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Blocking Disease Linked Lectins with Multivalent Carbohydrates

Marjon Stel and Roland J. Pieters

Department of Chemical Biology & Drug Discovery, Utrecht Institute for Pharmaceutical Sciences, Utrecht University,
PO Box 80082, 3508 TB Utrecht, the Netherlands

14.1 Introduction

Lectins are carbohydrate-binding proteins involved in various biological phenomena of life. Since every cell surface is covered with oligosaccharide structures, a layer called the glycocalyx, lectins play an important role in signalling pathways and cell adhesion [1,2]. Carbohydrate–lectin binding often depends on multivalent interactions because the affinity of one single binding event between a lectin and a monovalent carbohydrate ligand is often quite low. However, several binding interactions together can reach high affinities [3,4]. For this reason, synthetic ligands targeting lectins benefit from multivalency, as we will see in this chapter.

Inhibiting binding of these lectins to their natural ligands can possibly prevent the diseases they are involved in. In this chapter we will discuss a selection of lectins and their inhibitors. For example, the lectins LecA and LecB from the bacterial pathogen *Pseudomonas aeruginosa*, the hemagglutinin protein of the influenza A virus and the cholera toxin (CT) from the bacterium *Vibrio cholerae* are all involved in infectious disease. Human galectins play a role in inflammation and numerous cancer mechanisms. In this chapter, we will cover a variety of ligands that bind these proteins, aiming to inhibit their natural function which will hopefully lead to new therapeutics. In addition, we will cover Concanavalin A as one of the most studied lectins and, finally, a selection of propeller lectins that can have up to ten carbohydrate binding domains arranged symmetrically resembling a propeller.

Binding of multivalent ligands to lectins can occur through a number of mechanisms, the most important of which are mentioned here (Figure 14.1) [5,6]. Chelation of a ligand is possible when the ligand can bridge the distance between two binding sites on the same protein or protein complex. Crystal structures of these proteins provide detailed information on the distance between binding sites, allowing for the design of sufficiently long linkers and scaffolds. An alternative mechanism is statistical rebinding, where one carbohydrate binding site is freed up by its carbohydrate ligand, but due to multiple ligands presented closely together, another ligand can bind immediately after,

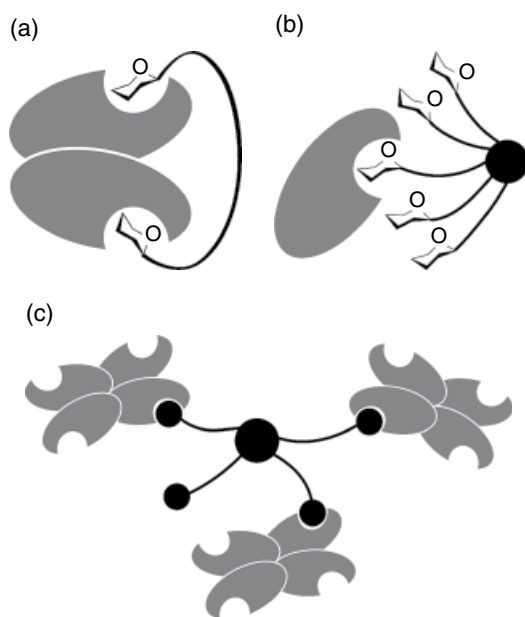


Figure 14.1 Three major binding mechanisms of lectin-carbohydrate systems: (a) chelation; (b) statistical rebinding; and (c) aggregation.

leading to low overall off rates. For this mechanism, only the ligand has to be multivalent, the protein does not, or may have multiple binding sites too far apart to be bridged. For these cases aggregation is also often observed, the ligand then bridges binding sites on different proteins, forming a network of proteins and ligands, leading to larger species that may precipitate out of solution. It is less clear what the possible contribution of the aggregation is to the inhibitory potency.

Detecting binding of ligands to these lectins can be done through widely used techniques such as isothermal calorimetry (ITC) providing thermodynamic parameters and stoichiometry, and surface plasmon resonance (SPR) providing insight into the association and dissociation rates. In the case of aggregation of ligands and lectins, sedimentation velocity analytical ultracentrifugation (SV-AUC) and dynamic light scattering (DLS) can provide information about the size of the particles formed. Atomic force microscopy (AFM) has been used for similar purposes, and is able to show the shape of the particles. NMR and X-ray crystallography provide atomic detail not only of the proteins themselves, allowing detailed design of ligands, but also of the specific interactions between protein and ligand. In addition, a variety of lectin specific assays have been developed [1,4]. As this chapter deals with carbohydrate-lectin binding, a short overview will be given here.

The haemagglutination inhibition assay (HIA) depends on the ability of lectins or viral particles that display lectins to agglutinate (aggregate) red blood cells. Adding a soluble ligand causes less precipitate to form, giving a minimum inhibitory concentration (MIC) or a concentration at the halfway point between fully aggregated and no aggregation at all (IC_{50}). The enzyme-linked lectin assay (ELLA) is a variant of the widely used ELISA (enzyme-linked immunosorbent assay). A natural carbohydrate ligand is immobilized on the surface of a microtiter plate, while the synthetic ligand and

lectin are in solution. The lectin is labelled with a fluorescent tag or an enzyme such as horse-radish peroxidase (HRP). HRP catalyses the conversion of a pro-dye to a dye, thus providing colour to the well proportional to the amount of lectin that is absorbed on the surface. This assay gives an IC_{50} as well, based on the curve-fitting of absorbance and the concentration range of the synthetic ligand used.

The solid-phase inhibition assay, specific to galectins, depends on immobilized asialofetuin (ASF) or glycosylated bovine serum albumin (BSA). Asialofetuin is a glycoprotein containing three triantennary glycans terminated with LacNAc. The galectins that can recognize the terminal galactose, are in solution, where ligands will prevent binding to the glycoprotein on the surface [7].

Finally, instead of immobilizing the protein or natural ligand, the synthetic ligand can be immobilized. In the context of a microarray, this allows for testing a large number of potential ligands and even determining a fingerprint for the binding specificity of a protein. For example, glycodendrimers (which will be discussed later in this chapter for various lectins) with a valency of one to eight were outfitted with various carbohydrates such as glucose, galactose and mannose. Testing a variety of fluorescently labelled lectins against this panel provided insight into the specificity and the effect of multivalency [8]. Besides chemical immobilization, a DNA-directed microarray has been used, where ligands contain a stretch of DNA that binds to the complementary strand of DNA on the microarray surface. This allows for screening a large number of ligands of which initially only a very small amount has to be synthesized [9].

In determining the effect of multivalency, the fold of improvement of the multivalent ligand is generally compared as improvement *per sugar*, compared with either the monovalent monosaccharide, or an equivalent monovalent ligand that contains a similar linker to the multivalent ligand.

14.2 Haemagglutinin

Haemagglutinin (HA) and neuraminidase (NA) are the two major proteins on the surface of influenza A viruses (IAVs). With 18 types of HA and 11 types of NA, IAVs are referred to through these subtypes. Pandemic strains, such as H1N1 (the Spanish flu in 1918), H2N2 (Asian pandemic in 1957) and H3N2 (Hong Kong flu in 1986), have caused millions of deaths. The annual influenza is fortunately milder than these pandemic strains, but there is concern over more recently reported H5N1 and other H5 and H7 strains, that are very pathogenic and could emerge as a new pandemic [10,11].

HA is a trimeric membrane protein, with three carbohydrate binding sites, evenly spaced on one side of the protein complex (see Figure 14.2 for a protein monomer) [12–14]. It recognizes sialic acids (*N*-acetylneuraminic acid, Neu5Ac) and linked LacNAc sections, and specificity for the sialic acid linkage to the linked galactose determines the ability of the virus to infect certain organisms. Avian HA recognizes α 2-3-linked sialic acids, while human HA recognizes predominantly α 2-6-linked sialic acids.

Vaccines have to be updated yearly due to the antigenic shift: as the virus mutates over the year, the vaccines lose their potency [10,15,16]. Current therapies, such as zanamivir (Relenza) and oseltamivir (Tamiflu), aim to block NA activity, which cleaves terminal sialic acids to facilitate the release of viral particles [17,18]. Resistance against these antivirals has been reported for H5N1 and H1N1 strains, among others [19,20]. Amantadine targets an M2 ion channel of IAV, which maintains a favourable

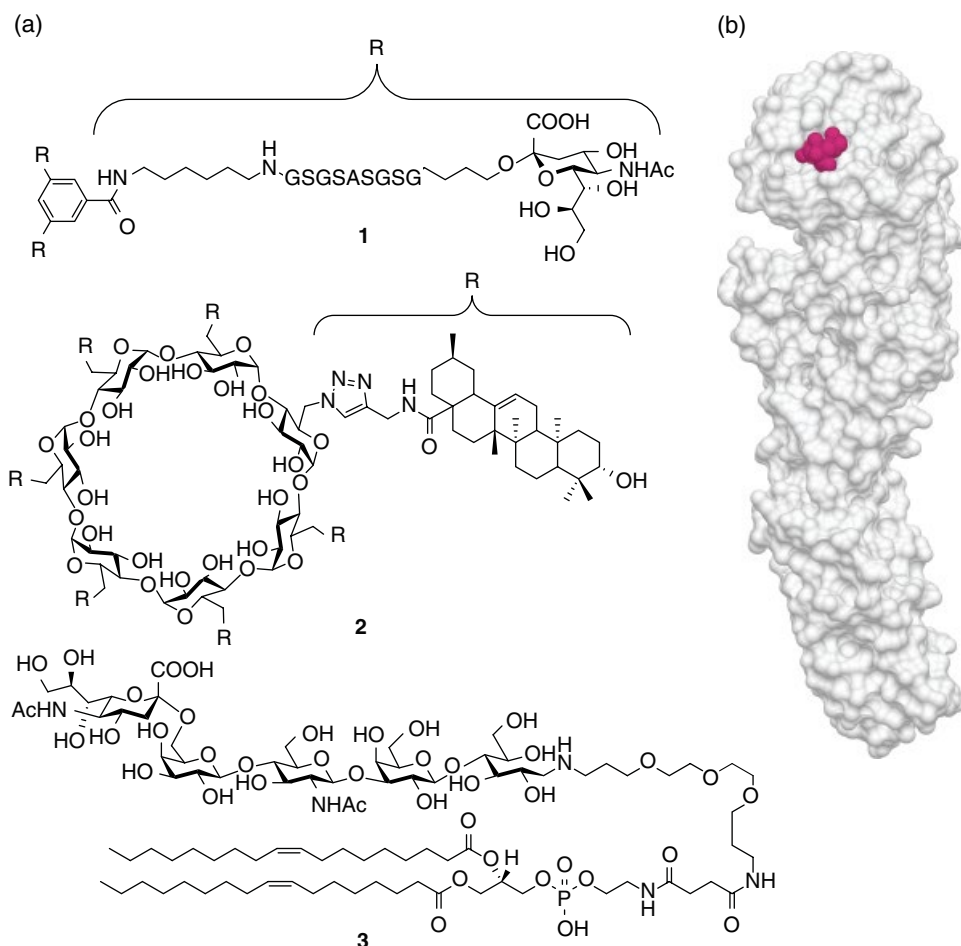


Figure 14.2 (a) Multivalent inhibitors against haemagglutinin. (b) A single H5 hemagglutinin with sialic acid (PDB: 1JSO). In the cell membrane, three haemagglutinin molecules associate and form a trimeric structure.

environment around the viral membrane, but many viral strains have become resistant to this antiviral drug [10]. No clinically approved therapies are available that target HA but blocking viral attachment might be advantageous to prevent infection in the first place and presents an opportunity to find new therapies.

The trivalent ligand 1 with a benzene core, alkyl-peptide linkers and sialic acids was presented to HA H5 from avian influenza [21]. SPR studies found a dissociation constant (K_d) of 450 nM for this ligand, which is a 4000-fold improvement compared with Neu5Ac- α 2Me. A shorter peptide or alkyl linker led to K_d s in the millimolar range, and showed little improvement compared with a monovalent equivalent ligand containing the same peptide and alkyl linker. This indicates the importance of designing a ligand with sufficiently long spacers. Using molecular dynamics (MD) simulations, the ligand was fitted to H7, and found to bind with all three sialic acids in the binding sites, with some movement still allowed in the linker regions.

The heptavalent ligand **2**, consisting of a β -cyclodextrin scaffold with triterpene ligands attached through CuAAC reaction, was found to be active against influenza A in the early stage of the viral lifecycle [22]. Further experiments indicated an interaction between HA1 and the ligand, with a K_d of 2.08 μ M as determined by SPR.

While most multivalent compounds discussed in this chapter are relatively small compounds, multivalency can also be achieved through supramolecular structures such as micelles and liposomes. Compound **3** containing an oligosaccharide terminated with an α 2-6 linked sialic acid, chosen for its ability to bind to a number of influenza HAs, could assemble into liposomes and present this oligosaccharide [23]. The monovalent compound showed no inhibition of influenza A infection, while liposomes containing 7.5 mol% of **3** were able to inhibit infection at an effective concentration of 10 nM sialic acid. This inhibition was specific for α 2-6 linked sialic acid bearing influenza A viruses. Liposomes and micelles containing an S-linked α 2-6-sialolactoside phospholipid were able to interfere with H1N1 influenza virus entry into MDCK cells [24].

Sialic acid-containing polyacrylamide polymers bound influenza A virus particles in an agglutination assay of erythrocytes in concentrations below 15 nM [25]. Brush-polymers with α 2-6-sialosides were also able to inhibit haemagglutination, where a longer spacer from scaffold to ligand was advantageous [26]. Having a larger amount of sialosides per polymer was beneficial. They were specific for influenza strains binding α 2-6, while α 2-3 influenza viruses in the haemagglutination assay were not inhibited.

Polyglycerol-based nanoparticles of 3–4 nm displaying approximately 20–35 sialic acids were found to have an IC_{50} in the micromolar range for a haemagglutination assay, while nanogels of 50 or 70 nm displaying 10 000–60 000 sialic acids had an IC_{50} in the low nanomolar range [27]. A study of sialoside-gold nanoparticles showed that the binding of a virus particle and nanoparticle depends on the size of this colloidal particle [28]. A small particle, smaller than the approximate 50 Å of a virion particle, benefits somewhat from multivalency. These small particles decorate the exterior of the virus. Large nanoparticles form clusters of virus and nanoparticles, thereby inhibiting cell adhesion and thus infection; in this study up to 70% inhibition of virus binding to erythrocytes.

14.3 LecA

LecA is one of the two lectins on the surface of *Pseudomonas aeruginosa* (PA) and is also known as PA-IL. LecA and LecB (or PA-IIL, discussed later in this chapter) are two important lectins in PA infection, a Gram-negative bacterium responsible for example for severe lung infections and forms a risk especially for cystic fibrosis patients [29,30]. LecA is a tetrameric, calcium-dependent lectin [31] (Figure 14.3) with a specificity for galactose and galactose derivatives, especially those attached to a hydrophobic aglycone [32–34]. In binding oligosaccharides on the cell surface of epithelial cells, it facilitates adhesion of PA to the patient's cell surface and the subsequent infection. Additionally, LecA is involved in the formation of PA biofilms [35], a protective polymeric matrix around the bacteria that makes it much more difficult for the immune system or therapeutics to reach the bacterial cells. Inhibition of both PA cell adhesion and biofilm formation can be achieved through LecA ligands, a promising approach to fighting these infections [36].

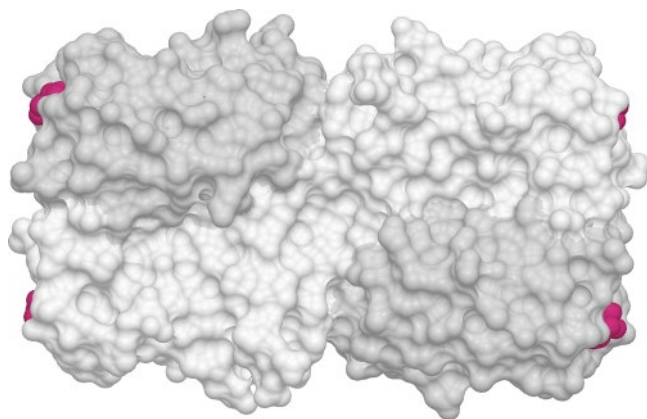


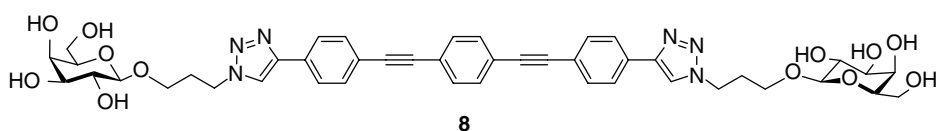
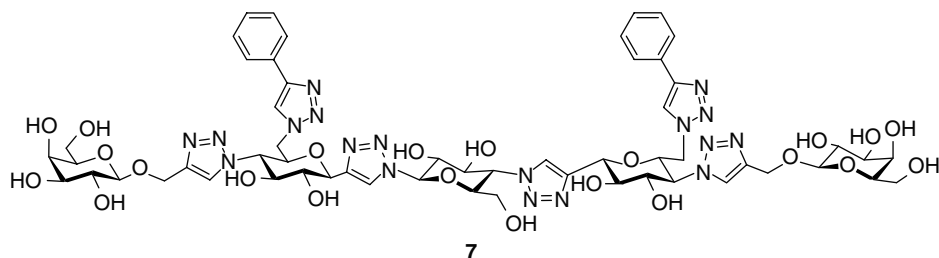
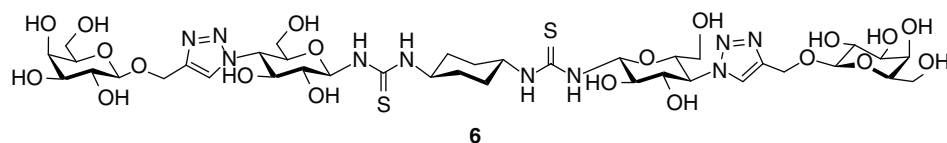
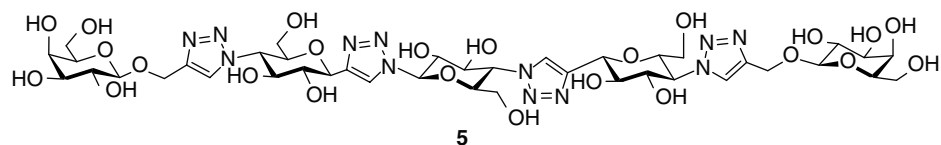
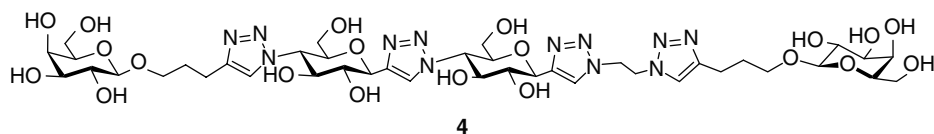
Figure 14.3 The structure of the tetrameric LecA with D-galactose in each binding site (PDB: 1OKO).

In order to facilitate a chelate binding mechanism of a multivalent ligand, the distance between the binding sites on the protein should be taken into account. The distance between the binding sites on LecA is approximately 26 Å [31], however, a flexible spacer such as poly(ethylene glycol) (PEG) needs to be about three times as long to bridge a given length [37]. By making the spacer much more rigid, a compound with a more defined length can be obtained. An initial exploration of these rigid spacers led to **4** (Figure 14.4a), where a series of glucose and triazole units were outfitted on both ends with galactose [38]. These were soluble in water, an important property for such ligands. The spacer length is crucial, especially for entirely rigid compounds, while a bit of flexibility by introducing some alkyl chains makes the ligand more forgiving for not being exactly the right length. The best binder **4** was found to have an IC_{50} of 220 nM, an improvement over the monovalent ligand of 545-fold. This assay used fluorescently labelled LecA in solution with a galactoside-functionalized chip surface.

Optimization of this divalent rigid ligand led to **5** which showed an improved IC_{50} of 2.7 nM and a K_d of 28 nM [39]. Molecular modelling showed the spacer had a length of 24 Å, a good match for the 26 Å between binding sites of LecA. An expected stoichiometry for all divalent compounds of 0.5 was found, indicating the chelate binding mechanism. Two types of variants of this compound were prepared. In one case, two triazoles were replaced by thiourea spacer units **6** [40], which had a K_d in the same range as **4**. Another series of modifications were at the 6 position of two of the spacer glucose moieties [41]. These substituents were positively and negatively charged, lipophilic and also aromatic as shown for compound **7** and were thought to interact with the protein surface. While found to be very good binders as well, they did not improve upon previous ones, suggesting that the groups were rotated towards the solvent, rather than the protein surface. A very different type of rigid ligand was **8** with alternating phenyl and alkyne moieties. The IC_{50} compared with the monovalent ligand improved 130-fold, as determined through an ELISA-like assay on a glycochip [42].

The binding mechanism of these divalent rigid ligands was confirmed by X-ray crystallography (Figure 14.4b) of **5** in which the entire molecule was visible, including the spacer. This is a clear indication that a more rigid spacer leads to a well-defined complex. The spacers in PEG linked multivalent ligands are rarely visible in X-ray

(a)



(b)

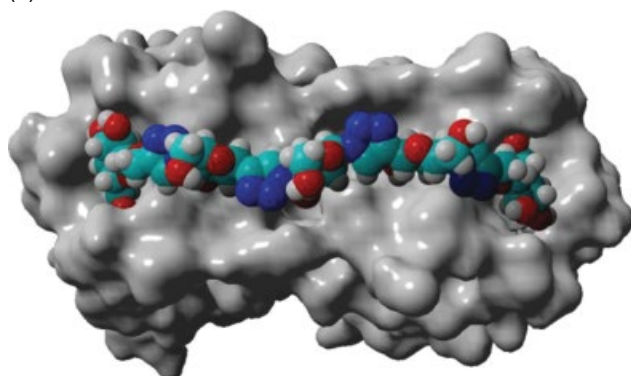


Figure 14.4 (a) Rigid divalent inhibitors of LecA. (b) X-ray structure of ligand **5** bound to LecA. See colour section.

crystallography. Interestingly, in the crystal structure a number of direct and water bridged hydrogen bonds between the protein and the spacer were observed. It is not yet clear how much these protein–spacer interactions contribute to the binding affinity [43]. These ligands did not lead to aggregation, as determined by SV-AUC. Unfortunately, these ligands do not inhibit biofilm formation.

Glycopeptide dendrimers that use lysine as a branching unit were synthesized and evaluated for binding to LecA [44–46]. They contained tripeptide spacers and galactose end-groups. Compound **9** (Figure 14.5), a tetravalent ligand, was found to be the best binder to LecA, with a K_d of 0.1 μM , a 219-fold improvement over galactose [44]. The multivalent effect was clear, as from a monovalent galactopeptide to the tetravalent ligand, the K_d improved with every step. The haemagglutination assay showed a 1000-fold improved inhibition for the tetravalent ligand, while the mono- and divalent ligands showed only a 40- to 50-fold improvement. More importantly in the context of infection, **9** can prevent formation of biofilms and disperse existing ones, but the dendrimers were not toxic by themselves.

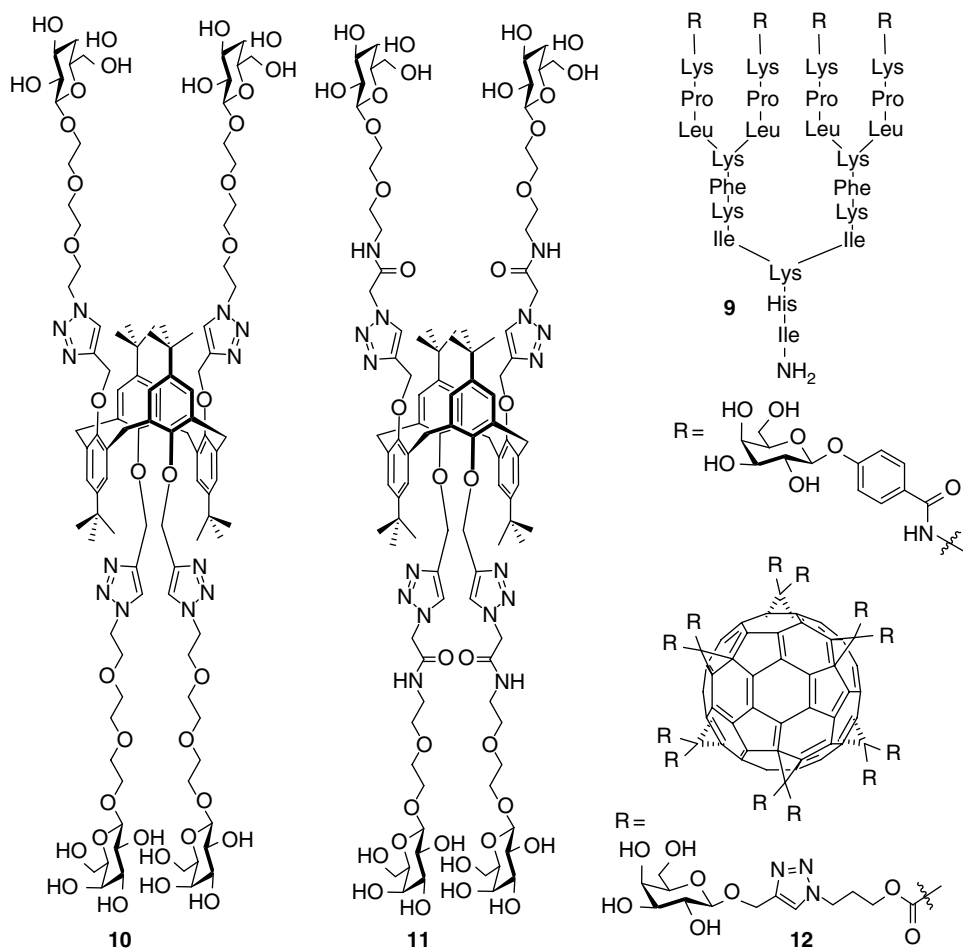


Figure 14.5 Inhibitors of LecA.

The X-ray structure of the monovalent galactose-tripeptide with LecA shows a T-shaped interaction of the phenyl with a CH of His50, explaining why LecA prefers aromatic galactosides, to the thioethyl galactoside linker dendrimers also synthesized [44]. An additional X-ray structure of the first generation peptide dendrimer found only the terminal tripeptide galactoside bound to LecA, while the rest of the dendrimer was not visible [43]. Optimization of the tripeptide linker, starting from **9** led to two galactose-tripeptides that have a slightly worse K_d and inhibition of biofilm formation, but improved biofilm dispersion, making them interesting from a therapeutic standpoint [45].

A study of calix[4]arene topology indicated that two ligand arms on either side of the calixarene scaffold was most advantageous **10** [47]. This 2:2 topology, with two carbohydrate ligands on one side of the calix[4]arene ring and two on the other side, had a K_d of 176 nM, a 852-fold improvement over the monovalent ligand. The 3:1 calix[4]arene showed a similar K_d of 200 nM, that is a very good ligand for LecA as well. Both had a stoichiometry of one ligand to one LecA monomer. This binding possibly occurs through a chelate binding mechanism, but some precipitation occurred during ITC experiments indicated aggregation as well. AFM studies using this 2:2 calix[4]arene revealed the formation of 90–500 nm filaments [48,49]. These filaments contain about 10–50 LecA tetramers linked by these glycoclusters. This supports the aggregative chelation mechanism of binding, likely in addition to chelation. Furthermore, it was found to inhibit cell adhesion between lung epithelial cells by around 70%, compared with 30% with the monovalent β -GalOMe, and additionally reduced biofilm formation of PA and reduced lung infection in an *in vivo* lung infection model [50].

In a somewhat more rigid linker between galactoside and calix[4]arene by introduction of amide bonds **11**, K_d again indicated this 2:2 topology as the most efficient ligand for LecA [51]. With a PEG-amide linker, a K_d of 90 nM was found.

Pillar[5]arene scaffolds displaying up to 10 galactosides reached nanomolar affinities [52,53]. Dodecavalent fullerenes improved binding, according to a HIA, by more than 12 000-fold, accounting for about 1000-fold improvement per sugar displayed. SPR and ELLA binding studies showed less dramatic improvements in binding [54].

Glycoclusters with a scaffold based on pyranosides allow for a variety of topologies and linkers to lead to generally tetravalent ligands. A broad approach, using mannose, galactose, and glucose as a central scaffold and a wide variety of phosphodiester linkers showed that having a mannose scaffold with sufficiently long linkers containing aromatic motifs was best for binding to LecA [55]. Following up with mannose as a scaffold, a variety of phosphodiester linkers containing thioethers, PEG, amides, and triazoles were tested for their influence on binding to LecA [56]. LecA prefers aromatic galactoside residues for binding, which was reflected in the performance of these linkers in a fluorescent microarray assay. The linker lacking this aromatic residue performed worse, and in general, solvated linkers were better than hydrophobic ones. However, the best binding was found with a previously synthesized linker containing a triazole moiety in addition to the phenyl, that is **13** (Figure 14.6). This was hypothesized to be due to the rigidity of the galactoside or the introduction of an interaction between Pro51 and the triazole in addition to the previously reported interaction of the aromatic moiety and His50. This interaction of an aromatic aglycone with Pro51 and His50 was confirmed when comparing a number of aromatic linkers. *O*-naphthyl and *O*-biphenyl showed the best affinity, compared with *O*- and *S*-benzyl and phenyl linkers [57]. Biofilm formation

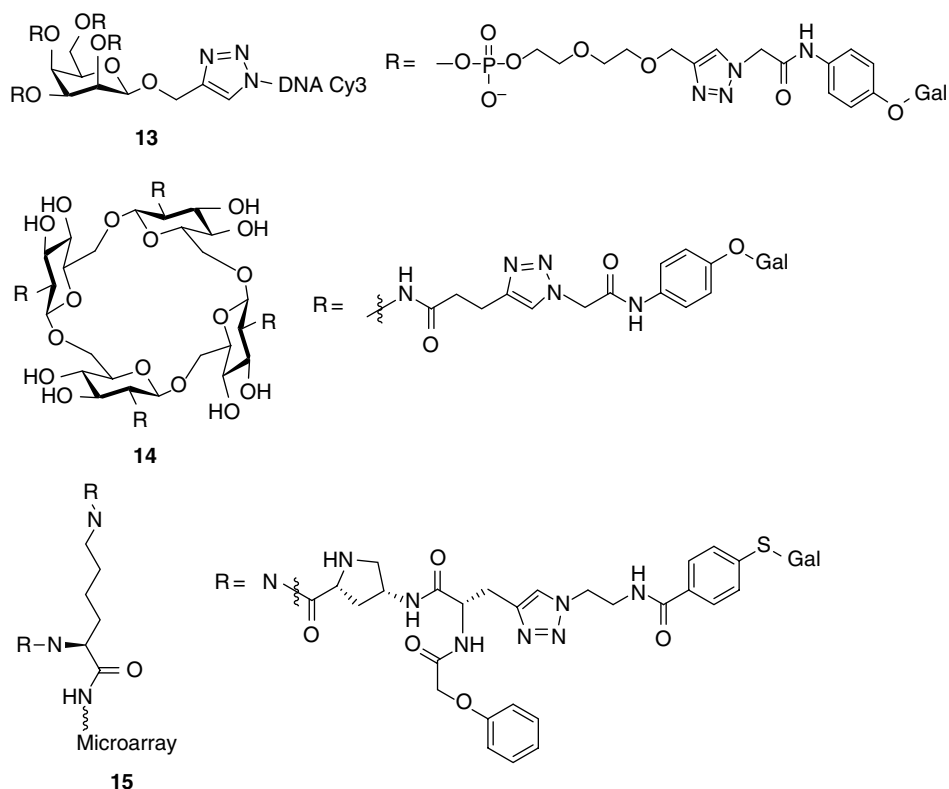


Figure 14.6 Inhibitors of LecA with mannose or cyclodextrin as a scaffold, and a divalent inhibitor found through the screening of a combinatorial library.

was inhibited with **13** up to 40%. A control experiment of a ΔlecA mutant of PA showed that biofilm formation cannot be entirely inhibited by this ligand, implying the role of other lectins in this process [9].

Divalent and tetravalent glycoclusters based on cyclic glycan **14** were found through ELLA to have IC_{50}s in the nanomolar range, with up to 1200-fold improvements compared with the monovalent compound [58]. Especially the rigid linker, containing phenyl and triazole moieties, contributed to a much better binding, findings that were reflected in HIA and ITC experiments.

Taking a combinatorial library approach, compound **15** was found to be a very good ligand to LecA with a K_d of 82 nM and remarkably showed a large reduction of the ability of PA to enter human epithelial lung cells [59]. Modelling suggests a chelate binding mechanism and interaction of the phenyl with His50.

14.4 LecB

LecB (or PA-IIL) is the other major soluble lectin produced by PA. Similar to LecA, this lectin is involved in cell adhesion and biofilm formation, and inhibition of both processes can be achieved through ligands targeting this fucose-specific lectin [60]. Like LecA, it

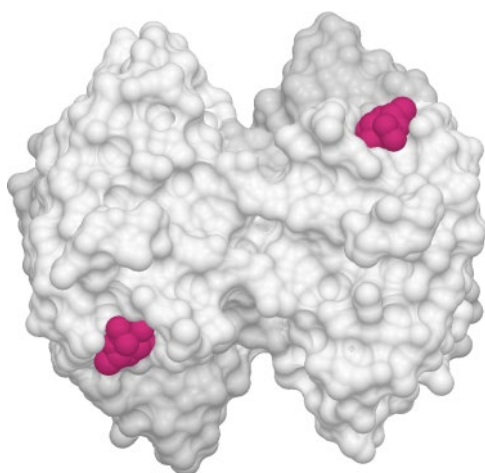


Figure 14.7 LecB binding α -L-fucose (PDB: 1OXC). Two binding sites are on the back of the protein in this view.

is a tetrameric protein with four carbohydrate binding sites that can bind both L-fucose and L-mannose [61–63] but in LecB these sites are more evenly spaced with approximately 40 Å between the sites [64–66], quite a distance to bridge with a multivalent ligand (Figure 14.7). An ELLA determined IC_{50} for α -FucOMe was found to be 430 nM [67], which is already a tightly binding ligand, so making even more tightly binding, multivalent ligands is not without challenge.

A number of tightly binding peptide dendrimers have been made with affinity in the nanomolar range (Figure 14.8). A C-fucosyl peptide dendrimer library led to the discovery of an octavalent **16** that has an IC_{50} of 25 nM as determined through ELLA, a 55-fold improvement per fucose. Lower valency showed much more modest improvements [68]. Molecular modelling showed that the peptide sequence did not interact extensively with LecB, implying that the multivalency was key to the improved potency. Dendrimer **17** has an affinity for LecB that is 46-fold higher than fucose and was shown capable of inhibiting biofilm formation. Since biofilm inhibition did not occur in a Δ *lecB* strain, this interaction between **17** and LecB seems crucial for biofilm inhibition [69]. Substituting the L-amino acids in **17** for D-amino acids altered the affinity for LecB, from 0.14 μ M for **17** to a slightly higher 0.66 μ M for **D-17** [70]. However, the D-variant was proteolytically stable while **17** was not and was still able to inhibit biofilm formation. A wide variety of tetravalent peptide dendrimers presenting both fucose and galactose, and Lewis^a trisaccharide were synthesized [46]. In the crystal structure of **18** with LecB, two binding sites are bridged by ligands fused through a Cys-Cys dimerization, while for the other two binding sites only the terminal arm is found. No chelate binding mechanism is observed. These dendrimers were able to disperse biofilms and inhibit their formation, doing so in sub-inhibitory concentrations when used in conjunction with the antibiotic tobramycin. A K_d of 28 nM and ELLA determined IC_{50} of 0.6 nM was found for **19**, a hexadecavalent α -fucosylated glycocluster [67]. The improvement compared to α -FucOMe was 65-fold per sugar. β -Fucosylated glycoclusters were notably worse ligands than the α -fucosides, however, multivalency benefits of these β -fucosylated glycoclusters were slightly larger, with a 86-fold improvement in IC_{50} for the best ligand.

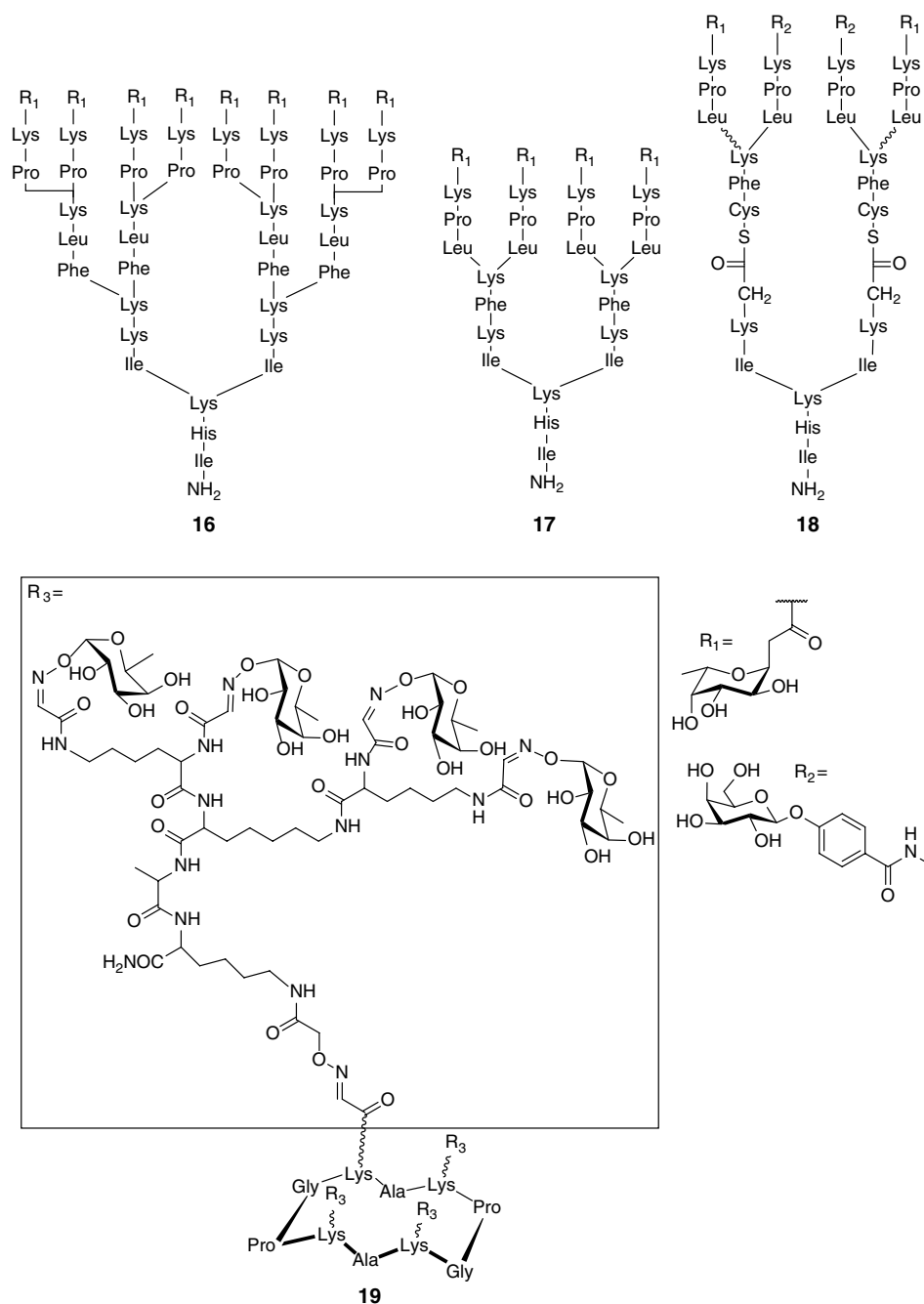


Figure 14.8 Multivalent peptide-based dendrimer ligands for LecB. The wavy lines indicate a bond with the side chain of the amino acid.

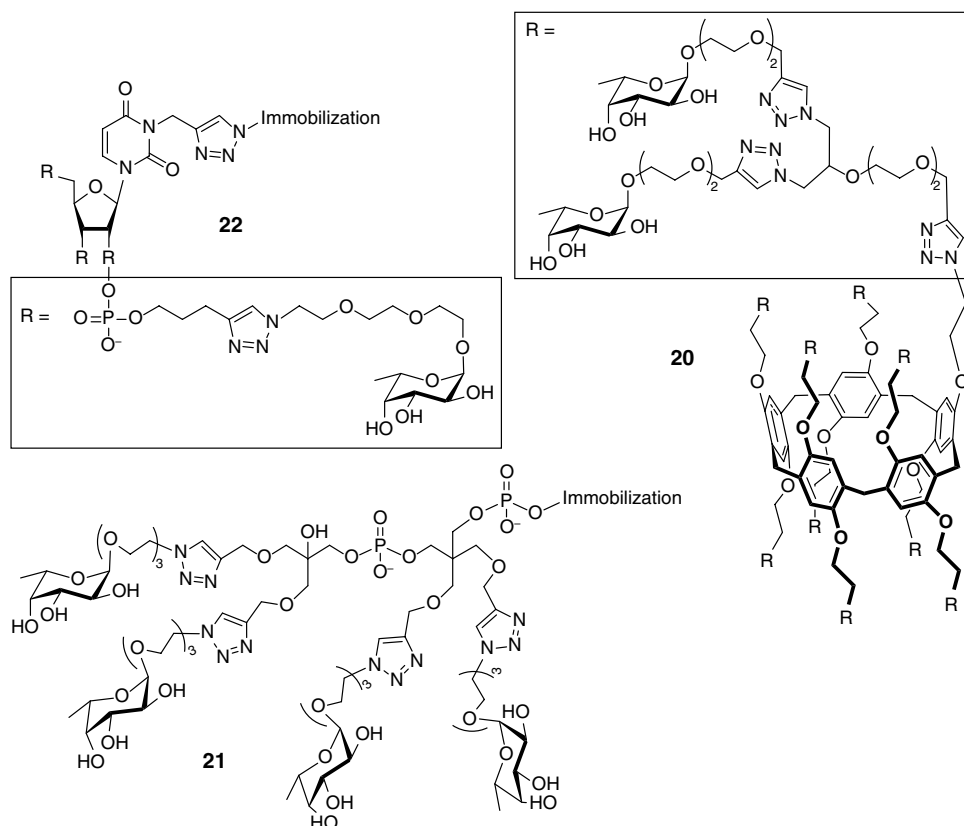


Figure 14.9 Multivalent ligands against LecB.

For the tetravalent 2:2 calix[4]arene **10** for LecB now presenting fucosides (Figure 14.5), a K_d of 304 nM was found with a stoichiometry of occupying three binding sites [50]. This compound cannot bridge the distances between LecB binding sites to facilitate a chelate binding mechanism, so a bind and jump mechanism was proposed [71], where LecB jumps from ligand to ligand, that is a rebinding mechanism. The calix[4]arene compound exhibited a 90% inhibition of PA adhesion to lung epithelial cells and reduced biofilm formation, but this happened in a LecB-independent manner as found out with the $\Delta lecB$ mutant. A similar structure is the pillar[5]arene **20** [52] (Figure 14.9). Aggregation of seven to eight LecB monomers to one decavalent ligand was found and ten LecB monomers for the icosavalent ligand. ELLA gave an IC_{50} of 6 nM, a 74-fold improved binding compared with the monovalent fucose; however, ITC experiments gave K_d s only up to a 3-fold improvement. C60 fullerenes were also effective, especially those presenting 24 instead of 12 fucosides, with a K_d of 23 nM [72]. Aggregation was found with a stoichiometry of three LecB lectins bound per fullerene, but only few of the fucoses present on the fullerene participate at any given time.

In varying the topology (antenna, linear or crown-like) of immobilized glycoclusters, high valency could be reached with **21** with a tetravalent antennary topology most preferred [73]. Since the glycoclusters were immobilized on a microarray, synthesis could be done on a picomole scale.

Using glycoclusters with various furanose-based cores, affinities between 56 nM and 95 nM were found [74], with ribose **22** and xylose-based scaffolds outperforming arabinose. Linking two mannose-centred ligands with a long linker performed slightly better on these microarrays than the single mannose-centred ligand [75].

14.5 Galectins

Galectins are a group of proteins that recognize β -galactosides and are widely spread throughout life [76,77]. Fifteen mammalian galectins are known and they fulfil a role in many cellular processes such as cell recognition [3,78], adhesion [79,80] and in signalling pathways [78,81], both inside and outside the cell. In humans, they are involved in the immune response [82,83] but especially their roles in infection [84] and cancer [81,85–87] have gained attention. The fifteen mammalian galectins are categorized into three groups based on their protein structure (Figure 14.10a). Prototypical galectins contain one carbohydrate recognition domain (CRD), a domain that is highly conserved among galectins consisting of two β -sheets. Galectins-1, 2, 5, 7, 10, 11, 13, 14 and 15 belong to this group, and they may form noncovalent dimers. Galectin-3 is the only chimeric galectin, where the CRD is connected to a tail consisting of a repeating pattern of amino acids. Galectin-3 can associate into pentamers when interacting with a multivalent ligand [88], otherwise it exists as a monomer (Figure 14.10b). Finally, tandem-repeat galectins (4, 6, 8, 9 and 12) contain two CRDs within a single polypeptide separated by an amino acid linker. Inhibitors against galectins can prevent their adhesion, and stop the signalling pathways in their tracks [76,89–92].

Monovalent inhibitors developed against galectins are usually based on a substituted galactose (Figure 14.11). Especially aromatic substituents on the C2 and C3 positions of galactose were found to have improved binding compared with free galactose or lactose. In galectins 1 and 3 (Gal-1 and Gal-3, respectively), this substituent has a favourable interaction with specific arginines. A large number of anomeric phenyl galactosides were synthesized to explore the role of the aryl–arginine interactions in Gal-1 and Gal-3 binding. Most had no or very little improved inhibitory properties compared with lactose, except **23**, which was around 160 times better than lactose [93]. Addition of a 4-phenoxyaryl group to the C3 of thiodilactoside **24** led to an inhibitor with almost 200 times improved binding

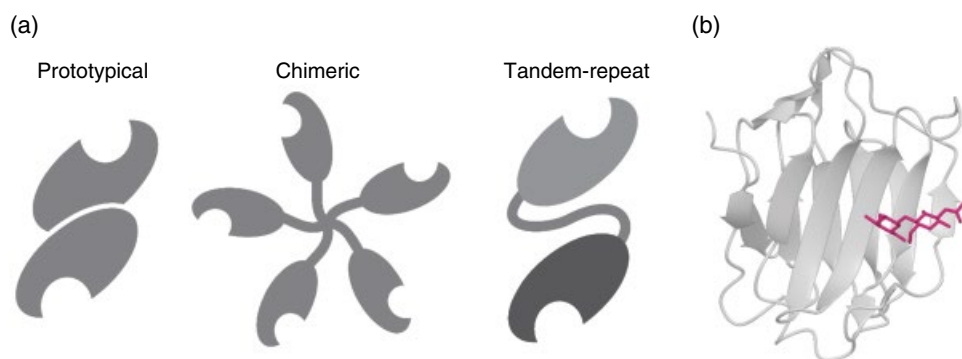


Figure 14.10 (a) Schematic representation of the three types of galectins. (b) A crystal structure image of galectin-3 in complex with LacNAc (PDB: 1KJL).

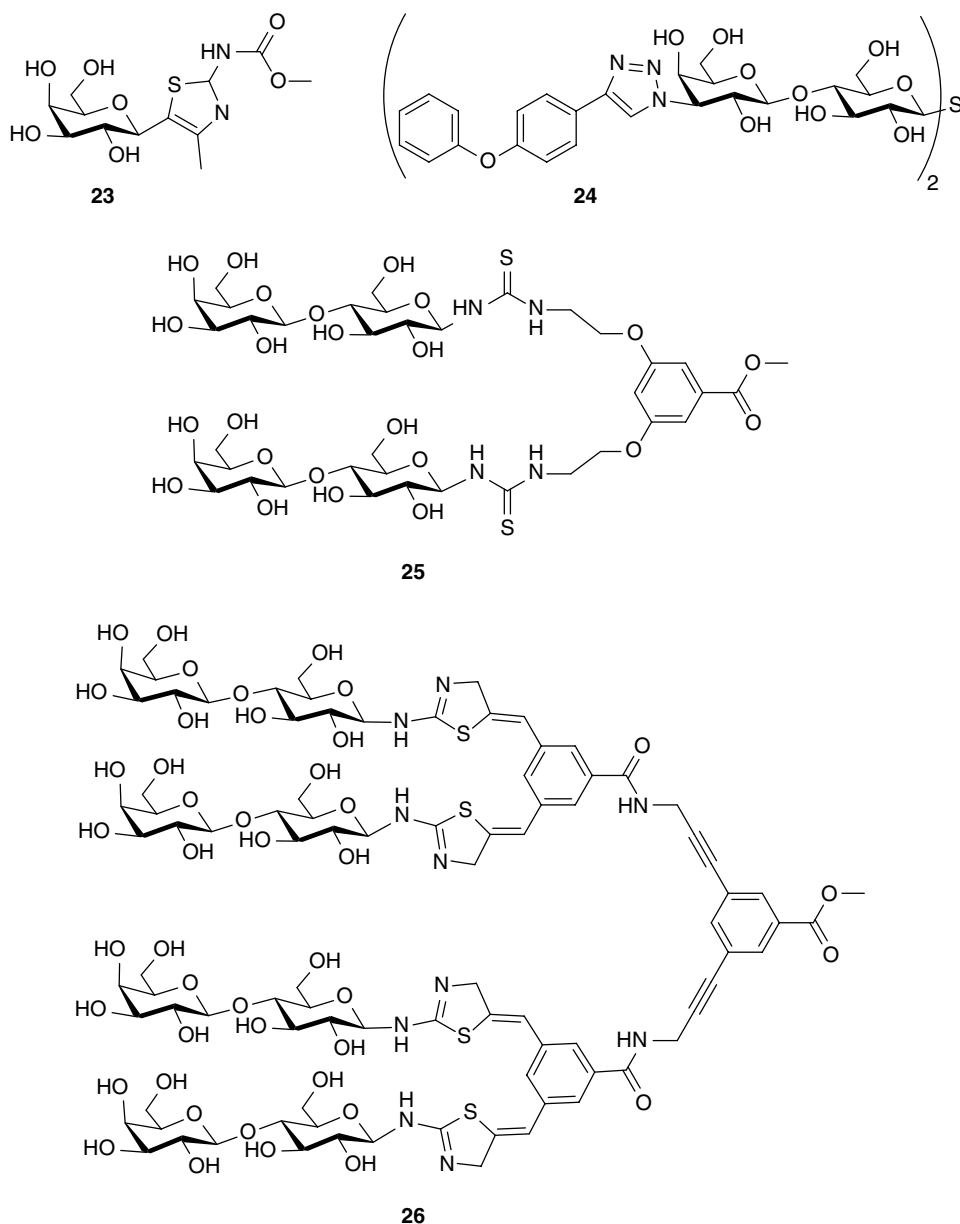


Figure 14.11 Monovalent and multivalent ligands for galectins.

to Gal-3 compared with Gal-1, while the parent thiodigalactoside showed no such selectivity [94]. Glycopeptides containing a threonine-*O*-lactoside improved binding up to 66-fold compared with free lactose [95]. Glycomimetic peptides, such as the pentameric WYKYW [96] or a number of peptides based on a Tyr-Xaa-Tyr [97] motif were found to have two to three times better binding compared with free galactose.

Dendrimers based on 3,5-di-(2-aminoethoxy)benzoic acid **25** up to the third generation found an improvement in IC_{50} of up to 144-fold per lactose in binding to Gal-3 [98]

in a solid-phase assay with immobilized glycoproteins. Improvements for these octavalent glycoclusters for binding Gal-1 and galectin 7 (Gal-7) were more modest, but for every step towards higher valency an improved IC_{50} and MIC for Gal-1, -3 and -7 was found.

Aggregation into nanoparticles of 500–2000 nm in diameter can be achieved for both Gal-1 [99] and Gal-3 [100] with sixth generation poly(amidoamine) (PAMAM) dendrimers presenting on average 130 lactosides. The ratio of galectin to dendrimeric ligand determines the size of the nanoparticles: a ratio of 9:1 Gal-1 or Gal-3 to ligand gave the largest particles (over 2 μ m), while 220:1 and 3:1 gave slightly smaller ones. Additionally, the higher the dendrimer generation and thus the valency, the larger the particles. Using these dendrimers [99] in a cell aggregation assay with human prostate cancer cells, each generation of dendrimer (G2–G6) inhibited cellular aggregation. These cancer cells express both Gal-1 and Mucin-1, a ligand for Gal-1, and this interaction is inhibited by the addition of these glycodendrimers. More epitopes is not necessarily better: the second generation dendrimer, displaying fifteen galactosides, was most efficient and inhibited at a concentration of 0.4 mM.

Rigid dendrimers based on a rigid phenyl-alkyne core **26** were synthesized [101]. While for Gal-1 little multivalent effect was seen, with both the divalent and tetravalent dendrimers showing similar IC_{50} s in a solid-phase assay with asialofetuin, Gal-3 and Gal-5 did benefit from the more chemically rigid structure of the dendrimeric ligand. Especially towards Gal-3, tetravalency brought a 1071-fold improvement in inhibitory capacity per lactose unit, down to an IC_{50} of 70 nM.

Calixarene geometry is very important for binding to galectins. A number of calixarenes were investigated to determine this effect: cone-shaped, flat or alternating side chains pointing to one or the other side of the calix[4, 6 or 8]arenes [102] were synthesized to present lactose **27** (Figure 14.12). Overall, most ligands improved for binding galectin 4 (Gal-4), and less so towards Gal-1 and Gal-3. A very rigid cone-like shape did not improve affinity, while the more flexible ones (where the arms were able to move more freely) did. Addition of aromatic side groups to the lactose ligands in cone-shaped calix[4]- and calix[6]arenes gave improved affinity even for the monovalent substituted lactose, and much better affinity for the multivalent ligand, going down to 0.15–3 μ M against Gal-3, -4 and -9 [103].

For Gal-1, having all four lactose moieties on one side of the calixarene improved affinity by 625-fold for **28**. Three lactosides on one side and one lactoside on the other, a 3:1 topology, saw some improvement, and a 2:2 calix[4]arene improved it only by 2-fold. However, these results were found with an HIA, while SPR showed a sequence of improvement that was the other way around [104]. Another study of a cone-shape and 1,3-alternate calix[4]arenes showed similar results: the cone-shape was a better binder than the 1,3-alternate [105].

A heptavalent cyclodextrin-based ligand **29** [106] successfully inhibited Gal-3, compared with Gal-1 and -7. This specificity was attributed to the ability of Gal-3 to form pentamers when in complex to a multivalent binder, while the Gal-1 and -7 binding sites are too far apart (~ 50 Å). Cyclodextrin galactosides that self-assembled like beads on an alkyl-phenyl string have a different architecture [107]. These fibres had a 30-fold improved binding to Gal-1 and were able to prevent cell adhesion in a T-cell agglutination assay.

LacdiNAc groups were attached to a thiourea linked alkyl spacer of varying length, to make a divalent ligand **30** [108] (Figure 14.13). In molecular modelling simulations the

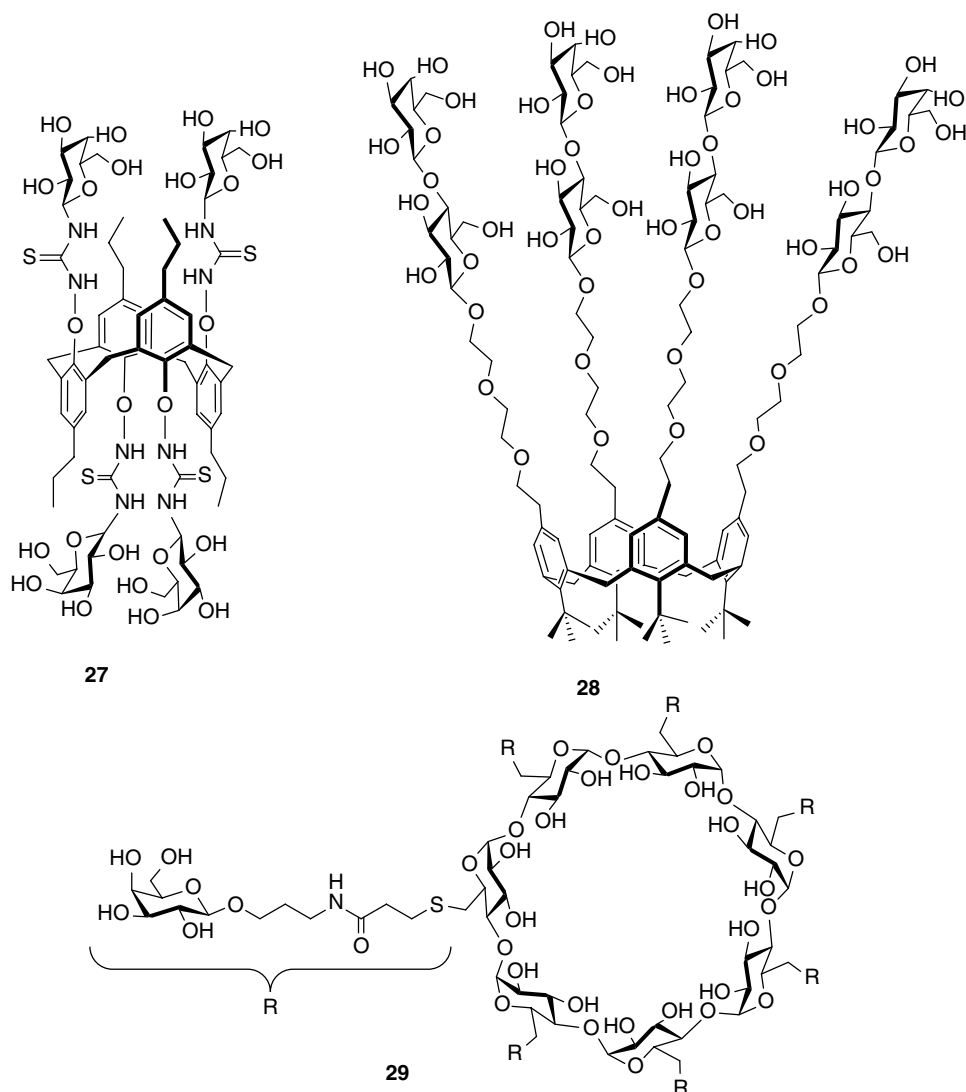


Figure 14.12 Multivalent ligands against galectins based on calixarene and cyclodextrin scaffolds.

optimal spacer length was slightly longer (eleven spacer units) than in ELLA assays (nine units). The ligand with this linker length was about three times as potent against Gal-3 with an IC_{50} of approximately 40–50 μM than a monovalent equivalent. Even ligands with a slightly shorter or longer spacer length (6–11 spacer lengths) were all about twice as potent as a monovalent control.

A variety of mono-, di-, tri- and tetravalent scaffolds with C-alkyl-lactosides were tested against Gal-3 [109]. Compound **31**, a divalent amino-derivative, showed the most improvement (21-fold, 10.8-fold per residue) in K_d (SPR, 15.8 μM) compared with lactose, and most multivalent ligands showed small improvements in affinity.

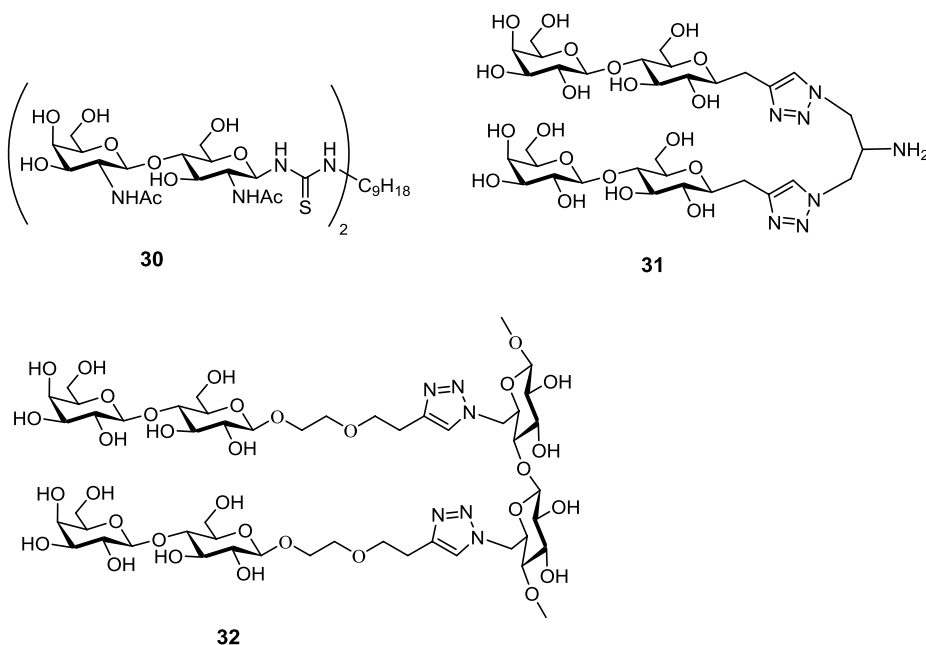


Figure 14.13 A variety of multivalent ligands against galectins.

With a chain of carbohydrates as the scaffold **32** [110], the effect of multivalency up to tetravalency and linker length between scaffold and the lactoside epitope was investigated against Gal-1 and -3. Linker length, in this case, had little effect on binding, and in MD simulations the linkers are so flexible that the epitopes stay close to the scaffold. They argue that the rebinding mechanism would probably not play a large role, as the length between lactose epitopes is too long to make it efficient. Instead, aggregation was shown to be a bigger aspect, where the linker length needs to have a minimum length to prevent steric hindrance between the binding galectins.

Self-assembled peptide-based β -sheet nanofibres presenting LacNAc attached to the N-terminal asparagine had a K_d 10 times lower than monovalent lactose when assayed with Gal-1 [111]. They were also able to inhibit tumour cell agglutination of Jurkat T cells. CdSeS/ZnS quantum dots with 108 LacNAc moieties presented to Gal-3 show a 184-fold improved binding compared with LacNAc, resulting in a K_d of 57 nM in an SPR assay [112]. Using human serum albumin tagged with azides as a scaffold, an alkyne-terminated N-glycan isolated from chicken was attached [113]. This glycoprotein containing approximately 30 glycans blocked binding of Gal-3 to cancer cells.

14.6 Concanavalin A

Concanavalin A (ConA) is a lectin from Jack bean and one of the first lectins discovered. It is a tetrameric lectin with four binding sites that recognizes α -mannosides, but can also bind α -glucosides [114] (Figure 14.14). The distance between the binding sites is very large compared with other lectins (ca. 70 Å) making a chelation binding

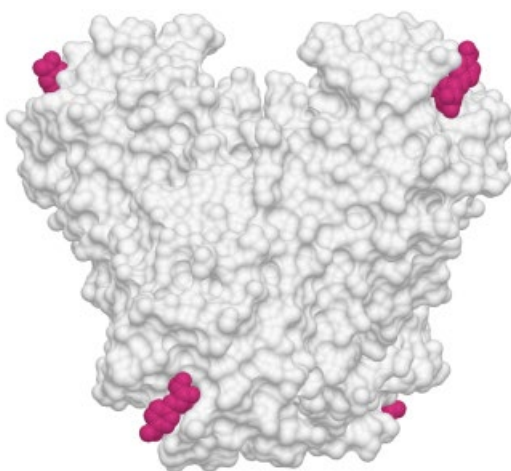


Figure 14.14 Structure of concanavalin A with 4-nitrophenyl- α -D-mannosides (PDB: 1VAM).

mechanism for a multivalent ligand difficult. A more recent sample of ConA ligands will be discussed here, as there is a long history of research on this lectin already [5].

An octavalent glycopeptide dendrimer **33** (Figure 14.15) with a branching core consisting of lysine residues and S-linked mannose residues was found to have an IC_{50} of 2.9 μ M using an ELLA assay, a 57-fold improvement per sugar over the monovalent mannose [115]. The octavalent ligand was clearly the better ligand compared with the tetravalent variants, where IC_{50} s in the 50–750 μ M range were found. A tyrosine residue close to mannose improved affinity. A different octavalent glycopeptide **34** had a slightly better IC_{50} of 2 μ M [116].

C60 fullerenes with 12 or 24 mannoses were efficient ligands with up to 15 ConA molecules attached to each fullerene molecule according to ITC measurements [117]. Cyclodextrins (cyclic α 1-4 oligoglucosides) have been used as multivalent scaffolds before, but novel cyclic α 1-6 octaglycoside presenting mannoses were used also where tetravalency showed to be the most efficient at binding to ConA [118]. Another scaffold was the so-called aromatic asterisk **35** where an IC_{50} of 89 nM was found in a HIA for this hexavalent ligand [119]. This was a case of a very efficient rebinding mechanism.

Hyperbranched polyglycerol polymers were able to form aggregates with ConA, with a higher valency leading to larger aggregates [120]. An IC_{50} of 35 nM, as measured by a competitive SPR assay, was found for the polymer presenting 60 α -Man moieties which is a 357-fold improvement over α -ManOMe. This 60-valent polymer, as well as the 33-valent one, each bind 2.9 ConA tetramers, and the linker between the polymer proper and the carbohydrate ligand needed to be of sufficient length to allow for a high affinity.

A glycopolymer with mono- or diantennary side groups **36** was synthesized (Figure 14.16). Strong aggregation was found when using the tetrameric ConA at neutral pH but even at a lower pH where ConA is dimeric, aggregation was observed. The diantennary polymer, containing both α -Man and non-binding β -Glc on its antennae, bound as strong as the polymer containing only mannose, with a association constant (K_a) of 30 μ M.

Artificial lipid rafts can be induced by addition of cholesterol to phospholipid vesicles. In this manner, the interaction of ConA with lipids presenting mannose could be

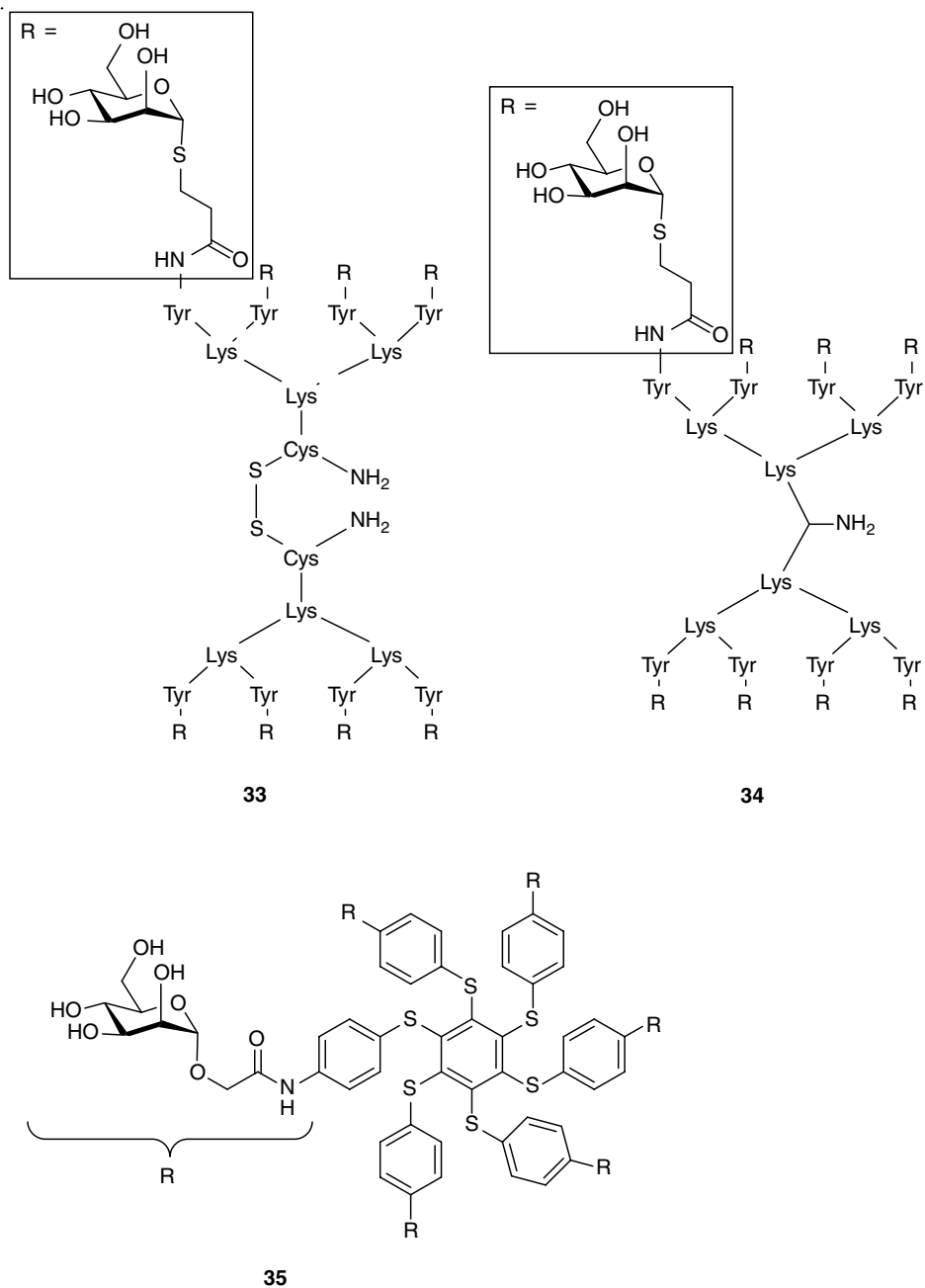
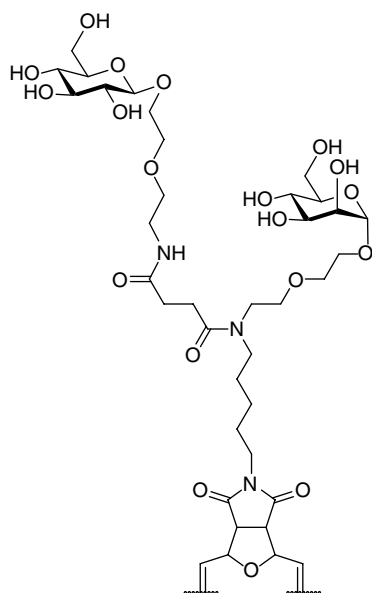
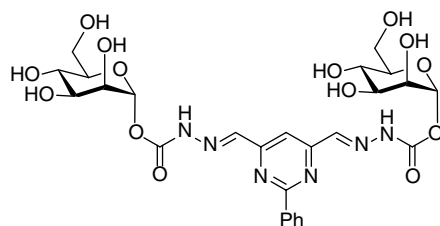


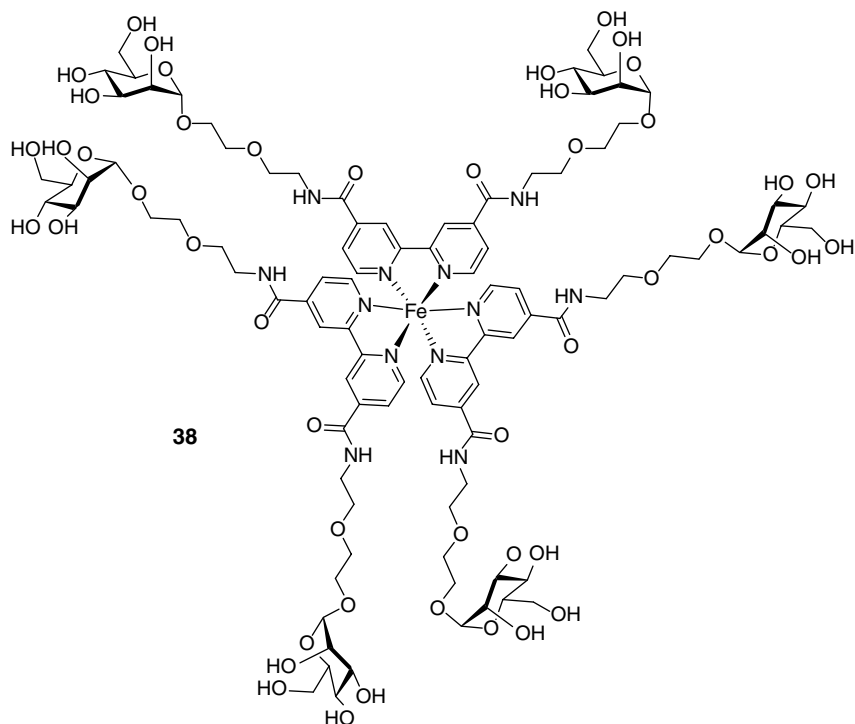
Figure 14.15 Multivalent ligands of concanavalin A.



36



37



38

Figure 14.16 More multivalent ligands of concanavalin A.

investigated with these groups dispersed over the vesicle surface or concentrated in a lipid raft [121]. Inducing lipid rafts did not significantly improve ConA binding compared with the dispersed ligands, possibly due to crowding of ligands on the surface. Only a higher concentration of dispersed mannosyl lipids slightly improved ConA binding, possibly due to some weak chelate binding interaction.

A dynamic combinatorial library of glycosylated bipyridine ligands **38** can form a complex with iron(II) [122]. Varying the ligands between mannose, fucose or galactose and exposing them to ConA, showed a bias for those self-assembling complexes containing mannosylated bipyridine ligands. An octavalent self-assembled system of ligand **37** containing zinc led to aggregation with ConA [123], forming a loose grid of ConA tetramers and the self-assembled complex.

14.7 Cholera Toxin

CT is produced by the bacterium *Vibrio cholera* and is the causative agent of cholera. Cholera is an often lethal disease that causes over 100 000 deaths annually across the world and is especially characterized by severe diarrhoea leading to major loss of fluids and nutrients [124]. Similar toxins exist and are collectively known as AB₅ toxins, with examples including Shiga toxin from *Shigella dysenteriae*, pertussis toxin from *Bordatella pertussis* and heat-labile enterotoxin from *Escherichia coli* [125]. All these toxins are taken up by the cell and are able to evade degradation through the normal cellular processes [126].

AB₅ toxins, and thus CT as well, consist of six subunits: the enzymatic A subunit, and five carbohydrate-recognizing B subunits that are evenly spaced and on one side of the protein [127]. The natural ligand for CT is the oligosaccharide GM1 that is present on the cell surface (Figure 14.17). CT-B binds to GM1os and is taken up by the cell, where

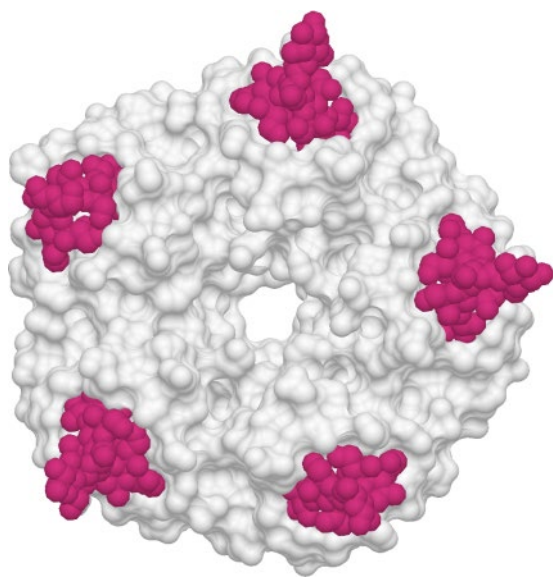


Figure 14.17 Structure of the pentameric cholera toxin B subunits in complex with GM1os (PDB: 3CHB).

the CT-A subunit induces a series of signals in the cell leading to secretion of fluids. Only one functional binding site is required for toxin activity [128].

Blocking the binding of CT-B to GM1os is an interesting approach for therapeutics to prevent the severe symptoms of cholera [129,130]. Two approaches towards multivalent ligands have been described: mismatch aggregation of non-pentavalent ligands and 1:1 binding of a pentavalent ligand to the pentavalent CT.

Dendrimers based on 3,5-di-(2-aminoethoxy)-benzoic acid, displaying lactose, galactose or GM1os, all show potent inhibition of CT [131–134] with an octavalent GM1os ligand showing the best IC_{50} of 50 pM of the variety of structures synthesized [134]. A study into the mechanism of the di- and tetravalent GM1os-presenting ligands investigated the aggregation of CT-B [135]. SV-AUC showed dimerization and higher aggregation upon addition of the divalent inhibitor.

To directly compare a tetra- versus a pentavalent dendrimer, an extra arm was attached to the dendrimer core to make a dendrimer-based pentavalent ligand **39** [136] (Figure 14.18). This arm was slightly shorter than the other linkers, to reach an approximate same distance from core to carbohydrate as the dendrimer proper. IC_{50} s of both the tetravalent and pentavalent compounds were in the same picomolar range. SV-AUC experiments indicate dimerization and larger aggregates for both compounds.

Taking the 1:1 approach, pentavalent and decavalent pentacyclen linked galactoside ligands were both shown to bind to CT with IC_{50} s in the low micromolar to nanomolar range [137,138] (Figure 14.19). Especially the decavalent ligand **40** with branched (divalent) ligand arms worked well, binding two CT pentamers at once, which was confirmed by X-ray studies. In the same vein, cyclic decapeptides that are slightly larger than the pentacyclen were found to have IC_{50} s in the same range as pentacyclen, with shorter linkers needed for optimal binding [139]. In this inhibition assay, GM1os was immobilized and both the inhibitor and CT were present in solution.

For the calix[5]arene presenting GM1os **41** an IC_{50} of 450 pM was found in the same type of assay as for the pentacyclen and cyclic decapeptide, which is a 100 000-fold improvement over the monovalent equivalent [140]. The importance of the terminal galactose for CT-B binding was confirmed, as a ligand lacking this sugar moiety had a much higher IC_{50} of 9 μ M. Similarly, the corrannulene-based pentavalent ligand **42** had an IC_{50} in the low nanomolar range, depending on the optimal length of the linker between the scaffold and GM1os ligand [141].

In another ingenuous approach CT-B itself is used as a multivalent scaffold, although an inactive mutant, by modifying N-terminal threonine residues on each CT-B subunit and linking them with GM1os [142]. This pentavalent ligand has an IC_{50} of 104 pM in an ELLA assay and SV-AUC showed predominant association of CT-B and this protein-based ligand in a 1:1 heterodimer.

14.8 Propeller Lectins

Propeller lectins are lectins containing β -sheet carbohydrate binding domains arranged as blades of a propeller. They can contain 4–10 blades. A number of these lectins have been reported over the years, from organisms ranging from bacteria and fungi to humans.

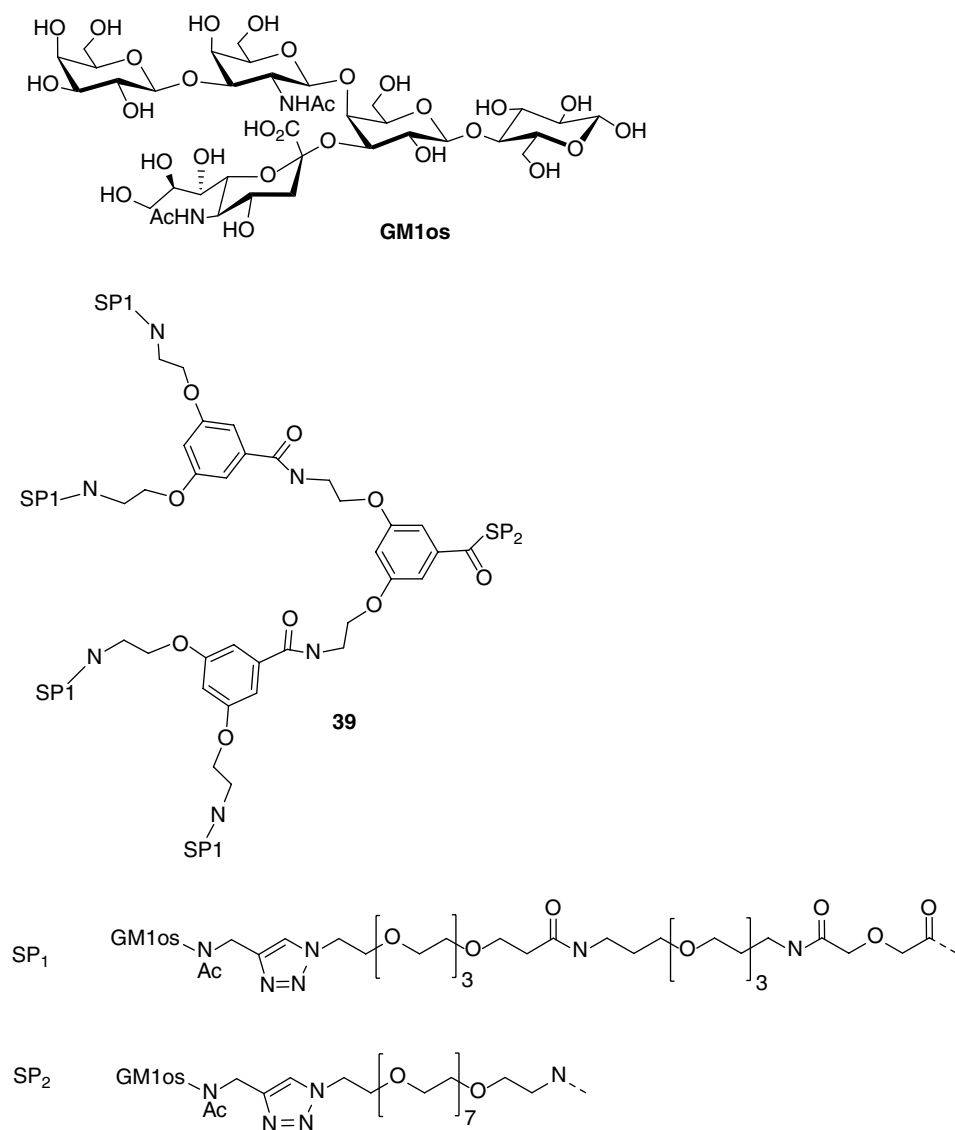


Figure 14.18 The structure of GM1os, the natural ligand of cholera toxin, and a pentavalent dendrimer inhibitor of cholera toxin.

Ralstonia solanacearum lectin (RSL) originates from a Gram-negative plant pathogen affecting many crops worldwide and binds L-fucose. RSL is a trimeric six-bladed lectin, with each monomer containing two β -sheet blades [143] (Figure 14.20a). RSL can be inhibited with a C60 fullerene presenting fucosides [72], as has been described earlier in this chapter for other lectins. While the monovalent fucoside already binds with a low K_d of approximately 300 nM, this can be improved through divalency **43** (K_d of 74 nM) or dodecavalency of a fullerene (K_d of 10 nM) (Figure 14.21). Due to steric hindrance many of the fucosides cannot reach the RSL binding sites, and as such the longer

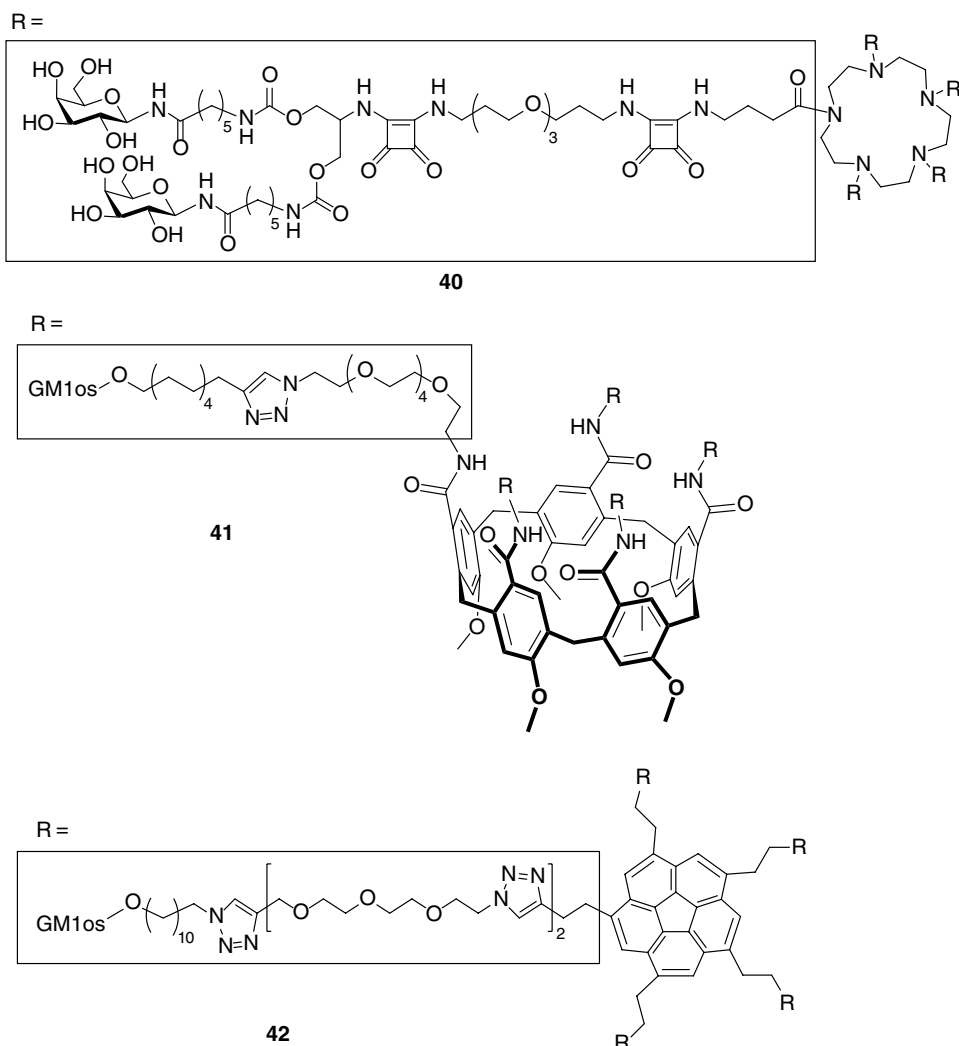


Figure 14.19 Multivalent inhibitors against cholera toxin, based on dendrimer, calixarene and pentacyclen scaffolds.

spacers have improved affinity compared with shorter spacers. In addition, the longer chains allow bridging between lectins and aggregation.

Burkholderia ambifaria lectin (BambL) is constructed in the same way as RSL as a trimer of tandem repeat β -sheets, resulting in a six-bladed lectin [144] (Figure 14.20b). It binds α - and β -L-fucose [145]. The Gram-negative bacterium has antifungal properties and protects plants from fungal infections, but can also pose a danger to immunocompromised patients, such as those with cystic fibrosis. They are capable of forming biofilms, as well, similar to PA [146]. Like other organisms discussed in this chapter, recognition by a lectin on the cell surface of a human cell is an important part of the infection. Several multivalent inhibitors have been developed against BambL.

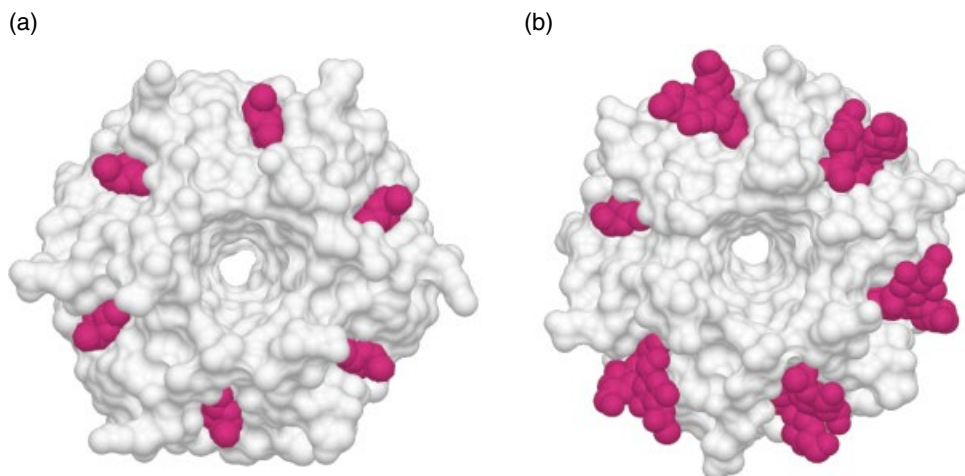


Figure 14.20 Structures of (a) RSL in complex with L-fucose in all five binding sites (PDB: 2BT9) and (b) BamBL with human H-type 2 tetrasaccharide bound in four of five binding sites, and α-L-fucose bound to the fifth binding site (PDB: 3ZZV).

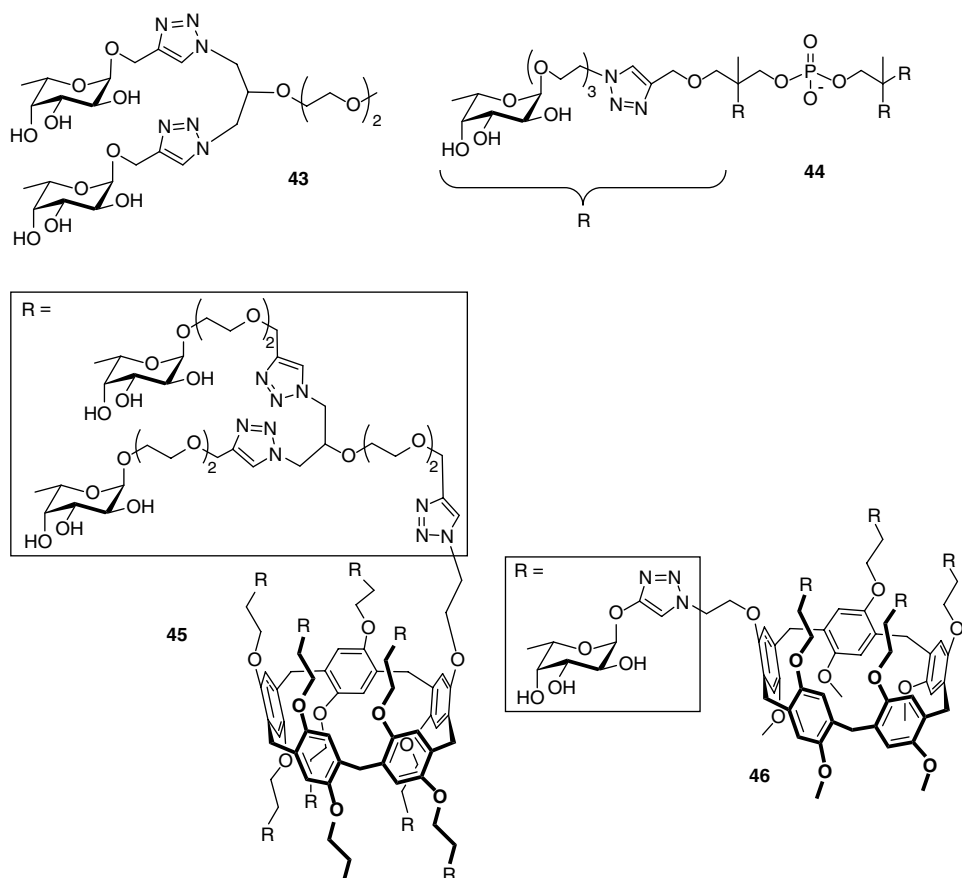


Figure 14.21 Multivalent inhibitors against propeller lectins.

The tetravalent ligand **44** was found to have an affinity of 43 nM, an improvement of 22-fold over the monovalent ligand [147]. A similar, but linear, decavalent ligand had a K_d of 13.8 nM [74]. Pillar[5]arenes, also tested for binding against LecA and LecB, were found to be very tight binders to BamBL [52]. Long linker arms and icosavalency **45** led to an IC_{50} of 70 pM in an ELLA assay and a K_d of 27 nM. Interestingly, lowering the valency to pentavalent, with all fucosides presenting on the same side **46**, gave an even lower K_d of 21 nM (53).

The discussion in this chapter thus far has been on multivalency of a ligand to bind to a multivalent protein, in order to block its natural function. The multivalency of the protein itself is also of interest, however. Through genetic studies, neolectins can be created to study the effects of multivalency in carbohydrate–lectin binding. By inactivating the binding sites of RSL, 13 neolectins were created [148], with all different geometries of active and inactive binding sites, with a valency ranging from zero to six of the wild-type. While ITC experiments showed no change in affinity, SPR experiments showed a limited effect on avidity. Important was the ability to disrupt the membrane, a crucial step in infection, which was only possible when adjacent binding sites were capable of binding carbohydrates. Having alternating binding sites active made the lectin incapable of invagination of the membrane and delayed its uptake in lung epithelial cells dramatically [149].

14.9 Conclusion

Multivalency is crucial in carbohydrate recognition between cells by lectins. Taking advantage of a wide variety of scaffolds, such as dendrimers, peptides and calixarenes, these lectins can be targeted for inhibition by synthetic ligands. Through optimization and investigating the details in the interactions between lectin and ligand, tightly binding inhibitors can be designed that have the potential for therapeutic use against pathogens such as PA, *Vibrio cholerae* and influenza A viruses, or targeting cancer and inflammatory responses.

Acknowledgements

The Netherlands Organization for Scientific Research is gratefully acknowledged for a PhD scholarship to M.S.

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