Glycopeptide dendrimers as *Pseudomonas aeruginosa* biofilm inhibitors†

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Synthetic glycopeptide dendrimers composed of a branched oligopeptide tree structure appended with glycosidic groups at its multiple N-termini were investigated for binding to the *Pseudomonas aeruginosa* lectins LecB and LecA. These lectins are partly responsible for the formation of antibiotic resistant biofilms in the human pathogenic bacterium *P. aeruginosa*, which causes lethal airway infections in immune-compromised and cystic fibrosis patients. Glycopeptide dendrimers with high affinity to the lectins were identified by screening of combinatorial libraries. Several of these dendrimers, in particular the LecB specific glycopeptide dendrimers **FD2** and **D-FD2** and the LecA specific glycopeptide dendrimers **GalAG2** and **GalBG2**, also efficiently block *P. aeruginosa* biofilm formation and induce biofilm dispersal *in vitro*. Structure–activity relationship and structural studies are reviewed, in particular the observation that multivalency is essential to the anti-biofilm effect in these dendrimers.

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† Part of the multivalent scaffolds in glycosciences themed issue.

1. Introduction

The spread of antibiotic resistant bacteria is one of the most pressing problems in human health today. The ubiquitous Gram-negative bacterium *Pseudomonas aeruginosa* infects immune-compromised and cystic fibrosis patients and causes lethal

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airway infections.¹ The formation of bacterial communities attached to biotic or abiotic surfaces, the so-called process of biofilm formation, forms a physical barrier to antibiotics and allows antibiotic resistance. Disrupting this process can restore antibiotic sensitivity and overcome resistance.² Biofilm formation is mediated in part by the galactose-specific lectin LecA (PA-IL)³ and the fucose-specific lectin LecB (PA-IIL).⁴ Deletion mutants lacking the *lecA* or *lecB* gene showed impaired biofilm formation,⁵ suggesting that the inhibition of the corresponding lectins might lead to biofilm inhibition and a therapeutic effect in controlling *P. aeruginosa* infections.

Lectins are ubiquitous carbohydrate-binding proteins with specific but often weak binding sites, whose potency is increased by multivalency leading to the so-called cluster effect.⁶ Multivalency occurs both on the side of the lectin (e.g. both LecA and LecB are tetrameric with four identical carbohydrate binding sites)⁷ and on the side of the glycosidic ligand such as multiply glycosylated glycoproteins. A variety of synthetic glycoclusters⁸ have been investigated for inhibiting LecA,⁹ LecB,¹⁰ or both,¹¹ and in selected cases for their effects in controlling *P. aeruginosa* infections *in vivo*.¹² This review focuses on a related effort to develop inhibitors of LecA and LecB on the basis of glycopeptide dendrimers.¹³

Dendrimers are regularly branched synthetic macromolecules¹⁴ particularly well suited for multivalent display of chemical groups and displaying useful properties for a broad range of applications such as catalysis¹⁵ and biomedical¹⁶ applications, including the multivalent display of carbohydrates for lectin binding.¹⁷ While most dendrimers are built of organic polymer building blocks, peptide dendrimers are assembled from proteinogenic amino acids using diamino acids such as lysine as branching points,¹⁸ which generates a protein-like structure whose properties can be fine-tuned by varying the length and composition of the peptide arms throughout the structure. Advantages of peptide dendrimers over most other dendrimers include: (a) a straightforward and modular synthesis by solid-phase peptide synthesis (SPPS) from commercially available building blocks yielding pure products rather than polymeric mixtures, (b) the possibility of tuning properties such as water solubility, hydrophobicity and charges by varying amino acids, and (c) a low potential for toxicity when applied *in vivo* due to the use of natural amino acid building blocks, although immunogenicity must be considered. Disadvantages include: (a) a relatively expensive synthesis by SPPS limited to multimilligram amounts at the research stage and (b) a high conformational flexibility given by the nature of the building blocks.

Appending the peptide arms with a glycosidic ligand within a combinatorial library set-up led to the discovery of the fucosylated peptide dendrimer **FD2**,¹⁹ a tetravalent ligand binding to the fucose-specific lectin LecB, and the related galactosylated dendrimers **GalAG2** and **GalBG2**, which target the galactose specific lectin LecA. These dendrimers are potent *P. aeruginosa* biofilm inhibitors (Fig. 1).^{20,21} Structure–activity relationship studies show that multivalency is crucial for biofilm inhibition and dispersal with these ligands.

Fig. 1 Structures of *P. aeruginosa* biofilm inhibitors **FD2**, **GalAG2** and **GalBG2**. **FD2** targets LecB, **GalAG2** and **GalBG2** both target LecA. Peptides written with one-letter codes for amino acids, K is a branching lysine with peptide chains extending at both the α - and the ϵ -amino groups. The C-terminus at the core is CONH₂.

2. Peptide dendrimers

2.1. Solid-phase peptide synthesis

Most dendrimers are symmetrical molecular trees consisting of a conserved branching network assembled from repetitive dendrons, on which functional end groups are grafted in a multivalent manner. We recently introduced a new class of dendrimers assembled from amino acid building blocks by solid-phase peptide synthesis.²² In these dendrimers the branches consist of variable amino acids connected by diamino acid branching points. Under optimized conditions using a relatively low resin loading (0.2 mmol g⁻¹) and double coupling (carrying out the peptide coupling step twice for each residue), the approach gives pure products in good isolated yields up to third generation dendrimers with eight end groups, typically containing 37 residues after 11 coupling cycles. In the divergent approach of SPPS, all arms are grown simultaneously, however orthogonal protection can yield asymmetric sequences.²³ Larger peptide dendrimers containing up to 341 amino acids and 64 end groups can be obtained by convergent ligation assembly²⁴ of smaller peptide dendrimer building blocks using the multivalent thioether formation between multiple N-terminal chloroacetamide groups of a “core” dendrimer and the thiol

group of a cysteine residue as the first residue of another peptide dendrimer, the so-called ClAc ligation.²⁵ Initial studies with enzyme models highlighted multivalency effects of histidine in esterase dendrimers,^{26–28} and of N-terminal proline in aldolase dendrimers.²⁹

2.2. Combinatorial libraries of protein-like peptide dendrimers

The possibility of varying amino acid side chains throughout the peptide dendrimer allows one to perform mutational adaptations similar to those in proteins and peptides. By selecting amino acids, one can define key properties such as defined ratios of hydrophilic and hydrophobic groups to promote solubility in various media, or the presence of key functional groups to enable binding or catalysis. Fine-tuning of these desirable properties can be reached by systematic variations using combinatorial chemistry capitalizing on split-and-mix synthesis of one-bead-one-compound libraries, the founding paradigm of combinatorial chemistry initially developed for linear peptides.³⁰ We introduced two new features to the SPPS protocol that rendered the combinatorial method more user friendly: (1) the decoding of the dendrimer sequence on polymer beads by amino acid analysis, which is an inexpensive, reliable and fast analytical method suitable to sequence hundreds of beads, a method which we later also adapted for linear peptides;³¹ (2) simplified on-bead and off-bead assays enabling fast and reliable identification of positive hits with various fluorescence assays.^{32,33} This combinatorial approach was successfully employed for the identification of cobalamin ligands,^{34,35} various dendritic esterases³⁶ and aldolase enzyme models,³⁷ iron binding peptide dendrimers³⁸ and antimicrobial peptide dendrimers.³⁹

Peptide dendrimers mimic natural proteins by the nature of their building blocks. The combinatorial library approach extends the analogy by making it possible to evolve the peptide dendrimers by sequence optimization, recapitulating the natural evolution of proteins by mutations and selection cycles. Although the peptide dendrimer sequence space is much smaller than that of a real protein, the dendritic topology avoids the necessity of folding by forcing the peptide dendrimer to adopt a globular structure. The dendritic architecture results in disordered conformations resembling the molten globule state of proteins,^{40–42} with a dynamic tertiary structure containing few native-like secondary structure elements such as α -helices and β -sheets.⁴³ In contrast to proteins, peptide dendrimers are entirely stable towards denaturation. The susceptibility of a peptide dendrimer towards proteolysis can be controlled by the degree of branching. Peptide dendrimers with three amino acids between the branching points may undergo proteolysis at protease cleavage sites, whereas only one amino acid between the branching points leads to complete resistance against proteases.⁴⁴

3. Glycopeptide dendrimers

3.1. Synthesis of glycopeptide dendrimers

Peptide dendrimers can be used to mimic glycoproteins by attaching glycosidic groups at the end of the peptide dendrimer branches.

Since the peptide dendrimer topology is in itself non-natural, we did not use the natural N- and O-glycosidic linkages but rather a more simple attachment chemistry compatible with SPPS and potentially high-yielding. Two approaches were investigated: (1) the oxidative cleavage of N-terminal serine residues with sodium periodate to generate a glyoxamide end group suitable for oxime ligation with an oxyamine-functionalized carbohydrate;^{45,46} (2) amidation of the multiple N-termini of the peptide dendrimer with a peracetylated glycoside containing a free carboxylic acid on the aglycone, followed by resin deacetylation by basic methanolysis prior to acidic cleavage. The two approaches were tested in a study of glycopeptide dendrimers as drug delivery dendrimers for colchicine,^{47,48} which showed that the amidation protocol as the last step of SPPS was the more practical method to prepare glycopeptide dendrimers. The approach was also applied to prepare concanavalin A ligands.⁴⁹

3.2. Selection of fucose lectin inhibitors from combinatorial libraries

Our initial approach to glycopeptide dendrimer lectin ligands was inspired by previous examples of combinatorial glycopeptide libraries.⁵⁰ In particular we used a previously reported enzyme-linked lectin assay (ELLA) to visualize binding of lectins to SPPS beads to identify binding sequences in a combinatorial library.^{50c} A 15'625-member library **L1** of G2 dendrimers with three amino acids per branch and lysines as branching diamino acids was prepared on tentagel beads and acylated at their N-termini with α -C-fucosyl-acetic acid **1** (Fig. 2). Amino acids were distributed in six variable positions to allow positive, negative, hydrophobic, small and polar or aromatic residues throughout the sequence, using each amino acid twice at two positions in successive branches to allow decoding by amino acid analysis.³² The library was assayed for binding to the fucose-specific lectin UEA-I lectin in the presence of 3 M free L-fucose, which reduced staining from 90% of the beads to less than 2% of the beads, allowing us to pick selective hits. The critical role of L-fucose was evidenced by the absence of lectin binding to the control N-acetylated dendrimer library.

A selection of hit sequences were resynthesized and their lectin binding characterized in detail by ELLA,⁵¹ which identified hit **FD2** as the most potent ligand with an $IC_{50} = 11 \mu M$ towards lectin UEA-I and a relative potency of 115 in reference to L-fucose, corresponding to a six-fold affinity enhancement per fucosyl group. Although the multivalency effect observed with **FD2** was modest, it was confirmed by the observation of much weaker binding in the divalent and monovalent glycopeptide analogs of the dendrimer. Valency variations in a dedicated library further confirmed that strong binding required at least four carbohydrate groups on the dendrimer.⁵²

In subsequent combinatorial experiments,⁵³ the fucosylated dendrimer library used to discover **FD2** was rescreened using a recombinant *P. aeruginosa* LecB⁵⁴ labelled with rhodamine B instead of UEA-I with the hope of identifying even stronger LecB ligands. Screening was performed with a 0.45 M fucose competitor in the presence of 1% bovine serum albumin to

Fig. 2 Synthesis and structure of glycopeptide dendrimer combinatorial libraries **L1** and **L2** used to identify **FD2**.

reduce non-specific binding. Beads retaining an intense fluorescent ring after extensive washing with fucose were considered as positive hits. These dendrimers contained predominantly cationic sequences similar to those identified using lectin UEA-I. Thus, six out of nine sequences carried multiple positively charged residues (Lys at X⁶ or Arg at X⁵). These positive charges do not seem to interact with the lectins, and might have been selected in the library experiment for providing good water solubility and allowing a favourable orientation of the glycosyl groups for lectin binding. On the other hand, one sequence was neutral and one was anionic, suggesting that charged side chains might not be necessary for optimal binding to LecB. Functionalizing the peptide dendrimer library with 4-carboxyphenyl- α -L-fucoside **2** at the N-termini to form library **L2** followed by screening yielded several new glycopeptide dendrimers such as **PA8** and **PA9** binding to LecB with comparable strength to **FD2**.⁵⁵ However these dendrimers were less soluble than **FD2** due to their more hydrophobic carboxyphenyl aglycone, and also did not yield substantially stronger biofilm inhibition effects.

Comparison of a series of dendrimers identified by screening as well as further multivalency variations showed that binding was strongly influenced by multivalency and by the presentation of the carbohydrate by the peptide backbone. An overview of the binding properties of fucosylated glycopeptide dendrimers to LecB as determined by ELLA is shown in Table 1. One particularly striking result was the observation of a very strong binding with dendrimer **2G3**, an octavalent analogue of **FD2**,

Table 1 ELLA data for binding to LecB^a

Cpd	<i>n</i> ^b	Sequence	IC ₅₀ (μ M)	Rel. pot. ^c	Fucose ^d
L-Fucose	1	—	11	1.0	1.0
2G0	1	cFuc-KPL	5.9	1.9	1.9
2G1	2	(cFuc-KPL) ₂ KFKI	2.7	4.1	2.0
FD1	4	(cFuc-KHV) ₄ (KHGA) ₂ KHI	0.43	26	6.4
FD2	4	(cFuc-KPL) ₄ (KFKI) ₂ KHI	0.14	79	20
FD3	4	(cFuc-KHL) ₄ (KGKI) ₂ KHI	0.19	58	15
FD4	4	(cFuc-KRD) ₄ (KSRA) ₂ KHI	0.54	20	5.1
FD9	4	(cFuc-SPD) ₄ (KEVA) ₂ KHI	0.63	18	4.4
FD10	4	(cFuc-ETD) ₄ (KTGA) ₂ KHI	0.25	44	11
PA5	4	(cFuc-GRV) ₄ (KEGL) ₂ KHI	0.75	15	3.7
PA6	4	(cFuc-GTV) ₄ (KHPT) ₂ KHI	0.30	37	9.2
PA7	4	(cFuc-GHT) ₄ (KTGD) ₂ KHI	0.28	39	9.8
PA8	4	(OFuc-KAD) ₄ (KSGA) ₂ KHI	0.11	100	25
PA9	4	(OFuc-EHD) ₄ (KEVA) ₂ KHI	0.35	31	7.8
PA10	4	(cFuc-GTV) ₄ (KHPT) ₂ KHI	0.21	52	13
PA10b	4	(cFuc-GTVH) ₄ (KPT) ₂ KHI	2.4	4.6	1.1
PA11	4	(cFuc-EHY) ₄ (KYGD) ₂ KHI	0.26	42	11
2G3	8	(cFuc-KP) ₈ (KLF) ₄ (KKI) ₂ KHI	0.025	440	55
2G3'	8	(cFuc-KP) ₈ (BLF) ₄ (BKI) ₂ BHI	0.94	12	1.5
10G3	8	(cFuc-ET) ₈ (KDY) ₄ (KGA) ₂ KHI	0.12	92	12

^a Linear peptide notation from N- to C-terminus as CONH₂ using one-letter codes for amino acids, *K* denotes a branching lysine and *B* a branching L-2,3-diaminopropanoic acid with both amino groups acylated, cFuc: c-fucoside from building block **1**, OFuc: O-fucoside from building block **2**; binding data from ELLA; all assays were run in triplicate. ^b *n* is the number of fucosyl groups in the ligand. ^c Rel. pot. = relative potency = IC₅₀(fucose)/IC₅₀(ligand). ^d Rel. pot. per fucose = rel. pot./*n*.

and simultaneously of a significantly weaker binding for its analog **2G3'** where the branching lysine residues were replaced by the more compact *Dap* (2,3-diaminopropanoic acid) branching amino acid. Interestingly, the positioning of the branching diamino acid was critical to binding, as illustrated by the observation that dendrimer **PA10b**, with the G2 branching point moved by one position inward to allow a more flexible and potentially further reaching the outer tetrapeptide arm, actually lost one order of magnitude in binding compared to the parent sequence **PA10**.

3.3. Biofilm inhibition with LecB ligands

The *P. aeruginosa* lectin LecB plays a role in biofilm formation, as evidenced by the impaired biofilm formation in Δ LecB mutant strains and in the partial inhibitory effect of *p*-nitrophenyl fucoside (NPF) on wild type biofilms but not on Δ LecB biofilms. Glycopeptide dendrimer **FD2** and analogs were evaluated for biofilm inhibitory activity against *P. aeruginosa* biofilms using the steel coupon assay.⁵⁶ In this assay bacterial biofilms are grown on stainless steel coupons and stained with acridine orange. At a concentration of 50 μ M, all tested dendrimers were able to reduce biofilm formation significantly, but only **FD2** inhibited biofilm formation completely. All dendrimers were also tested for their ability to disperse already existing biofilms, which is more similar to the *in vivo* situation. In both the biofilm inhibition and the dispersal assay, all the dendrimers showed a remarkable activity. The effect was much stronger than with the monovalent control α -NPF. The high biofilm inhibitory activity of **FD2** was also observed on several clinical isolates of *P. aeruginosa*. **FD2** did not show any effect on the

Fig. 3 *P. aeruginosa* biofilm inhibition assay. (A) Inhibition of *P. aeruginosa* PAO1 biofilms. (B) Biofilm inhibition of PAO1 and three *P. aeruginosa* clinical isolates. Biofilm inhibition was performed using the steel coupon assay on the wild-type strain PAO1 without additive (Ref.), or with added α -NPF (0.5 mM) or dendrimers (0.05 mM). See ref. 57 for details.

deletion mutant Δ *LecB*, giving evidence that the biofilm inhibitory effect is *LecB*-mediated.

Since **FD2** contains several protease cleavage sites and is conformationally flexible, we synthesized **D-FD2** (cFuc-kpl)₄-(Kfki)₂Khi-NH₂, a stereoisomer of **FD2** containing D-amino acids, with the hope of obtaining an analog retaining biological activity but more stable towards proteolytic degradation.⁵⁷ The affinity of **D-FD2** to *LecB* determined by ELLA was 5-fold weaker than that of **FD2**, indicating that the amino acid chirality has some influence on binding affinity, an effect accompanied by a smaller secondary structure content as measured by CD and a less compact structure compared to **FD2** as measured by the hydrodynamic radius. Nevertheless **D-FD2** showed similar potency in inhibiting the biofilm formation of *P. aeruginosa* strain PAO1 and was also able to disperse already existing biofilms. When tested on clinical isolates of *P. aeruginosa*, **D-FD2** was however less active than **FD2** (Fig. 3). Proteolytic treatment of **D-FD2** with trypsin or chymotrypsin for 48 h showed no degradation, whereas **FD2** was completely degraded after 30 min under the same conditions. **D-FD2** was also stable in human serum.

3.4. *LecA* ligands and biofilm inhibitors

Since **FD2** was identified as being a high-affinity ligand to the fucose-specific lectin *LecB* and a potent *P. aeruginosa* biofilm inhibitor, we tested if the peptide sequence of **FD2** may also be suitable for inhibitors of the galactose-specific *P. aeruginosa* lectin *LecA*. Considering that galactosides with hydrophobic groups at the sugar anomeric position show enhanced affinity

Table 2 Hemagglutination assay and ITC data for binding to *LecA*^a

Ligand	Name or sequence	<i>n</i>	MIC (μ M)	r.p./ <i>n</i>	<i>K</i> _d (μ M)	r.p./ <i>n</i>
D-Gal	D-Galactose	1	3100	1	88	1
NPG	<i>p</i> -Nitrophenyl- β -galactoside	1	550	6	14	6
IPTG	Isopropyl- β -thiogalactoside	1	1100	3	32	3
GalAG0	GalA-KPL	1	80	40	4.2	20
GalAG1	(GalA-KPL) ₂ KFKI	2	31	50	0.5	90
GalAG2	(GalA-KPL) ₄ (KFKI) ₂ KHI	4	0.8	1000	0.10	220
GalBG0	GalB-KPL	1	2500	1.3	52	1.7
GalBG1	(GalB-KPL) ₂ KFKI	2	630	2.5	2.1	20
GalBG2	(GalB-KPL) ₄ (KFKI) ₂ KHI	4	130	13	0.4	60

^a Peptide sequences indicated with one-letter codes for amino acids, *K* = branching lysine. The C-terminus in CONH₂. MIC = minimal inhibitory concentration for the hemagglutination assay. *n* = number of galactose residues per ligand, r.p./*n* = relative potency per galactose residue = (MIC(D-galactose)/MIC(ligand))/*n* or (*K*_d(D-galactose)/*K*_d(ligand))/*n*.

to *LecA*,⁵⁸ the 4-carboxy-phenyl β -galactoside (GalA) was used as a galactosyl group at the terminus. To evaluate the effect of the linker to affinity, a second set of glycopeptide dendrimers bearing carboxypropyl- β -thiogalactoside (GalB) as an N-terminal glycoside was also prepared. For a better understanding of the effect of multivalency on binding, the monovalent linear tripeptide arms **GalA/BG0** and the divalent dendrimers **GalA/BG1** were also considered. Characterization of the binding interaction of *LecA* with the various ligands by hemagglutination inhibition assay⁵⁹ and isothermal titration calorimetry indicated a strong multivalency effect on both the GalA and the GalB series (Table 2). The tetravalent dendrimer **GalAG2** showed the strongest binding.

The *LecA* specific glycopeptide dendrimers not only bound tightly to *LecA*, but also inhibited *P. aeruginosa* biofilms (Fig. 4). The G2 dendrimers showed strong biofilm inhibition, while the lower multivalency analogs and the control N-acetylated dendrimers were essentially inactive. The strong biofilm inhibition by **GalBG2**, a relatively weak but tetravalent ligand, showed that multivalency was essential for biofilm inhibition, while the actual binding constant was not essential. These data were consistent with a *LecA*-mediated inhibition of *P. aeruginosa* biofilms by the dendrimers.

Fig. 4 Inhibition of *P. aeruginosa* biofilms by glycopeptide dendrimers binding to *LecA*. Biofilm assays were performed using the steel coupon assay⁵⁶ at 20 μ M galactose endgroup concentration.

Fig. 5 Models of glycopeptide dendrimer lectin complexes obtained from MD simulations. The protein is shown in cartoon representation and the glycopeptide dendrimers in CPK. The Ca^{2+} ions are shown in magenta and indicate the location of the carbohydrate binding sites.

3.5. Structural studies

Structural studies were carried out to gain an insight into the interaction of the tetravalent glycopeptide dendrimers **FD2**, **GalAG2** and **GalBG2** with their lectins. While crystallization attempts with the tetravalent dendrimers and the lectins all failed, single crystals were obtained for complexes of the terminal tripeptides **2G0** in complex with LecB,²⁰ and **GalAG0** and **GalBG0** in complex with LecA.²¹ In these structures the terminal glycosidic group was observed to bind at the expected position. In the case of the LecB complex with **2G0** and the LecA complex with **GalBG0** the tripeptide portion of the ligands was disordered. In the case of **GalAG0** however, the tripeptide was well resolved. The most striking interaction concerned the interaction of the 4-carboxyphenyl group with residues His50 on the lectin, which interacted *via* a histidine-T-stack interaction. This interaction, which probably explains the stronger binding of aromatic galactosides to LecA, was also observed in the corresponding nitrophenyl galactoside LecA complex and was unprecedented for ligand–protein interactions.²¹

Lectins LecA and LecB both have a typical tetrameric structure with four carbohydrate binding sites. Multivalency effects on lectin binding are often interpreted in terms of a multivalent ligand binding simultaneously to two or more carbohydrate binding sites on the same lectin. A molecular dynamics (MD) study was carried out to gain an insight into the possible cause of the multivalency effects that might explain the stronger binding of **FD2** to LecB and **GalA/BG2** to LecA as compared to their monovalent ligands. Models of the dendrimers bound to their respective lectin were constructed by fusing the MD simulated dendrimer structures with the experimentally determined tripeptide–lectin complexes **2G0.LecB**, **GalAG0.LecA** and **GalBG0.LecA**, followed by energy minimization and simulations for 10 ns. In all three cases the dendrimer arms were clearly too short to enable bridging between two carbohydrate binding sites on the same lectin tetramer, ruling out chelation type multivalency effects as a cause for the increased binding (Fig. 5).

The structural models provided an insight into the interactions of the glycopeptide dendrimers with their lectin. In all cases

protein–ligand contacts were visible outside the carbohydrate binding site, some of which were also confirmed in docking studies with the terminal tripeptides.⁵³ Such contacts probably explain the fact that the amino acid sequence of the glycopeptide dendrimers significantly influences lectin binding as observed in the extensive study with various glycopeptide dendrimer ligands to LecB (Table 1). On the other hand the structures cannot explain the biofilm inhibition effect observed, in particular the fact that several strong binding tetravalent ligands such as **PA5** and **PA6** (binding LecB) fail to exert a significant effect on the biofilm, while other weaker ligands such as **GalBG2** (binding LecA) are potent biofilm inhibitors. One possibility of explaining these discrepancies might involve localization effects relative to the site of action of the lectins.⁶⁰ The formation of supramolecular aggregates might also play a role. Indeed both LecA and LecB are believed to enable tissue attachment and biofilm formation by multivalent cross-linking between glycosidic groups on the bacterial surface and in the tissues. Certain multivalent lectin ligands might lead to the formation of insoluble aggregates that effectively take the lectins out of the system, while others could act as cross-linking agents between lectins to form a higher multivalency complex still capable of acting as a cross-linking lectin and therefore promoting biofilm formation. Additional studies will be required to explain the biofilm inhibition mechanism of the glycopeptide dendrimers.

4. Conclusion and outlook

A straightforward and easily tunable SPPS and combinatorial library approach to glycopeptide dendrimers was implemented to rapidly discover potent multivalent ligands to the *P. aeruginosa* lectins LecA and LecB. Variations in branching sequences and multivalency were carried out leading to an extensive structure–activity landscape for lectin binding. Further optimizations are in progress concerning the nature of the tripeptide arms in contact with the lectin and the degree of multivalency as possibilities to increase lectin binding and biofilm inhibition.

Many questions are still open concerning the biofilm inhibition effect observed with the glycopeptide dendrimers. While multivalency is clearly crucial to obtain biofilm inhibition, strong binders are not necessarily good biofilm inhibitors, which might indicate the formation of lectin complexes still functional for promoting biofilm formation. Biofilm inhibition might also depend on localization of the ligand within the biofilm and not only on binding. Further studies will be necessary to identify the site of action of the lectin inhibitors. Potency optimization will also be required by further amino acid and valency variation to reach sub-micromolar levels not only in lectin binding but also in biofilm inhibition.

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