2) performed better than those with shorter linkers (e.g. MAN3\textsubscript{Fuc}). Despite good activity and a multivalent presentation, the influence of multivalency seems to be less pronounced, as expected, in the case of LecB, as no chelate-binding was observed.

SAR of a library of 25 DDI-immobilised galactomimetics, based on linear and hexose-centred scaffolds allowed the influence of linker-length and topology on LecA-binding to be explored. The modular components of this library also allowed the role aromatic galactoside-aglycons to be assessed efficiently.[91] An on-surface competitive fluorescence assay was used to obtain IC\textsubscript{50} values, using lactose as competitive inhibitor (Table 4). MAN3\textsubscript{Gal}, with EG\textsubscript{3}-aglycon gave relatively...
poor binding. By contrast, four compounds, with phenyl aglycons excelled. Two tetravalent Man-centred clusters, MAN4 and MAN5, where L1 = propylene or EG2, respectively (Figure 8), and octavalent MAN6, all showed high affinity for LecA and nanomolar Kd determined on the microarray. A pentavalent linear cluster based on repeating DMCH units, DMCH6G, was also an effective LecA-inhibitor. These four standout compounds all feature O-phenyl aglycons. As discussed in Section 2.1, it was clear from the data that aromatic aglycons increase affinity considerably, and phenyl aglycon outperforms Tz analogues.

Non-immobilised analogues of the highest-affinity tetravalent systems MAN4-a and MAN5-a showed similar affinity trends in solution, and were demonstrated to inhibit PAO1 biofilm formation by 40%, at 10 and 5 μM, respectively. These compounds could also disperse established biofilms.

Man-centred clusters had higher affinity for LecA in general (compared to Gal or Glic), indicating that scaffold-topology is important, however an interesting observation was made on the relationship between the nature of the linker and the scaffold: with shorter linkers Man-clusters were better, however with longer more aliphatic linkers Glc-clusters had higher affinities. This indicated that linker-length and presentation-topology, act together rather than separately to influence affinity. In general, however, longer more hydrophilic linkers at L1, such as oligoethyleneglycols, give better results. Higher valency is also beneficial in this case, as demonstrated by the 2.4-fold increase in affinity of MAN4 vs MAN6, respectively tetra- and octavalent clusters with the same combination of features.

The importance of carbohydrate-scaffold topology was further explored by synthesis of two further families of clusters: trivalent furanose-based systems (arabinose, ribose and xylose); and open chain penta- and decavalent mannitol systems. A total of 9 galacto- and 9 fuco-clusters, were synthesised modularly, and Kd determined. In this study, all glycosclusters screened exhibited nanomolar affinities, and activity differences were subtle. For LecA-targeting clusters, xylose-based cores were generally preferred, and longer hydrophilic linkers at L1 were slightly more advantageous (Figure 8). Benefits of specific topologies over “cluster” effects are also much more evident as the decavalent mannitol cluster ManniGal and the best trivalent cluster XyloGal had almost equivalent Kd (Table 4). For LecB-targeting clusters, ribose and xylose-based cores exhibited very similar behaviours, but arabinose was markedly disfavoured. Linkers had little to no effect and multivalency was proven irrelevant by the fact that the decavalent cluster ManniFuc was a worse inhibitor than any furanose-based cluster. Among fucoamidomimetics, RiboFuc was best, illustrating well the more pronounced importance of multivalency effects when targeting LecA vs LecB.

Enhancement of affinity for LecA upon incorporation of either S- or O-linked aromatic aglycons was again seen in a study of linker impact on Man-centred clusters (L1 and L2, Figure 8). These compounds showed nanomolar Kd, but ligands with shorter EG2-linkers at L1 alongside naphthalene and diphenyl derivatives (e.g. MAN7) showed highest affinity (Kd 20 nM). This structural combination maintains the total length between triazole and the epitope’s anomeric oxygen close to the optimum of ca. 25 atoms, the same length as was achieved with phenyl-aglycons at L2 and a longer linker at L1 (e.g. MAN4). Linking carbohydrates to mannose scaffolds through phosphodiester rather than phosphorothioate-linkages seems to also have beneficial effects on Kd. Overall it was determined that, of the four aglycons used, the order of influence on affinity is benzyl < phenyl < biphenyl < naphthyl. Further structural optimization in this vein screened 27 compounds, with a wider range of linkers (L1) and aglycons (L2), concluding that EG3 is the best L1, as it offers hydrophilicity, flexibility and optimum length in most combinations.

Nine different aglycons at L2 explored influence of ring-size, heteroatoms, distance between ring and epitope, and regio-isomerism of substitution. The influence of L2 on binding affinity was greater than L1, because L2 can interact with the lectin binding-site. MAN8, with tyrosine-derived aglycon possessing a free carboxylate was a low-nanomolar LecA ligand, outperforming the regioisomeric compound with a free amine group, likely due to carboxylate interaction with His50 rather than π-stacking (as observed for phenyl aglycons). Docking simulations of MAN8 with LecA reveal two additional possible stabilising interactions with Pro38 and Glu39.

Multiple functionalisations of phosphodiester moieties were only implemented in octavalent compound MAN6, where Angeli et al explored whether adding additional chains would affect binding through complementary lectin-interactions in the binding-site. Hydrophobic and hydrophilic chains with amide and amine functionalities were utilised. It was challenging to achieve significant affinity increases in this way when aromatic aglycons were present, but in structures with a non-aromatic aglycon (i.e. EG3 at L2), the impact of a secondary chain could improve affinity to the levels of clusters with aromatic aglycons. For instance, the Kd of cluster MAN9 was enhanced from 395 nM to 48 nM upon introduction of a [C6H5O]C2H5NH3+ chain, indicating that this alternative strategy for mannose-centred clusters also has merit.

3.2. Peptide and β-peptoid-based scaffolds

A significant number of PA lectin-inhibitors based on glycopeptide dendrimer scaffolds are reported, with lysine residues acting as branching points (Figure 9a). This approach (up to 2013) was thoroughly reviewed by Reymond and co-workers.

In the seminal article, a library of >15,000 fucosylated glycopeptide dendrimers (up to 4 generations) were rapidly synthesized via solid-phase peptide synthesis (SPPS), followed by screening on solid-support beads with Rhodamine B-labeled lectins. Lead candidates from this library were re-synthesised, cleaved from the solid support to determine lectin-affinity and anti-biofilm activity; data for some of the best-performing examples are given in Table 5. Tetrameric 2nd-generation dendrimer FD2 (Figure 9a) was determined to have affinity for LecB almost 80-fold that of Fuc. FD2 also showed very potent
Figure 9. Selected dendrimer structures: a–b) Glycopeptide dendrimers represented using one letter codes for L-amino acids, wobbly bonds mark side-chain lysine connectivity, and lysine (K) indicated in italics are branching-points. Various generations of dendrimer synthesis are indicated in different colours for clarity. c) β-peptoid glycoclusters and polyproline helices; d) Non-peptide dendrimers.
activity for biofilm-inhibition and dispersion of established biofilms with both PAO1 and also antibiotic-resistant clinical isolates. In knockout strains, not containing the lecB gene, FD2 showed no effect, supporting the hypothesis that activity is LeCB-mediated. The analogue of FD2 with unnatural β-amino acids (β-FD2) was more resistant to proteolytic degradation than FD2, but its LeCB affinity was 5-fold weaker and it was less active against clinical isolates. A second library, bearing hydrophobic aromatic residues next to Fuc was also studied, but this modification was not found to significantly enhance affinity for LeCB, in contrast to observations for other lectins.

Having identified potent LeCB-inhibitors, analogous system of comparable activity against LeCA were also sought, leading to identification of GaLA2G and GaLBG2 with nanomolar-affinity and potent biofilm-inhibition activity (Table 5). GaLA2G possessed hydrophobic aromatic aglycons and, as expected (see Section 2.1) these dendrimers had enhanced LeCA-binding compared to GaLBG2, which possessed carboxypropyl-β-thiogalactoside groups instead (Figure 9). This key role of the aglycon on affinity was observed across multiple families of glycopeptide dendrimers. However, the authors also point out that GaLBG2, a higher-valency compound with a relatively weaker Kᵦ was actually a better biofilm inhibitor compared to lower-valency but higher affinity ligands. This indicates that multivalency plays a larger role in biofilm-inhibition than simply lectin binding constant.

Further work attempted to optimise the peptide sequence, linkers used, as well as probing effects of multivalency, presentation, and even number of ionic residues in the sequence. Structural screening, wherein all amino acids in the sequence of the core scaffold (save branching lysines) were sequentially replaced by alanines, revealed little-to-no change in affinity and biofilm-dispersal. Consequently, they focused on the terminal tripeptide of dendrimer arms, in order to interactions of these with the lectin. Computational docking identified 26 tripeptides, which were synthesised as monovalent ligands for HIA screening. Two leads identified (GaLa-KPY and GaLa-KRL) both showed higher affinity in ITC than reference GaLA0 (Section 2.1). A general trend identified was that tripeptides featuring aromatic residues at the first position had corresponding 2nd-generation (G2) dendrimers were made. G2-dendrimers, G2KPY and G2KWPW had enhanced biofilm-dispersal activity and similar affinity to GaLA2G (Table 5). This demonstrates that amino acid sequences can be fine-tuned to increase

| Compound Ref. | Scaffold | Lectin | Kᵦ [nM] | Kᵦ [nM] | r.p/n | Activity against PA | Biofilm Dispersal[a] |
|---------------|----------|--------|---------|---------|-------|---------------------|---------------------|
| FD2           | [63,97,102] | Peptide dendrimer | LeCB | 66[a] | 140[a] | 19.7[a] | 20 > 30 | 100% |
| PA8           | [63]     | Peptide dendrimer | LeCB | 110[a] | 25[a] | 50 | 35% |
| -FD2          | [100]    | Peptide dendrimer | LeCB | 660[a] | 4.2[a] | 80% |
| 2G3           | [98]     | Peptide dendrimer | LeCA | 100[a] | 219[a] | 30 | 100% |
| GalIG2        | [53,102] | Peptide dendrimer | LeCA | 400[a] | 60[a] | 20 | 60% |
| GalIBG2       | [53,102] | Peptide dendrimer | LeCA | 500[a] | 22[a] | 20 | 60% |
| GalA-KPL      | [101]    | Monovalent tripeptide | LeCA | 4300[a] | 20[a] | 30 | 100% |
| GalA-KPY      | [101]    | Monovalent tripeptide | LeCA | 2700[a] | 34[a] | 30 | 100% |
| GalA-KRL      | [101]    | Monovalent tripeptide | LeCA | 2700[a] | 33[a] | 30 | 100% |
| G2KPY         | [101]    | Peptide dendrimer | LeCA | 1700[a] | 30 | 80% |
| G2KWPW        | [101]    | Peptide dendrimer | LeCA | 830[a] | 20 | 70% |
| Het4G2        | [102]    | Peptide dendrimer | LeCA | 120[a] | 153[a] | > 45 | > 45 | 0% |
| Het2G2        | [102]    | Peptide dendrimer | LeCB | 75[a] | 20[a] | > 30 | 80% |
| Het2G2        | [102]    | Peptide dendrimer | LeCB | 292[a] | 1.93[a] | |
| FucG2C2       | [102]    | Peptide dendrimer | LeCB | 121[a] | 0.9[a] | 9 | > 20 | 100% (at 30 μM) |
| LeCG2G2       | [102]    | Peptide dendrimer | LeCB | 39[a] | 2.8[a] | 30 | 88% |
| GalAVG3       | [103]    | Peptide dendrimer | LeCA | 2.5[a] | 148[a] | 9 | 100% |
| RNFuc         | [104]    | Cyclopeptide | LeCB | 145[a] | 1[a] |
| RDNuFuc       | [104]    | Cyclopeptide | LeCB | 28[a] | 6.6[a] | 64.6 |
| RFRuFuc       | [104]    | Cyclopeptide | LeCB | 7.6[a] | 5[a] |
| RDRuFuc       | [104]    | Cyclopeptide | LeCB | 213[a] | 109[a] | 40 |
| 4GalG1        | [105]    | Cyclopeptide | LeCA | 91[a] | 412[a] | |
| 4GalG2        | [105]    | Cyclopeptide | LeCA | 22[a] | 1705[a] | |
| HGC1          | [105]    | Cyclopeptide | LeCA | 34[a] | 0.8[a] | |
| HGC2          | [105]    | Cyclopeptide | LeCA | 35[a] | 55[a] |
| P,Cyc         | [106]    | Cyclic β-peptoid | LeA | 296[a] | |
| P,Lin         | [106]    | β-peptoid | LeA | 1800[a] | |
| Pro1          | [107]    | Peptide | LeA | 442[a] | |
| Pro2          | [107]    | Peptide | LeA | 808[a] | |
| Pro3          | [107]    | Peptide | LeA | 136[a] | |

[a] ITC; [b] ELLA; [c] Dispersal at 50 μM, unless otherwise stated; [d] HIA; [e] SPR (5 % DMSO for solubility).
LecA-affinity, particularly by having one hydrophobic and one cationic residue in the terminal tripeptide, but again that multivalency plays a much larger role in biofilm-dispersal activity.

FD2’s core-pptide-sequence was employed as a scaffold for hetero-clusters, such as Het4G2, which has \( K_0 \) of 120 nM against both lectins. However homo-dendrimers FD2 and GalAG2 have higher affinities for their specific lectin than hetero-systems.\(^{106}\) Despite high lectin-affinities, Het4G2 had inferior biofilm-dispersion and minimum biofilm-inhibitory concentration (MBIC), while other hetero-glycodendrimers (e.g. Het2G2) with more cationic peptide sequences were comparable to the parent homo-dendrimers. This counterintuitive result prompted investigation into the activity of non-glycosylated dendrimers, finding that highly cationic peptides have inherently bactericidal effects comparable to those of antibiotics polymycin-B and tobramycin, whereas most glycosylated dendrimers, particularly those with more neutral sequences were non-toxic to bacteria even at relatively high concentrations. Thus the mechanism of biofilm-inhibition for cationic dendrimers involves toxicity, whereas biofilm-inhibition by neutral compounds (e.g. Het4G2 and FD2) is dependent on selective carbohydrate-lectin interactions. Synergistic addition of sub-inhibitory doses of both FD2 and tobramycin can be used to achieve similar anti-biofilm results as with the full dose of each individual component, thereby lowering the \textit{de facto} dosage of each. This effect was also observed with FucC\(_6\)G\(_2\) (Figure 9). This may be seen because biofilm-dispersion by non-toxic dendrimers facilitates entry of the toxic antibiotic into cells. Similar effects were observed combining a non-toxic and a toxic dendrimer.

Lewis’s\(^{4}\) natural high-affinity ligand for LecB, and analogue Le\(_7\)C\(_6\)G\(_2\), functionalised with this glycan indeed had heightened affinity, but lower biofilm-inhibition than FD2 (Table 5).\(^{102}\) This again underscores that higher-affinity ligands do not directly correlate with good anti-biofilm activity, and that structural consideration are also at play; fucoside analogue FucC\(_6\)G\(_2\), showed improved biofilm-inhibition properties.

Synthesis of higher-generation dendrimers by direct SSPS was hindered by steric crowding. Despite low isolated yields of 2G3 and analogues, these octavalent systems demonstrated 55-fold increase in r.p./n.\(^{104}\) Convergent synthesis was used to more efficiently make 3\(^{rd}\)-generation analogues of GalAG2 and GalBG2 to probe any multivalency enhancements on LecA-binding.\(^{103}\) In terms of r.p./n, gains are not dramatic on expanding from 2\(^{nd}\) to 3\(^{rd}\)-generation, while higher generations proved detrimental. Furthermore solubility issues and formation of precipitates hampered ITC. However, octavalent GalAxG3 did show modestly higher biofilm-inhibition and -dispersal than tetravalent GalAG2.

XRD of tetravalent fucoclusters with LecB demonstrated cross-linking of two tetramers,\(^{103}\) while the structure of octavalent GalAxG3PS shows aggregative chelate binding with LecA (Figure 5c): all 8 epitopes were bound to different LecA tetramers creating a checker-board pattern, rationalising the tendencies of higher-generation dendrimers to form precipitates in ITC.

Renaudet and co-workers expanded peptide-scaffold strategies to include their ‘RAFT’ cyclopeptide scaffolds\(^{109}\) in addition to lysine-based dendrimers to obtain multivalent clusters.\(^{104}\) Tetra-, hexa- and hexadecavalent scaffolds were synthesised by efficient oxime conjugation in all possible combinations of cyclopeptide and peptide dendrimer components (e.g. RD\(_{16}\) and RR\(_{16}\)). Tetravalent presentation of \( \alpha \)-fucosides (R\(_{4a}\)Fuc) did not lead to significant enhancement by the multivalent glyocluster effect, with increases simply mirroring concentration effects. For the hexadecavalent compounds, RD\(_{16}\)Fuc with the rigid cyclopeptide core and more flexible lysine-dendron arms was found to best enhance binding potency, with r.p./n of 64.6 (contrasting with only 5 for all-cyclopeptide structure RR\(_{16}\)Fuc), pointing to more favourable epitope spatial arrangements. This illustrates that structural parameters such as orientation and distribution can be more important than simple multivalency effects. Similar enhancements were also seen for hexadecavalent \( \beta \)-fucosides, e.g. RD\(_{16}\) Fuc, but none outperformed \( \alpha \)-fucoside analogues (IC\(_{50}\) 51 to 109 nM vs 0.6 to 11 nM). This is in keeping with the known lectin selectivity. ITC of both RD\(_{16}\)Fuc and RD\(_{16}\) Fuc revealed lectin-binding was enthalpy-driven, with strong entropic barriers in both cases. The \( \alpha \)-fucoside has strong binding-enthalpy (\( \Delta H^\circ = -223 \) kcal/mol) arising from this anomer better fitting the binding-site. As such, RD\(_{16}\)Fuc has \( K_0 \) tenfold higher than the \( \beta \)-fucoside. Stoichiometric information from ITC points to interaction with only 3–6 monomers of LecB by glyoclusters, suggesting not all 16 sugars are involved. An aggregative chelate binding mode was proposed.

In addition to oxime ligation, CuAAC was also used to functionalise cyclopeptide scaffolds.\(^{103}\) Comparison of tetravalent galactoclusters 4Gal\(_{16}\) and 4Gal\(_{16}\) showed significant enhancements in LecA binding (\( K_0 \) 22 vs 91, Table 5), with triazolyl-derivatives having a remarkable r.p./n of 1705, consistent with the established binding benefits of aromatic aglycons.\(^{107}\) Orthogonal use of oxime ligation and CuAAC produced a library of related hexadecavalent hetero-glyoclusters with combinations of fucosides, mannosides and galactosides, e.g. HGC1 and HGC2. These mixed compounds, containing a mix of \( \alpha \)-fucoside and \( \beta \)-galactoside, retain nanomolar affinity for both LecA and LecB, and the presence of and crowding by the non-specific epitope doesn’t detrimentally effect binding affinities, when compared with homo-glyocluster analogues.\(^{105}\)

In a related, but simplified approach, \( \beta \)-peptoids were also used as scaffolds for LecA-targeting by Cecioni et al.\(^{106}\) Both linear and cyclic tetravalent \( \beta \)-peptoids, Figure 9c, showed low-micromolar activity. Cyclic scaffold presentation seen in P\(_4\)Cyc had higher affinity than the linear equivalent P\(_4\)Lin, with a 25-fold increase in r.p./n by SPR, and 500-fold by ITC. (Table 5). Cyclic scaffolds were less sterically constrained, allowing all four epitopes to interact with LecA binding-sites.

Wang and co-workers used the rigid helical polyproline structure as a scaffold to control the spacing and presentation of galactosides. Initially, a microarray was prepared by immobilising various functionalised polyproline peptide helices on a fluorous surface to assess LecA-interactions.\(^{105}\) Each three-
residue turn of this peptide helix spaces a conjugated functional group by multiples of 9 Å on the same face of the scaffold. Galactoside-derivatives, synthesised by SSPS and CuAAC, with spacing of 9, 18 and 27 Å between glycans were immobilised and their binding with fluorescently tagged LeCα was studied. At an appropriate surface-distribution, fluorescence binding data clearly suggested that glycopeptides can differentiate the spatial specificity of LeCα, with the 27 Å spacing (K_d,surface = 354 nM) being very well matched to the known distance between LeCα galactose-binding sites.\(^{[71]}\)

Compound PProA, Figure 9c, was analogous to the fluoros-immobilised peptide and in SPR experiments with LeCα showed K_d of 442 nM (compared to 808 nM for shorter spaced PPro2, Table 5), confirming that appropriately-spaced epitopes gave similar binding-enhancement in solution. As expected, introduction of aromatic aglycons into these structures (PPro3) further improved binding affinity.\(^{[105]}\)

3.3. Other dendrimers

In addition to glycopeptide dendrimers, other dendrimer classes are also reported as PA lectin-inhibitors.\(^{[118]}\) A library of glycodendrimers synthesised by a convergent approach using CuAAC included DenAFuc and DenAFucGal.\(^{[111]}\) Turbidimetric assays showed rapid formation of insoluble complexes, indicating cross-linking with LeCβ (for fucodendrimer) and both lectins (for hetero-dendrimer). No quantitative lectin-binding assessments were reported.

Another class of dendrimer was supramolecularly self-assembled from amphiphilic glycoconjugates possessing azobenzene hydrophobic tail groups, which have capacity for light-activated trans-cis isomerisation.\(^{[112]}\) Amphiphiles (e.g. DenB) aggregated into cylindrical micelles with effective diameters ~ 100 nm (DLS). Lectin-inhibition was assessed by competitive FP binding assay and results were disappointing, with large degrees of multivalency (hundreds of sugars per dendrimer) leading to no sizable enhancement of micromolar IC_{50} when compared to monosaccharide references. This is perhaps due to excessive flexibility in dendrimer structure. Manno-dendrimers, however, did show 30–80-fold binding-enhancement compared to Me-αMan (IC_{50} 2.7 μM for LeCβ). Unfortunately, no significant photomodulation was seen for either dendrimer aggregation or lectin-inhibition, likely due to low photo-isomerisation yields (perhaps owing to densely packed aggregate geometry impeding micelle light penetration).

The first use of Saturation Transfer Difference-NMR to assess LeCα-ligand interactions was reported for 18-valent galactodendrimer DenGal, showing both Gal epitopes and dendrimer scaffold interacting with LeCα in solution (K_d 41 μM). Man- or non-glycosylated analogues demonstrated no lectin interactions. DenGal inhibited PAO1 biofilm-formation in vitro, giving a 1.8-fold decrease in biofilm mass at 250 μM.\(^{[113]}\)

3.4. Aromatic macrocycle scaffolds

Macrocycles, including calixarenes and others (Figure 10), are attractive scaffolds for lectin-targeting mainly due to their versatility in achieving varying topologies and valencies,\(^{[114]}\) and several glycocluster families based on macrocylic cores are reported.

Imberty, Matthews and Vidal reported what would be the first in a series of papers exploring calix[4]arene scaffolds, CalixA-C, as PA lectin-inhibitors.\(^{[75]}\) In this seminal study, effects of varying presentation-topologies of carbohydrates were...
assessed. Varying degrees of propargylation on either the macrocycle’s lower rim or both rims allowed EGl-linked galacto- and mannosides to be coupled to scaffolds by CuAAC. The standard “cone” calix[4]arene geometry was utilised, as well as the 1,3-alternate conformer to explore the relevance of differing standard “cone” calix[4]arene geometry was utilised, as well as and mannosides to be coupled to scaffolds by CuAAC. The mined that trivalent cluster topologies, in addition to multivalency. ITC with LecA deter-

leads to 2-fold $K_r$ enhancement, with the “2:2” presentation (CalixB$^{22}$, Figure 10) being the most potent inhibitor ($K_r = 176 \text{ nM}$, r.p./n 144). Observed stoichiometry of CalixB$^{22}$ ($n = 0.26$) indicated that it could chelate two binding-sites on one LecA tetramer as well as two further sites on a second tetramer, leading to aggregative-chelate complexes (Figure 5b). The hypothesis was supported by AFM studies where an equili-

Special attention was given to the self-assembly structure. This is a powerful tool for more precisely describing glyocluster binding mode.115)

In direct comparisons with $P_c$Cyc and $Zn$Por (Sections 3.2 and 3.6, respectively), all of which had TzEG, Gal arms, calixarene-derivatives like CalixB$^{22}$ had higher LecA-affinity.116) Hexavalent Calix$^{6}$ performed even better, and a “bind-and-jump” mechanism for its interaction with LecA tetramers was proposed to explain the 1:3 stoichiometry calculated from ITC.

CalixB$^{22}$ analogues with five different linker-arms were designed to explore variations in length, rigidity and hydrophobicity on tetravalent scaffolds.37) Analysing arm-precursors as monovalent ligands, GalPhNAz-N$_2$ was by far the best inhibitor ($K_r = 5.8 \mu M$), as expected, with the presence of aromatic aglycons giving stronger enthalpy of binding. However, due to the added hydrophobicity and rigidity of the linker, this arm led to major solubility issues when coupled with nearly all scaffolds studied, resulting in limited data from biological assays. Even against a larger library, CalixB$^{22}$ continues to outperform other structures in SPR, including its analogue with $E_G$NAz- linker CalixD$^{22}$ (Table 6). This study illustrated very well the importance of balancing increased potency (which hydrophobic aglycons can provide) with solubility, linker-length and presentation topology. CalixB$^{22}$ seems to combine these elements in a serendipitous way. It strikes the balance to excel in all measures (HIA, ELLA, SPR and ITC) both compared against its own derivatives, other calix[4]arene topologies and also entirely different scaffolds.

Calix[4]arenes functionalised at the upper rim, Calix$E$, were immobilised onto oligonucleotide scaffolds (discussed in Section 3.1) to assess their LecA-recognition.116) Unexpectedly, these were not found to bind Alexa 647-labelled LecA, in contrast to trivalent linear presentation modes, potentially due to steric crowding. This however does not correlate well to the excellent results seen with lower-rim decorated tetravalent CalixA$^{22}$.

| Compound | Ref. | Scaffold | Lectin | $K_r$ (nM) | $k_{\text{on}}$ (nM) | r.p./n |
|----------|------|----------|--------|-----------|----------------|-------|
| CalixA$^{10}$ | [75] | Calix[4]arene | LecA | 2050$^a$ | 6040$^a$ | 24$^a$ |
| CalixA$^{10}$ | [75] | Calix[4]arene | LecA | 420$^b$ | 2500$^b$ | 89$^b$ |
| CalixB$^{22}$ | [75] | Calix[4]arene | LecA | 176$^c$ | 500$^c$ | 213$^c$ |
| CalixC$^{2,10}$ | [75] | Calix[4]arene | LecA | 200$^c$ | 1700$^c$ | 188$^c$ |
| CalixD$^{2,10}$ | [57] | Calix[4]arene | LecA | 90$^c$ | 1000$^c$ | 284$^c$ |
| CalixE$^{2,10}$ | [106] | Calix[4]arene | LecA | 140$^c$ | 800$^c$ | 179$^c$ |
| CalixF$^{2,10}$ | [118] | Calix[4]arene | LecB | 48$^c$ | 1.5$^c$ | |
| CalixG$^{2,10}$ | [118] | Calix[4]arene | LecB | 2267$^d$ | 700$^d$ | 78.5$^d$ |
| RES | [119] | Rescoricinarane | LecA | 700$^d$ | 2600$^d$ | 16.9$^d$ |
| PillarA$_{Gal}$ | [120] | Pillar(S)ene | LecA | 413$^d$ | 26000$^d$ | 19.1$^d$ |
| PillarA$_{Gal}$ | [120] | Pillar(S)ene | LecA | 366$^d$ | 218000$^d$ | 15$^d$ |
| PillarA$_{Gal}$ | [121] | Pillar(S)ene | LecA | 931$^d$ | 29000$^d$ | 23.8$^d$ |
| PillarA$_{Gal}$ | [121] | Pillar(S)ene | LecA | 586$^d$ | 9000$^d$ | 0.04$^d$ |
| PillarB$_{Gal}$ | [120] | Pillar(S)ene | LecB | 990$^d$ | 90$^d$ | 0.19$^d$ |
| PillarC$_{Gal}$ | [121] | Pillar(S)ene | LecB | 220$^d$ | 30$^d$ | |
| PillarD$_{Gal}$ | [120] | Pillar(S)ene | LecB | 1402$^d$ | 15$^d$ | |
| PillarE$_{Gal}$ | [120] | Pillar(S)ene | LecB | 150$^d$ | 6$^d$ | 0.29$^d$ |
| Axle$_{Gal}$ | [122] | Divalent axle | LecA | 158$^d$ | | |
| Axle$_{Gal}$ | [122] | Divalent axle | LecA | 112$^d$ | | |
| Rotax1 | [122] | Pillar(S)ene | LecA | 261$^d$ | | |
| Rotax2 | [122] | Pillar(S)ene | LecB | 279$^d$ | | |
| CalixC$_{Gal}$ | [123] | Calix[4]arene | LecB | 5360$^d$ | | |
| CalixC$_{Gal}$ | [123] | Calix[4]arene | LecB | 249$^d$ | | |
| Rotax3 | [11] | Pillar(S)ene | LecA | 171$^d$ | | |

[a] ITC; [b] SPR; [c] ELLA; [d] FP assay.
The first fucosylated calix[4]arene ligand to target LecB, CalixF, displayed four acetylglycylamido-linked epitopes on the macrocycle's upper rim, and this showed biofilm-inhibition of up to 80% at 200 μM. This behaviour was dose-dependent and CalixF biofilm-inhibition always exceeded the activity of a non-fucosylated analogue. However, modest activity of this analogue points to possible non-specific anti-biofilm interactions in addition to lectin binding.[117]

Fucosylated analogue of CalixB(2:2), was also found to be an excellent LecB-inhibitor ($K_d$ 48 nM).[118] ITC studies of the thermodynamics of CalixB(2:2)$_{Fuc}$ binding, and the unimpressive increase in r.p./n (1.5-fold), pointed to a “bind and jump” mechanism of the lectin around the glycocluster structure. A chelating mode is not possible for LecB with this structure, since its binding sites are more distant from each other than in LecA (40 vs 29 Å, see Figure 1).

This multi-team study provides a remarkably complete set of data to assess PA lectin-inhibitors as anti-infection agents, surpassing much that preceded it. Both CalixB(2:2) and Calix(2:2)$_{Fuc}$ were evaluated for various biological effects relevant to the virulence of PA. Their impact on bacterial cell aggregation was estimated with wild-type PAO1, as well as PAO1ΔlecA and PAO1ΔlecB (isogenic knockout mutants not expressing LecA and LecB, respectively). At concentrations of 100 μM, both tetravalent calix[4]arene glycoclusters led to aggregation of the wild-type bacteria, but not of knockout strains. These results and various control experiments indicate aggregation behavior is lectin-dependent. A dose-dependent inhibition of PA-adhesion to A549 human epithelial cells was also seen for both glycoclusters, reaching 70% and 90% for galactosides and fucosides respectively. This effect was lost in knockout strains, although galactocluster CalixB(2:2) still had some activity at higher concentrations, indicating the potential presence of targets other than LecA. At concentrations of 5 mM, both calix[4]arene clusters demonstrate significant inhibition of biofilm-formation by all three strains of PA. This result is counterintuitive, since activity is seen even in the absence of target lectins, but is specific to Gal- and Fuc-derivatives, and not seen for Glc- and Man-analogues. Finally, these compounds (at millimolar concentrations) were shown to be effective at protecting against bacterial lung-injury in an in vivo mouse model. Bacterial load in lungs and spleen was also decreased in test subjects. Importantly nanomolar affinities in vitro did not translate directly to in vivo studies, with higher concentrations required, likely due to competition from other adhesins, lectins and proteins in more complex systems. To date these remain the only multivalent PA lectin ligands evaluated in vivo.[118]

Apart from calixarenes, other macrocyclic scaffolds have also been used, including attempts to use a porphyrin macrocycle-scaffold for multivalent glycoconjugates through CuAAC,[106] but the macrocycle sequestered copper, interfering with this strategy. Instead the Zn(II)-porphyrin complex ZnPor was used to form square planar tetrasaccharidic ligands (see Section 3.7). Resscorcin[4]arenes RES were also prepared by CuAAC, but displayed $K_{IC}$ values lower than calix[4]arenes and thermodynamic insight into decreased affinity couldn't be obtained due to solubility issues.[119] Pillar[S]enes are applied as densely-functionalised scaffolds, with a series of decavalent (PillarA-B)[120] and pentavalent (PillarC-D)[121] ligands, functionalised with carbohydrates with varying linkers. Difficulty separating the diastereoisomers of pillar[S]enes is an issue with this class of glycocluster, which is not yet resolved; nonetheless studies have been performed on 1:1 isomeric mixtures. Galactosylated pillar[S]ene clusters were strong LecA-ligands, with increased linker-flexibility from Pillar$_{A}$gal to Pillar$_{B}$gal leading to enhanced $K_d$ (Table 6). Increased multivalency between decavalent and pentavalent systems, however, yielded only moderate improvements. This is rationalised through stoichiometries observed in ITC, where only five LecA tetramers interact with decavalent clusters, and thus little advantage arises from extra Gal-epitopes. Their binding behaviour, nonetheless compared unfavourably to tetravalent CalixB(2:2). This leads to the conclusion that for macrocyclic scaffolds, increased multivalency leads to enhanced LecA binding, but only up to a point, with linker-length, flexibility and presentation also playing important roles.

LecB affinity of fucosylated pillar[S]ene ligands depended on multivalency, and to a lesser extent, linker-length. In ITC, pentavalent ligands Pillar$_{C}$gal gave disappoointing millimolar affinities,[121] while decavalent compounds saw a significant $K_d$ increase upon addition of $EG_2$-spacers (220 nM for Pillar$_{A}$gal vs. 990 nM for Pillar$_{B}$gal, Table 6). This difference is perhaps due to steric crowding limiting optimal Fuc interactions with LecB. Extension of these structures to flexible 20-valent cluster Pillar$_{E}$gal with triazolyl aglycons, gave the highest affinity ligands ($K_d = 150$ nM), representing very potent LecB-inhibitors.[120]

In an imaginative extension of this work, PillarA were also employed as the “wheel” component of mechanically-interlocked [2]rotaxane molecules, where the “axle” is a divalent glycoconjugate (Axle$_{Gal}$ or Axle$_{Fuc}$), assembled by CuAAC. Sugar motifs on the axle act as “stoppers” to prevent de-threading.[122] This strategy resulted in heteroclusters Rotax1-2, where axle and wheel were functionalised with alternate saccharides (β-Gal or α-Fuc). The divalent axle compounds and their respective [2]rotaxanes showed similar binding affinities to their target lectin, indicating that the pillar[S]ene has no dramatic negative impact on their binding. As seen already for above structures, LecA-binding is sensitive to multivalency effects, due to chelate-binding, and Rotax1 has 268-fold increase r.p./n. This [2]rotaxane can thus inhibit both LecA and LecB at similar submillimolar $K_d$ values (Table 6), without the non-specific carbohydrate impeding binding. Stoichiometries determined from ITC suggest this sophisticated system could cluster LecA tetramers and also aggregate LecB. However, these systems were not effective at biofilm-inhibition.

3.5. Carbohydrate-based macrocycles

Mazzaglia et al. report amphiphilic β-cyclodextrin derivatives can aggregate into nanoparticles in solution. The macrocyclic core was decorated with thiogalactosides, connected by oligo(ethylene glycol) spacers, and these formed particles with...
diameters of ~100 nm. Their ability to bind LecA was supported by new peaks in mass spectrometry, and more detailed physical studies showed that the lectin’s dynamics (as determined by light-scattering) are slowed down upon interaction with as little as 2 equivalents of Cd. A characteristic and persistent decrease in LecA’s intrinsic fluorescence upon addition of galactosylated cyclodextrin-derivative was measured, as well as decrease in time-resolved fluorescence lifetimes and increase in steady-state fluorescence anisotropy. None of these changes were observed for glucosylated control compounds.\[^{[124,125]}\]

Cyclic oligo-(1–6)-β-D-glucosamine scaffolds were used for 15 glycoclusters with various valencies and linkers, including examples CGA (Figure 11).\[^{[126]}\] Increasing multivalency of compounds with flexible EG₃-linkers, e.g. from CGA-TEG-3Gal to CGA-TEG-4Gal, led to increases in binding-affinity as expected, however, the r.p./n gain was not noteworthy (Table 7). Significant affinity enhancement was achieved by using rigid hydrophobic phenyltriazole-linkers instead. A marked advantage of this scaffold over calixarene scaffolds (Section 3.4) is that they do not present solubility issues when functionalised with epitopes featuring phenyltriazole linkers.\[^{[57]}\] Best results were obtained with tetravalent galactosides cluster CGA-Ph-4Gal. With Kᵣ of 79 nM, it was among the best ligands tested to that point (by ITC, ELLA and HIA). This indicates that these macrocyclic carbohydrate-derived scaffolds present a versatile and effective platform for glycocluster design.

### 3.6. Nanomaterials and nanoparticles

Nanomaterials and nanoparticles present potential as platforms for highly-multivalent glycoclusters. This has not been ignored by researchers targeting lectins, exploiting their potential to provide glycocalyx-like surfaces for presenting carbohydrates.\[^{[128]}\] So-called “fullerene sugar-balls” were synthesised using CuAAC chemistry to graft unprotected sugar-derivatives onto alkyne- or azide-functionalised C₆₀. This yields glycoclusters with globular topology, where carbohydrate epitopes are almost equidistant with overall tetrahedral

![Figure 11. Selected structures of multivalent glycocluster carbohydrate-based macrocycles, nanomaterials and metal complexes.](image-url)

**Table 7.** Affinity of selected compounds from Sections 3.5–7.

| Compound | Ref. | Scaffold | Lectin | Kᵣ [nM]\[^{[a]}\] | IC₅₀ [nM] | r.p./n | HIA MIC [μM] |
|----------|------|----------|--------|-----------------|----------|--------|---------------|
| CGA-TEG-3Gal | \[^{[126]}\] | Cyclic glucosamine | LecA | 460 | 270\[^{[b]}\] | 50\[^{[a]}\] \(63\[^{[b]}\]\) | 98 |
| CGA-TEG-4Gal | \[^{[126]}\] | Cyclic glucosamine | LecA | 310 | 150\[^{[b]}\] | 56\[^{[a]}\] \(69\[^{[b]}\]\) | 49 |
| CGA–Ph-4Gal | \[^{[126]}\] | Cyclic glucosamine | LecA | 79 | 57\[^{[b]}\] | 222\[^{[b]}\] \(303\[^{[b]}\]\) | 1.2 |
| FulGal1 | \[^{[127]}\] | Fullerene | LecA | 688\[^{[b]}\] | 27\[^{[b]}\] | 250 | (3\[^{[b]}\]) |
| FulGal2 | \[^{[127]}\] | Fullerene | LecA | 40\[^{[b]}\] | 458\[^{[b]}\] | 0.78 | (1068\[^{[b]}\]) |
| FulGlc | \[^{[127]}\] | Fullerene | LecA | 233 \(\times 10^{[0]}\) \[^{[b]}\] | 0.08\[^{[c]}\] | 2\[^{[a]}\] | 63 |
| GNP2 | \[^{[128]}\] | AuNP | LecA | 5800 | | | |
| GNP3 | \[^{[128]}\] | AuNP | LecA | 760 | | | |
| GNP6 | \[^{[128]}\] | AuNP | LecA | 50 | | | |
| 100%-GalNP | \[^{[129]}\] | Polymer NP | LecA | | | | 6.31 \(1495\[^{[c]}\]\) |
| 50%-GalNP | \[^{[129]}\] | Polymer NP | LecA | | | | 3.15 \(992\[^{[c]}\]\) |
| ZnPor | \[^{[106]}\] | Zn-porphyrin | LecA | 332 | 500\[^{[b]}\] \(1400\[^{[b]}\]\) | 113.5\[^{[a]}\] | 63 \(159\[^{[a]}\]\) |

\[^{[a]}\] ITC; \[^{[b]}\] ELLA; \[^{[c]}\] SPR; \[^{[d]}\] relative potency by HIA.
symmetry. Such systems were studied as carbohydrate-processing enzyme inhibitors and FimH inhibitors before their interactions with LecA were studied. Dodecavalent fullerene FulGal1 and FulGal2 had nanomolar IC₅₀ for LecA (Table 7), with potency enhanced by the “glycoside cluster effect”. Higher affinity of the latter was rationalised in terms of the aromatic Tz group adjacent to the galactose, available for interactions with hydrophobic residues in LecA's binding site. An unexpected result was obtained with a Glc analogue of these systems, FulGlc, acting as a modest inhibitor for LecA, on a similar scale to the monosaccharide reference, indicating the possibility of additional mechanisms for “sugar-balls” to inhibit LecA even through non-specific binding.

Gold nanoparticles with a core diameter of < 2 nm were functionalised with thiol-containing glycoconjugates. Different presentation densities were assessed, by adding varying ratios of galactoside and glucoside arms (Figure 11) to give GNP2, GNP3 and GNP6 (among others) with galactose presentation densities of 17%, 33% and 100%, and valencies of 12, 15 and 67 respectively. Interactions with LecA were studied by qualitative HIA, and quantitatively by SPR and ITC. All three techniques showed similar trends, with ligands presented on nanoparticles demonstrating increases in avidity for LecA, compared to free glycoconjugates in solution. This further increased with presentation density; GNP6 had nearly a 3000-fold affinity enhancement (Kₐ 50 nM, Table 7). ITC pointed to reduction in entropic penalties being key to enhanced binding, with presentation density important for increasing enthalpic contributions. Increase in ligand-activity was explained by both increased effective concentration of Gal, as well as structural contributions. Instead, the coordination geometry and appropriate linker-length is proposed to be favourable for chelating an active therapeutic role for the metal in this anti-biofilm activity. The coordination geometry and appropriate linker-length is proposed to be favourable for chelating adjacent Gal binding-sites in LecA, as was seen for calix[4]arene structures of similar geometry, CalixB[113,114]. Clusters based on various carbohydrate epitopes were tested for their ability to inhibit PAO1 biofilm-formation. Tetravalent galactocluster RuBTP1 with flexible EG₃-spacers inhibited biofilm-formation at 5 mM, while neither the precursor ligand, nor complex RuBTP2 (without spacer) did. None of the Ru(II)-complexes tested were bactericidal or bacteriostatic, discounting an active therapeutic role for the metal in this anti-biofilm activity. Instead, the coordination geometry and appropriate linker-length is proposed to be favourable for chelating adjacent Gal binding-sites in LecA, as was seen for calix[4]arene structures of similar geometry, CalixB[113,114]. Use of metal ions of different coordination geometry to tune the activity of multivalent glycoconjugates presents opportunities for future developments.

3.7. Metal complexes

Among the large variety of multivalent scaffolds, the use of metal complexes in this field is very rare, with only two examples to the best of our knowledge. A propargylated porphyrin scaffold was selected by Cecioni et al. for comparison with CalixB[128] and P₃Cyc, but CuAAC reactions were unsuccessful for the unmetallated scaffold (due to copper-complexation). Consequently, the scaffold was complexed with Zn(II) before successful triazole formation, giving ZnPor (Figure 11), a tetravalent galactocluster with flexible EG₃-arms. Here the Zn(II) ion has no structural or functional role, but seems to be present only for synthetic simplicity. ZnPor is a more potent HIA inhibitor for LecA than either calix[4]arene or β-peptoid derivatives in the same study, owing perhaps to its square planar geometry. Similar evidence of LecA-affinity was seen in SPR. ITC measurements showed that ZnPor had a Kₐ of 332 nM and the rigid and planar topology seems to be conducive to lowering entropy costs of binding, allowing for 1:2 complexes to form with the lectin (but not for all four epitopes to engage in aggregative-chelate binding).

Byrne and co-workers recently reported use of Ru(II)-coordination chemistry to template formation of metal-centred tetravalent glycoconjugates from lower valency ligands, namely divalent bis[(triazolyl)pyridine glycoconjugates.[129,130] Clusters based on variables carbohydrate epitopes were tested for their ability to inhibit PAO1 biofilm-formation. Tetravalent galactocluster RuBTP1 with flexible EG₃-spacers inhibited biofilm formation at 5 mM, while neither the precursor ligand, nor complex RuBTP2 (without spacer) did. None of the Ru(II)-complexes tested were bactericidal or bacteriostatic, discounting an active therapeutic role for the metal in this anti-biofilm activity. Instead, the coordination geometry and appropriate linker-length is proposed to be favourable for chelating adjacent Gal binding-sites in LecA, as was seen for calix[4]arene structures of similar geometry, CalixB[128,113,114]. Use of metal ions of different coordination geometry to tune the activity of multivalent glycoconjugates presents opportunities for future developments.

4. Coupling Lectin Targeting with Additional Functionality

Among the most inspiring recent examples of PA lectin inhibitors are those which couple this strategy with further innovative functionality, in search of therapeutic or diagnostic tools. Some complimentary anti-virulence, imaging and antimicrobial examples are highlighted here (Figure 12), along with their future outlook.

Two different approaches have been reported, attempting to simultaneously target lectins and hijack PA’s siderophore pathway, which uses iron-chelators to transport Fe(III) to the cell membrane for metabolic activity. In addition to siderophores produced by the bacterium, PA can recruit exogenous siderophores. Calix[4]arene-clusters CalixG (Figure 12, Table 7),
with hydroxamic acid arms, put PAO1 cells under stress under iron-limiting incubation conditions, causing increased production of endogenous fluorescent siderophore pyoverdine-I, as PA competes with chelating CalixG for Fe(III). Moreover bacterial growth of a siderophore-deficient PA strain was significantly disrupted by CalixGMan and to a weaker extent by CalixGcat indicating that calix[4]arenes were not recruited as exogenous iron-chelators. Biofilm-inhibition results of these systems, however, were not as expected, with fucosides being inactive but CalixGMan giving reduction of 70% at 50 μM. Curiously, a tested α-glucocluster analogue, demonstrated very strong biofilm-inhibition (92% at 20 μM). This raises serious questions about whether lectin-binding is key to anti-biofilm effects, since glucose does not interact with LecB. Possibly, the hydroxamic acid arms play a more significant role, either due to anionic charge or their propensity to release nitric oxide, which is known inhibit biofilms. This study highlights the importance of adequate control experiments before antipseudomonal activity is unambiguously attributed to lectin-inhibition.

Carbohydrate-centred clusters derived from MAN4 (Section 3.1) functionalised to include catechol or hydroxamate arms for iron-chelation were also studied with PAO1 (and several knockout strains), indicating that catechol-appended galactoclusters penetrated the bacterial envelope, exploiting the siderophore pathway in a “Trojan horse” strategy. Despite evidence for localisation at the cell membrane (where 5% of LecA is located), no decrease in virulence was observed. Protection assays of human pulmonary cell cultures against PAO1 demonstrated 70% protection with MAN10 (Section 3.1) functionalised to include catechol or hydroxamate arms for iron-chelation were also studied with PAO1 (and several knockout strains), indicating that catechol-appended galactoclusters penetrated the bacterial envelope, exploiting the siderophore pathway in a “Trojan horse” strategy.

Encouragingly, accumulation of these conjugates in the biofilm was significantly from the parent glycosides, and binding-affinity of conjugates for LecA and LecB didn’t differ significantly from the parent glycosides, and CipHyb (Figure 12) effectively bound LecB from both PAO1 and PA14 strains. Encouragingly, accumulation of these conjugates in the biofilm was higher than ciprofloxacin alone, corresponding with increased concentration of lectins in this difficult-to-drug environment. This observation is key to supporting the hypothesis that lectin-targeting allows conjugates to further penetrate biofilm. Unfortunately both strains of PA showed lower antimicrobial-susceptibility to conjugates than to ciprofloxacin. Decreased antibiotic activity is also seen upon initial penetration of 2.4 μM. Such anti-biofilm activity was previously shown for other bacteria but suffered from a lack of specificity. This heteroglycocuster leads to targeted effects for PA; anti-biofilm activity is not seen when the ΔlecA ΔlecB knockout strain was used, nor for Staphylococcus aureus. This confirms that

**Figure 12.** Selected examples of glycocusters coupled with additional functionality. Lectin-targeting components are shown in blue, and active components in red.
activity is driven by lectin-binding. These rotaxane anti-virulence compounds were non-bactericidal and were found not to damage red blood cell membranes. These impressive results marry lectin-targeting with separate anti-virulence effects and offers much scope for additional development.\textsuperscript{[11]} The future of this field will rely on translation of lectin-targeting to agents, like those outlined in this section, to be effective in a clinical setting.

5. Conclusions

The main considerations which have been shown to impact inhibitor affinity and effectiveness for anti-biofilm and antiadhesive effects are: (a) the correct choice of carbohydrate epitope; (b) the role of linker length, hydrophobicity and rigidity; and (c) carbohydrate presentation and multivalency (scaffold topology).

(a) Native monosaccharide ligands for PA lectins serve as a reliable starting point for building more sophisticated higher-affinity glycoconjugate inhibitors. For LecA, almost all reported examples are based on \( \text{D-Gal} \) epitopes. For LecB, the situation is slightly more complex. Many fucosides and fucoclusters are reported with high affinities for LecB, and indeed LecB has unusually high affinity for L-Fuc, by comparison to other C-type lectins. However it is less selective, as also recognises \( \text{D-Man} \) (with a similar affinity to LecA’s binding with \( \text{D-Gal} \) and \( \text{D-arabinose} \). The challenge of selective targeting has been addressed to give molecules with drug-like properties, by incremental rational design of bespoke glycomimetics (Section 2.1), combining structural aspects of \( \text{D-Man} \) and L-Fuc which promote strong protein-ligand interactions, giving selectivity and affinities comparable to natural glycans.

(b) LecA-inhibition benefits significantly from careful tuning of the properties of the linker between several epitopes due to two key structural factors. Firstly, the His50 residue in LecA’s binding pocket can engage in “T-shaped” CH–π interactions: suitably-positioned hydrophobic aromatic aglycons (particularly phenyl-derivatives), greatly enhance affinity in numerous examples detailed above. Hydrophobic linkers however, in several cases lead to solubility limitations contrasting with more flexible hydrophilic \( \text{EG}_\text{L} \)-linkers, meaning this consideration must be balanced with other structural features of the inhibitor. Secondly, the distance between adjacent binding-sites (\( \sim 29 \) Å) allows for careful design of linker-length and rigidity to result in strong chelate-binding. Combining these considerations has led to divergent systems with low-nanomolar affinities. For LecB, which has a longer inter-binding site distance, the impact of linker-tuning is less dramatic. The ability to cross-link two tetramers of LecB in an aggregative mode plays a greater role, and a chelate-binding mode is seldom proposed.

(c) Multivalency increases avidity for many inhibitor-lectin interactions, but LecA-targeting systems benefit most markedly from glycose cluster effects, resulting in enhanced potency per epitope beyond simple concentra-

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(but no biofilm-inhibition), and targeted anti-biofilm activity of heteroglycocluster [2]rotaxanes at low-micromolar concentration. These examples represent exciting steps forward and we anticipate increased development in the near future for applications of well-designed PA lectin-targeting molecules in medicinal chemistry.

**Abbreviations**

ADMET | absorption, distribution, metabolism, excretion and toxicity
---|---
CF | cystic fibrosis
CuAAC | copper(I)-catalysed azide-alkyne cycloaddition
Cy3 | cyanine3 dye
DDI | DNA-directed immobilisation
Gal | α-galactose
Glc | α-glucose
Man | β-mannose
IC50 | isothermal titration calorimetry
HIA | hemagglutination inhibition assay
EG | ethylene glycol
ELLA | enzyme-linked lectin assay
FP | fluorescence polarisation
H-bonding | hydrogen-bonding
MBIC | minimum biofilm-inhibition concentration
MIC | minimum inhibitory concentration
PrOF-NMR | protein-observed fluorine nuclear magnetic resonance
PA | *Pseudomonas aeruginosa*
\(r_p/n\) | relative potency per carbohydrate epitope (compared to a monovalent control compound)
SAR | structure-activity relationship
SPR | surface plasmon resonance
Tz | triazole
XRD | X-ray diffraction

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords:** carbohydrate-protein interactions - glycoconjugates - lectins - *Pseudomonas aeruginosa* - biofilms

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