Chem Soc Rev

RSC Publishing

REVIEW ARTICLE

Bridging lectin binding sites by multivalent carbohydrates†

Cite this: *Chem. Soc. Rev.*, 2013, **42**, 4492

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Carbohydrate–protein interactions are involved in a multitude of biological recognition processes. Since individual protein–carbohydrate interactions are usually weak, multivalency is often required to achieve biologically relevant binding affinities and selectivities. Among the possible mechanisms responsible for binding enhancement by multivalency, the simultaneous attachment of a multivalent ligand to several binding sites of a multivalent receptor (*i.e.* chelation) has been proven to have a strong impact. This article summarizes recent examples of chelating lectin ligands of different size. Covered lectins include the Shiga-like toxin, where the shortest distance between binding sites is *ca.* 9 Å, wheat germ agglutinin (WGA) (shortest distance between binding sites 13–14 Å), LecA from *Pseudomonas aeruginosa* (shortest distance 26 Å), cholera toxin and heat-labile enterotoxin (shortest distance 31 Å), anti-HIV antibody 2G12 (shortest distance 31 Å), concanavalin A (ConA) (shortest distance 72 Å), RCA_{120} (shortest distance 100 Å), and *Erythrina cristagalli* (ECL) (shortest distance 100 Å). While chelating binding of the discussed ligands is likely, experimental proof, for example by X-ray crystallography, is limited to only a few cases.

Received 4th March 2013

DOI: 10.1039/c3cs60089k

www.rsc.org/csr

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† Part of the carbohydrate chemistry themed issue.

1. Introduction

Carbohydrate recognition is increasingly recognized as an important phenomenon for biological processes.[1,2] Most cell surfaces are decorated with glycans that make contact with complementary proteins either on another surface or free in solution.

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Professor Valentin Witmann was a chemistry student at the Goethe University in Frankfurt and the Technical University of Munich. At the latter place he also obtained his PhD with Horst Kessler. After post-doctoral stays in Frankfurt (with Christian Griesinger) and at The Scripps Research Institute (with Chi-Huey Wong) he started independent research in Frankfurt. In 2003 he moved to the University of Konstanz where he is currently a professor of organic and bioorganic chemistry. His main research area is the chemical biology of carbohydrates including structural aspects of multivalent carbohydrate ligands, ligation reactions, metabolic oligosaccharide engineering, and RNA-targeting antibiotics.

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Since the individual protein–carbohydrate interactions are weak it often takes a multitude of simultaneous interactions, *i.e.* multivalency, to produce a biological effect.[3–7] Since blocking the carbohydrate binding proteins or lectins may be beneficial for treating certain diseases, the aspect of multivalency cannot be ignored. In fact, incorporating multivalency into the design of inhibitors may even be necessary to achieve sufficient inhibitory potency. Many researchers have taken this lesson to heart and have designed and synthesized multivalent inhibitors with a large variety of architectures.[8,9] It is currently well accepted that both bridging and non-bridging systems can exhibit multivalency effects with a suitable target lectin. Strong effects of several orders of magnitude can be observed with systems of low valency capable of bridging binding sites, also called the chelation mechanism.[8] Creating well-designed ligand systems is expected to result in both dramatically enhanced potency and also selectivity. The selectivity aspect is attainable with a more rigid and well-designed system that binds strongly only to a single target with the matched interbinding site distances, yet leaves all other lectins with the same carbohydrate specificity untouched. Besides the bridging ones, non-bridging systems can also be effective. The mechanism leading to their enhanced potency is often called 'statistical rebinding',[10,11] where a nearby ligand quickly replaces the bound ligand due to its proximity, leading to reduced off-rates. These effects are often observed when binding sites of a lectin are spaced far apart or when only a single binding site is present. In specific cases, high valency systems[12] or a spherical geometry[13] were shown to be successful. This review focuses on bridging systems although, especially when bridging large distances with large high-valent systems, both mechanisms may operate simultaneously.[8] The following sections discuss multisite lectins, in most cases of medical relevance, where the interbinding site distances increase from section to section. For the Shiga-like toxin the shortest distance is *ca.* 9 Å whereas in the final section lectins with interbinding site distances of *ca.* 100 Å are discussed. Clearly different design solutions are required for these very different systems. We did not aim to be comprehensive, rather chose striking, mostly recent examples especially if they illustrate the bridging theme. The assays that were used to determine the potency enhancement effects are mentioned in the discussion. Progress has been made across the whole spectrum, but the full potential has only been harnessed in a few cases, with more to be explored and exciting developments to be expected.

2. Shiga-like toxin

The Shiga toxins are part of the AB_5 toxins and are produced by *Shigella dysenteriae* but also by enterohemorrhagic *E. coli* (EHEC) that produces the so-called Shiga-like toxins (SLT-I and SLT-II). The Shiga-like toxins can lead to diarrhea but also to the serious hemolytic uremic syndrome (HUD) after the toxin enters circulation from the gut.[14] Blocking the toxin is of medical interest and most likely too late in the intestine and therapeutically more viable in circulation.[15]

There are also reasons to make the toxin the target for treatment rather than the bacterium that produces it. This approach induces less resistance and the toxins may still cause symptoms after their producer is cleared.[14] The Shiga toxins are an interesting group of lectins for the design and evaluation of multivalency effects because of their numerous carbohydrate binding sites. They contain three binding sites of unequal affinity for its natural Gb3 ligand (Galα1,4Galβ1,4GlcβCer) per monomeric B-subunit. The shortest distance between binding sites appears to be as low as 9 Å,[16] *i.e.* one of the shortest distances present between lectin binding sites. However considering the large number of binding sites numerous distances are present ranging up to *ca.* 50 Å. For this reason the toxin binds well to most multivalent systems that can achieve a certain degree of chelation, possibly supplemented by binding multiple toxins *i.e.* aggregation into higher order structures.

The first to study the possibility for chelation with the Shiga like toxin were Toone and coworkers[17] They noted distinct potency enhancements for divalent ligands using peptidic spacers. The enhancements in an ELISA type assay were up to 95-fold (48-fold per sugar). It was curious that the binding stoichiometry varied with the use of a hydrophobic or hydrophilic spacer, clearly indicating an additional role of the spacer.

Multivalent inhibitors were prepared by Bundle *et al.* who used a pentavalent core structure.[18] In compound **1** (Fig. 1) ten globotriose moieties were attached to the five arms that were linked to the glucose-based core structure. The shape of the construct led to its name Starfish. The globotriose units were linked *via* their O2′ after inspection of the X-ray structure. Very high inhibitory potencies were observed for SLT-I, with a multivalency enhancement of 875 500, as determined in an ELISA-like assay with immobilized toxin. The design included two globotriose units per arm to bind to two of the binding sites per subunit, however in the crystal structure of the complex it was observed that a hamburger shaped 2 : 1 complex was formed in which two toxins bound to one inhibitor and the two globotriose units per arm each occupied a binding site in a different toxin. A similar approach was based on a carbosilane dendritic scaffold to which copies of globotriose were linked.[19] For **2**, one member of this so-called Super-Twig family, the K_ds for Stx1 and Stx2 were 110 and 210 nM, respectively, *i.e.* a major improvement over the typical monovalent K_d of *ca.* 1 mM.[17] Gold glyconanoparticles that contain the globotriose sugar were also prepared.[20] Particles with sizes ranging from 4 to 20 nm were reported containing between 60 and 2000 sugars on the surface. These particles were tested as inhibitors of the SLT-I B-subunit binding to an SPR chip surface. The particles proved to be very potent inhibitors with relative potencies per sugar ranging from 1300 for the smallest particles to 228 000 for the larger **3**. The enhanced potencies were also used for the purpose of a sensitive detection method. Polymers with attached globotriose units (**4**) were also prepared and tested as inhibitors of SLT-I.[21] Enhancements of 5000-fold per sugar were determined in this case. In a recent study chitosan was used as the scaffold polymer for globotriose conjugation, which resulted in a K_d as low as 50 nM per trisaccharide ligand, while

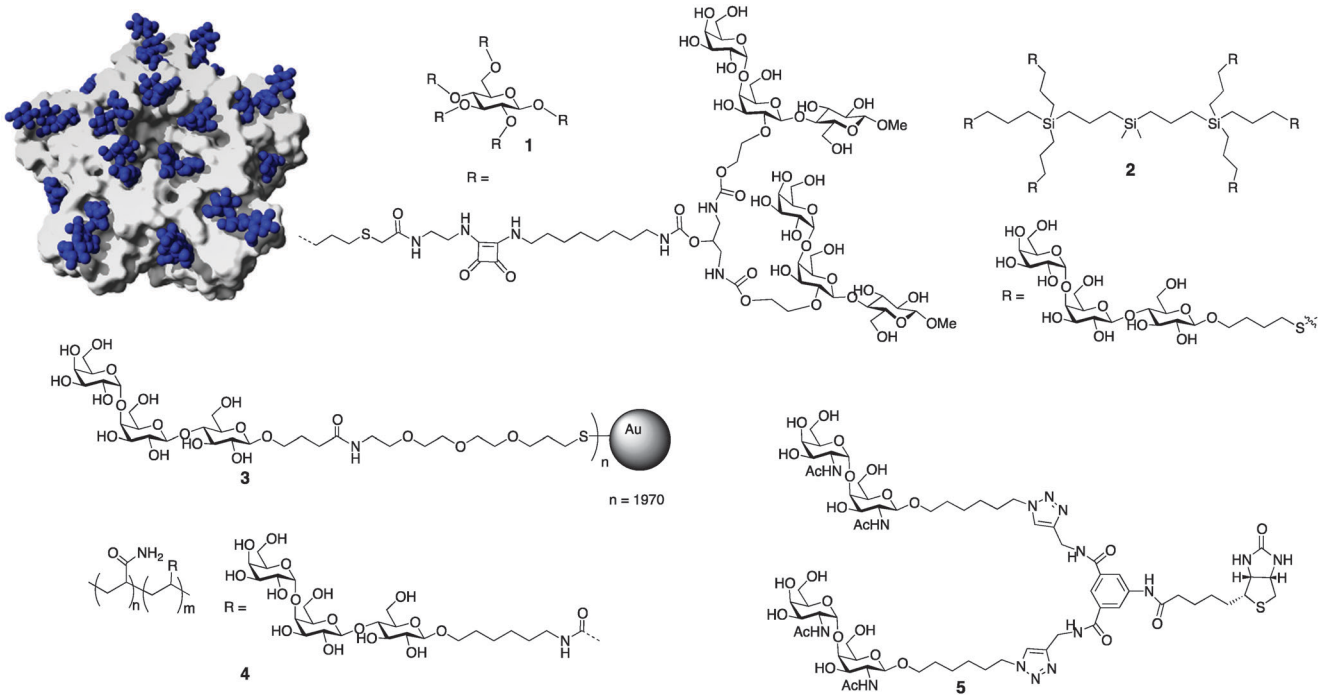

Fig. 1 X-ray structure of Shiga like toxin (SLT-1) in complex with its ligands and drawn structures of Shiga like toxin binders.

the monovalent ligand showed essentially no binding at 162 μM.[22] Oral activity in a mouse model was demonstrated.

Detection of the Shiga-like toxin is also of importance for diagnostic and public health purposes. Weiss, Iyer and coworkers reported an analytical system based on the recognition of the divalent **5**.[23] Surprisingly, the use of GalNAc instead of Gal makes the molecule specific for the more toxic Stx2. The C6 spacers in the arms were shown to perform better than their C12 counterparts. An alternative detection system was based on the recognition of a BSA–galabiose conjugate. From a biological sample an immobilized antibody was able to capture the toxin and the visualization of the BSA construct binding led to selective toxin detection.[24]

Most multivalent systems bind well to the Shiga-like toxins. This is likely due to the many binding sites they contain and the many interbinding site distances that can be accommodated. All binding sites are also present on the same side of the protein complex, to facilitate simultaneous binding. This chelate-type binding is almost certainly the origin of the very strong inhibition by even relatively low valency inhibitors.

3. Wheat germ agglutinin (WGA)

WGA is one of the 'classical' lectins used for studying multivalent carbohydrate–protein interactions. It is enriched in the seeds of wheat (*Triticum vulgaris*) and occurs in three closely related isoforms.[25,26] WGA is specific for terminal *N*-acetylneuraminic acid and GlcNAc and has been shown to inhibit fungal growth through interaction with fungal cell-wall components[27,28] and to agglutinate transformed cells *in vitro*.[29,30] The lectin exists as a stable homodimer with a twofold symmetry axis. Each monomer consists of four 43-amino acid residues hevein domains (A–D) that are arranged in tandem upon "head to tail" dimerization of the monomers. The monomer/monomer interface accommodates eight functional sugar binding sites (four unique sites due to the twofold symmetry axis) between contacting domains with side chains of both monomers interacting with the saccharide ligands.[31] These ligands are GlcNAc or β-(1,4)-linked GlcNAc oligomers (chitooligosaccharides, such as *N*,*N*′-diacetylchitobiose, *N*,*N*′,*N*″-triacetylchitotriose,...). The binding sites are labeled as pairs of capitals (each followed by the number of the monomer it belongs to) indicating the hevein subunit which provides the aromatic residues and the polar residues, respectively. In D, the polar residues are absent, so that the sites are A1, B1C2, C1B2, D1A2, A2, B2C1, C2B1, D2A1. The binding sites involving A and D exhibit lower affinity than those formed from B and C. Consequently, the sites involving the B and C domains have been termed "primary" binding sites and the ones involving the A and D domains "secondary" binding sites. The closest distances between pairs of adjacent binding sites (B1C2/C2B1, C1B2/B2C1, A1/D2A1, D1A2/A2) are 13–14 Å (distances between anomeric oxygens of two bound GlcNAc residues).[32] Linear distances between other pairs of primary binding sites range from 29 Å (C1B2/C2B1) to 37 Å (B1C2/B2C1). However, ligands supposed to bridge these pairs need to be considerably longer due to the presence of protruding protein parts.

Several multivalent ligands have been described that are expected to bind in a chelating binding mode (Fig. 2). Zanini and Roy reported glycodendrimers with valencies up to 8 that were investigated by an enzyme-linked lectin assay (ELLA).[33] The best ligand was octavalent **6** with an IC_{50} value of at least 170 times lower than that of GlcNAc-α-OAll corresponding to an

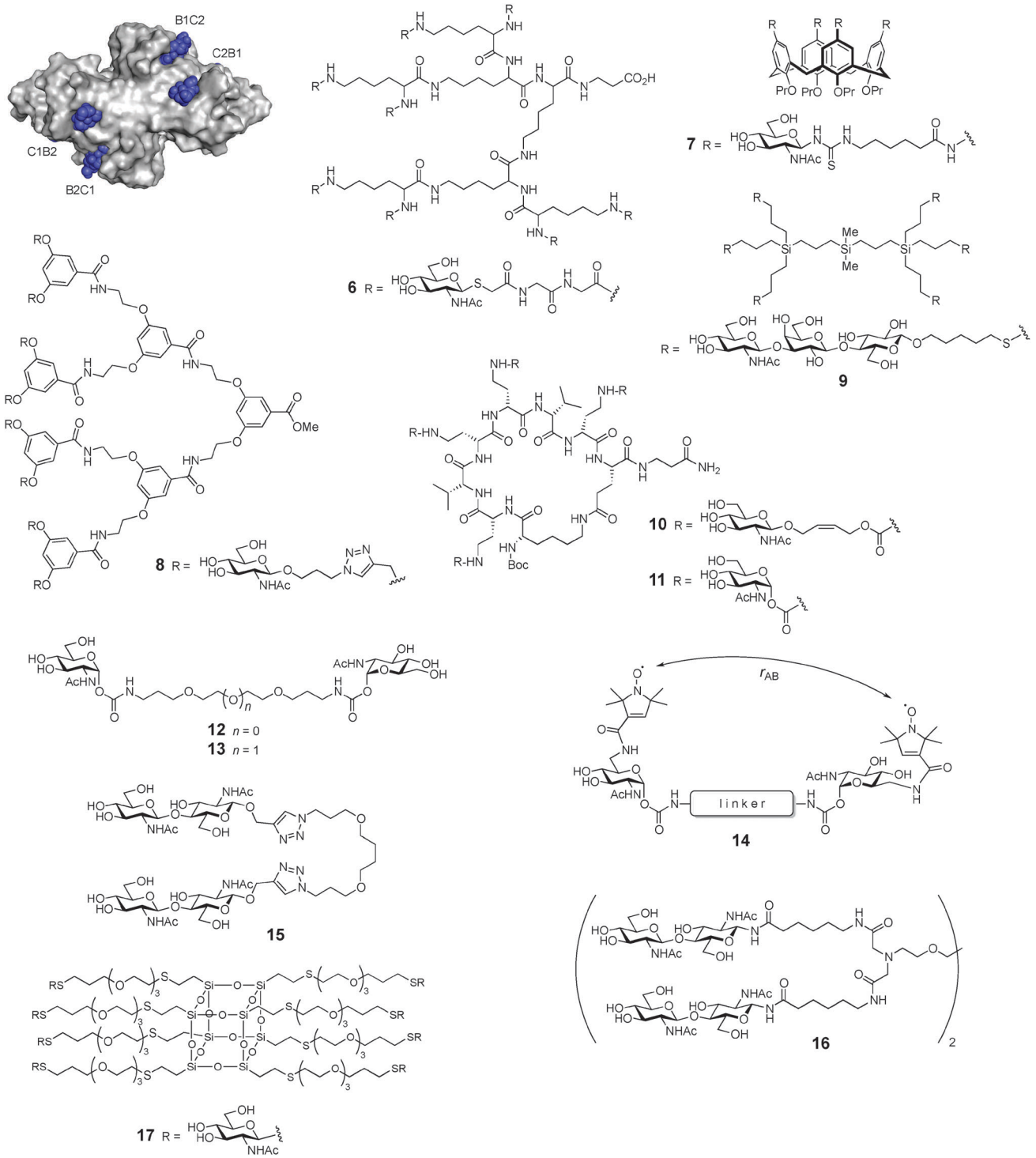

Fig. 2 X-ray structure of the WGA dimer (isolectin 1) in complex with GlcNAc (PDB-ID: 2UVO). Visible are the four primary binding sites. The secondary binding sites are located on the backside of the protein. Reported WGA ligands.

increased binding potency of more than 21-fold per sugar. Interestingly, although the distances between the GlcNAc moieties within **6** are considerably longer than 14 Å, these numbers are moderate. Much higher multivalency effects were observed with calix[4]arene **7** (ref. 34) and glycodendrimer **8**.[35] The tetravalent glycoconjugate **7** inhibited hemagglutination 312-fold better per contained sugar than free GlcNAc and for octavalent **8** a potency enhancement of 4700-fold per contained sugar was determined by an ELLA. Hexavalent **9** is an example of a multivalent lactotriose derivative.[36] Its binding enhancement was 424-fold per saccharide compared to a single trisaccharide as determined by a fluorescence-based binding assay.

Interestingly, a dodecavalent analog was less effective both in absolute numbers (K_d = 0.77 μM *versus* 0.36 μM for **9**) and in terms of binding enhancement per lactotriose moiety (98-fold). GlcNAc-modified quantum dots have also been employed as WGA ligands and showed binding enhancements of 3–4 orders of magnitude over the corresponding monovalent ligand.[37]

When comparing binding potencies of ligands, it is important to keep in mind that the determined potencies are strongly dependent on the assay, a phenomenon that has often been observed.[38–40] The reason behind this observation is that most assays do not provide K_d values but rather IC_{50} values that are dependent on the assay setup.

As an example we determined the WGA binding potency of tetravalent neoglycopeptide **10** by two types of ELLA employing either porcine stomach mucin (PSM)[41] or covalently immobilized GlcNAc[40] as the matrix on a microtiter plate. Whereas with the PSM matrix an IC_{50} value of 380 μM (corresponding to a binding enhancement of 54 per sugar) was noted, the GlcNAc matrix led to an IC_{50} of 16 μM (360-fold enhancement per sugar). Thus, binding potencies should only be directly compared when determined by the same assay.

To allow a chelating binding mode of a multivalent ligand to become effective, a careful design of the spacer between individual sugar epitopes is needed. For cases in which a design is not possible due to missing structural information, we employed a combinatorial approach with one-bead one-compound libraries.[42] Using cyclic peptides as scaffolds, a library of almost 20 000 glycoclusters was prepared and screened for WGA ligands.[41] Interestingly, the best ligand within this library contained fewer than the maximum possible number of sugar residues. Later, we developed the tetravalent neoglycopeptide **11**.[32] An IC_{50} value of 0.9 μM (corresponding to a binding enhancement of 6400-fold per sugar) was determined by an ELLA.

A remarkable multivalency effect was also observed with the simple divalent ligand **12** (1170-fold binding enhancement per sugar) featuring an α-glycosidic linkage as in **11**.[32] X-ray crystallography of the complex of WGA and the somewhat weaker, closely related **13** explained this activity. Four divalent molecules simultaneously bind to WGA with each ligand bridging adjacent binding sites. This showed for the first time that all eight sugar binding sites of the WGA dimer are simultaneously functional. The structure also illustrated that the α-glycosidic linkage is a key feature of the spanning ligands because the axial glycosidic bonds of bound GlcNAc pairs point towards each other. With β-glycosidic linkages, a considerably longer linker would be required. X-ray crystal structure analysis of the complex of **11** bound to WGA revealed that indeed **11** binds to the lectin in a chelating binding mode with two sugars bridging two primary binding sites.[32] Comparison of the X-ray structure and the NMR-derived solution structure of **11** suggests that the cyclic glycopeptide is preorganized in solution in a way that supports divalent binding.

Since binding mechanisms of multivalent interactions may differ in a densely packed crystal from those in solution, we employed distance measurements in the nanometer range using EPR spectroscopy of spin-labeled WGA ligands **14** to study multivalent binding in solution.[43] Distributions of distances r_{AB} between the nitroxide labels in the presence and absence of WGA provided, for the first time, structural evidence for chelating binding to the protein in (frozen) *solution*. In addition, it was possible to observe successive binding of labeled monovalent ligands to the multivalent protein, thereby identifying differences in binding affinities among the primary binding sites.

Since the WGA binding affinity of *N*,*N*′-diacetylchitobiose is significantly higher compared to that of GlcNAc, multivalent ligands containing up to four chitobiose moieties were designed and synthesized.[44,45] Divalent **15** was investigated by an ELLA and has a binding potency that is 500-fold per chitobiose moiety higher than that of chitobiose.[45] Although this enhancement is lower than that of divalent GlcNAc ligand **12**, **15** is the better ligand in terms of the IC_{50} value (0.6 μM for **15** *versus* 9.8 μM for **12** determined by the same ELLA). Molecular modeling supported a chelating binding mechanism for **15**. The best ligand in this series was a trivalent chitobiose-containing ligand with an IC_{50} value of 220 nM.[45] A very similar IC_{50} (180 nM) was determined for tetravalent ligand **16**.[44] However, this value was the result of a hemagglutination inhibition assay.

Interestingly, tetravalent **16** led to precipitation of WGA in a certain submillimolar concentration range whereas this was not observed for **15**. The world record in potency enhancement is currently held by the octavalent GlcNAc derivative **17** featuring an octasilsesquioxane scaffold.[46] The ELLA-determined IC_{50} value of 3 nM corresponds to a binding enhancement of an impressive 6 orders of magnitude per sugar, likely due to a chelating binding mode.

In summary, WGA inhibition studies have provided important insights into chelation type of multivalent interactions. The potency enhancements were large even for low valency systems. Importantly, both in solutions (EPR probes) and in the solid state (X-ray) a chelation type of binding was confirmed for the first time.

4. LecA

The lectins LecA and LecB (or PA-IL and PA-IIL) are of medicinal interest and challenging targets for the design of multivalent inhibitors. These proteins are adhesion proteins and virulence factors of the problematic pathogen *P. aeruginosa*.[47] Unlike most adhesion proteins these adhesins are not covalently linked to the cell surface. Instead they are soluble proteins that can bind non-covalently to the cell surface using at least one of their four carbohydrate binding sites.[48] Besides playing a role in the infection process the lectins are also involved in biofilm formation.[49]

LecA is specific for galactosides and is a tetramer. Two of the binding sites are relatively close together at *ca.* 26 Å while the next closest distance is *ca.* 80 Å.[50] For a chelation type of interaction the two most closely spaced sites are of interest. Strikingly, from a study on calixarene conjugates the most potent isomer was the 1,3-dialternate as in **18a** which seems

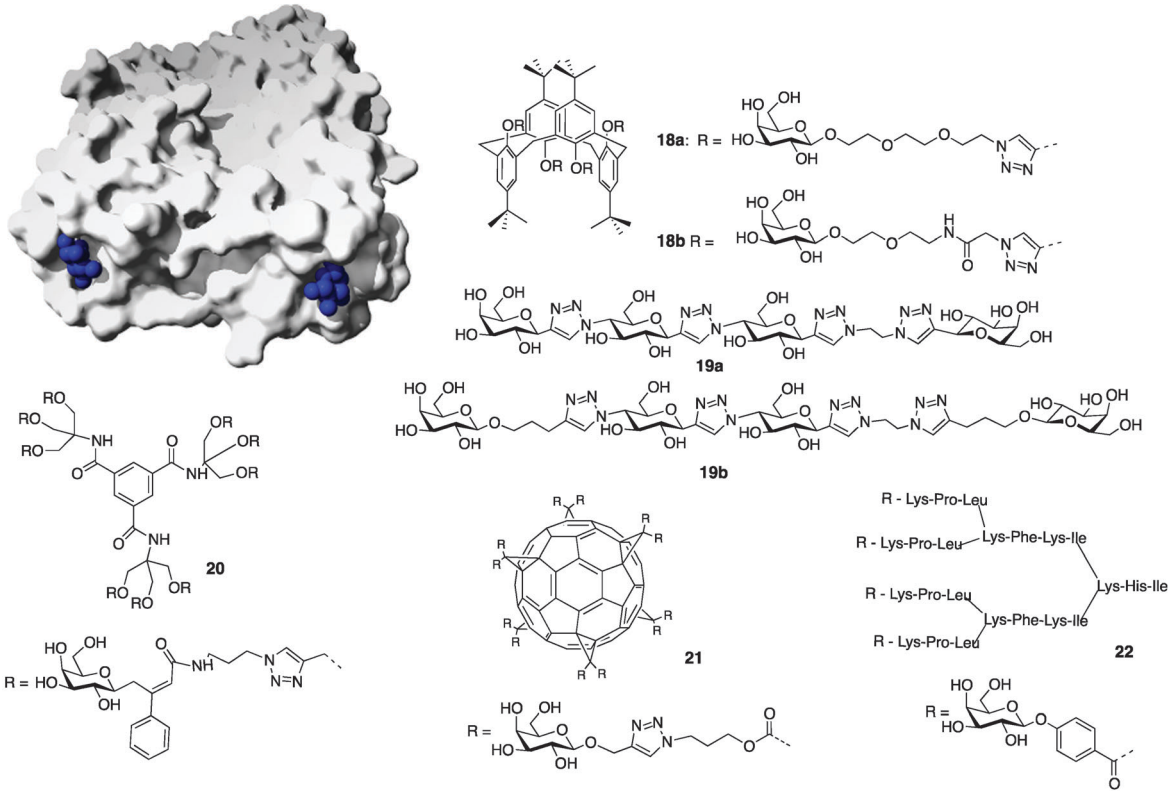

Fig. 3 Structures of LecA and reported inhibitors.

to be preorganized to chelate the two nearby binding sites (Fig. 3). It did so with a K_d determined by ITC of 176 nM, *i.e.* an 852-fold (212-fold per sugar) binding enhancement in comparison with a relevant monovalent reference compound.[51] Interestingly, the compound can simultaneously bind two LecA tetramers due to its symmetry. The result is that a polymeric fiber-like aggregate forms containing alternating LecA subunits and tetravalent **18a** molecules. These fibers were observed by atomic force microscopy.[52] Further attempts were undertaken to optimize the potencies and a slight reduction in spacer arm flexibility led to an additional two-fold improvement for **18b** (IC_{50} = 90 nM) with a relative potency of 1138 (285-fold per sugar) when compared to just the monovalent 'arm' molecule.[53]

The sugar ligand structures of **18** reach their neighboring binding sites with the calixarene allowing adjustment in the spacing due to a hinge-like action. While obviously good results can be obtained from such a design we opted for a different design. This design entailed a straight bridge between the binding sites consisting of a rigid spacer and at the termini some more flexible sections to allow the necessary adjustability.[54] A series of compounds was prepared and yielded both very poor and very potent inhibitors, in line with the predictions dictated by the design. In comparison with a monovalent reference compound, the divalent **19a** showed no improvement in an ELISA type assay.[35] This suggests that its spacer was too short to allow binding at both binding sites simultaneously.

The somewhat elongated **19b** was a 545-fold (272-fold per sugar) better inhibitor than the reference compound with an IC_{50} of 220 nM. Molecular modeling supported the experimental findings.[54] This example shows a hint of what is possible with spacer design and how potencies can skyrocket with minor spacer alterations, with likely similar benefits in selectivity. The spacer of **19a** and **19b** consists of a repeating pattern of glucose moieties linked in a β-1,4 fashion to triazole units. Cu-catalyzed azide–alkyne cycloaddition (CuAAC) was used to elongate the spacer and the multiple hydroxyl groups ensure good aqueous solubility. In this design rotation is possible but the overall shape of the spacer remains mostly linear. In another study by Roy, Imberty and coworkers, C-galactosides were optimized as monovalent ligands and subsequently incorporated into multivalent systems.[55] The most potent was the nonavalent **20** (K_d 230 nM), which was on a per sugar basis a 45-fold more potent ligand than the β-Gal-O-Me reference compound, as determined by ITC. Based on modeling and determination of the solvodynamic diameter of their multivalent constructs the authors concluded that the systems could not reach both binding sites simultaneously and that chelation would therefore not occur. The enhanced potency was therefore attributed to aggregative phenomena.

Gold nanoparticles outfitted with galactosides were also shown to be potent LecA binders.[56] The size and epitope density are important factors in the potency and also the assay method showed some variability. Particularly striking was the K_d of 50 nM per sugar of a particle displaying *ca.* 67 galactosides as determined by ITC. The particle is easily large enough to allow chelation, but in addition its spherical nature and high

density display of glycans likely make the rebinding mechanism very efficient and this likely further enhances the overall potency. Similarly, linking of 12 galactosides to a fullerene (**21**) resulted in very high potencies, with the most striking result being an IC_{50} of 40 nM representing a relative potency per sugar of 458 fold.[57] While no mechanistic insights were given, a combination of statistical rebinding with some degree of chelation seems the most likely. The tetravalent glycopeptide inhibitor **22** was described by Reymond *et al.* that showed a K_d of 100 nM, *i.e.* a 35-fold enhancement per sugar when compared to *p*-nitrophenyl-β-galactoside.[58] Based on molecular simulation studies, the authors concluded that chelation was not possible with this system, but simultaneous binding of multiple proteins was possible.

From the above it is clear that there is a major amount of recent activity dedicated to finding multivalent LecA inhibitors. These compounds vary from linear dimers to gold glyconanoparticles and all have yielded major potency enhancements. Relatively few claim chelation to be the driving force for the enhancement. In some cases a rebinding or aggregative mechanism was coined while mixed mechanisms may be involved in certain cases. Instead of using a trial and error approach, a well-defined system like LecA is ideal for the design of inhibitors using the interbinding site distance as a ruler. In cases where medicinal applications are the goal, selectivity is important and the differentiation between related compounds **19a** and **19b** suggests that this can be achieved.

5. Cholera toxin and heat-labile enterotoxin

The cholera toxin (CT) is a relative of the Shiga-like toxin in the AB_5-toxin family. In this case the 5 B-subunits contain only 5 carbohydrate binding sites and the spacing between the binding sites is *ca.* 31 Å.[59] The toxin is the causative agent of the cholera disease. The heat-labile enterotoxin of *E. coli* (LT) is the causative agent of 'traveller's diarrhea' and is closely related to CT with *ca.* 80% sequence homology. The 5 binding sites of these toxins are located on the same face of the protein pentamer, which makes simultaneous binding to the nascent GM1 gangliosides on the intestinal cell surface very favorable. This multivalent binding is followed by internalization of the toxin and disease progression due to the A-subunit, resulting in massive diarrhea.

The work by Hol, Fan and coworkers clearly showed the promise of a multivalent approach of inhibition by using a pentavalent pentacyclen core structure with 5 appendages terminating in galactoside moieties.[60] This team has brought the spacer length issue to the forefront of the design of the multivalent carbohydrate field. They applied the theory of Kramer and Karpen,[61] who showed the importance of matching the spacer length with the interbinding site distance, *i.e.* spacers can be too short or too long. Furthermore, for flexible spacers, such as PEG, the average length of the spacer is far shorter than the extended conformation, due to folding. This so-called effective length is proportional to the square root of the number of PEG monomers. A similar rationale is to use the Flory radius which can readily be calculated for PEG based spacers.[62] Using pentacyclen and also cyclic peptides as pentavalent core structures and galactose as the ligand (**23a**,**b**, **24**, Fig. 4), it was shown that the potency enhancements varied a great deal with the spacer length, and optimal lengths were obtained, in line with expectations based on the effective lengths. Compound **23a** (n = 4) was the most potent LTB_5 inhibitor in a series (n = 1–4) with a potency enhancement of almost 9000-fold (1786-fold per sugar) over a relevant reference compound.[60] Similarly **23b** (n = 4) was the best in its series (n = 2, 4, 6, 8) for CTB_5 inhibition.[63] For the larger cyclic peptide scaffold of **24** (n = 1–4), it was the shorter arm that was the most effective (n = 2), with optimal inhibitory potencies being similar to those of **23a**,**b** *i.e.* in the low micromolar range. Taking the spacer design criteria of Hol and Fan into account we used a dendritic scaffold to which long spacer arms were attached as is shown for **25a**,**b**. Both the natural pentasaccharide ligand, the GM1 oligosaccharide (GM1os), and a more simple galactoside ligand were linked to the dendrimers. Valencies of 2, 4, and 8 were used. For **25a** a potency enhancement was observed over 30 000-fold (7600-fold per sugar) when compared to a monovalent GM1os derivative and an IC_{50} of 230 pM.[64] The multivalent galactoside **25b** was also tested and found to be as effective as a monovalent GM1 derivative in the ELISA-like inhibition assay (IC_{50} in a low micromolar range).[65] The multivalency effect was able to overcome the many orders of magnitude difference when comparing GM1os to the weakly binding galactoside monovalent ligand. Besides the multivalency approach a structure-based approach of optimizing monovalent ligands has also been developed.[66]

Recently such an improvement strategy was combined with the attachment of the newly built ligands to a multivalent polymeric backbone such as dextran.[67] Very potent compounds resulted from the study as exemplified by **26**, which surprisingly contained an α-linked galactoside, in contrast to the natural ligand GM1os which contains a β-linkage to the terminal non-reducing galactoside. In summary, the cholera toxin is a highly suitable target for the exploration of multivalent ligands. From a mechanistic point of view it is more complicated than *e.g.* LecA which only has two nearby binding sites. Due to the five binding sites numerous interbinding site distances are available. The chelation mechanism can easily be mixed with cross-linking or aggregation-type mechanisms as was observed *e.g.* for **25a**.[68] The valency mismatch between the toxin and the ligand was the likely cause of the aggregation, but it is unclear if that contributed to the high inhibitory potency.

6. HIV neutralizing antibody 2G12

2G12 is a broadly neutralizing antibody against several human immunodeficiency virus type 1 (HIV-1) isolates. It binds to a cluster of mannose moieties on the gp120 envelop glycoprotein.[69,70] The crystal structure of 2G12 in complex with the oligosaccharide $Man_9GlcNAc_2$ **27** revealed that the antibody forms a tightly packed interlocked dimer *via* a three-dimensional swap of the V_H domains resulting in two additional (secondary)

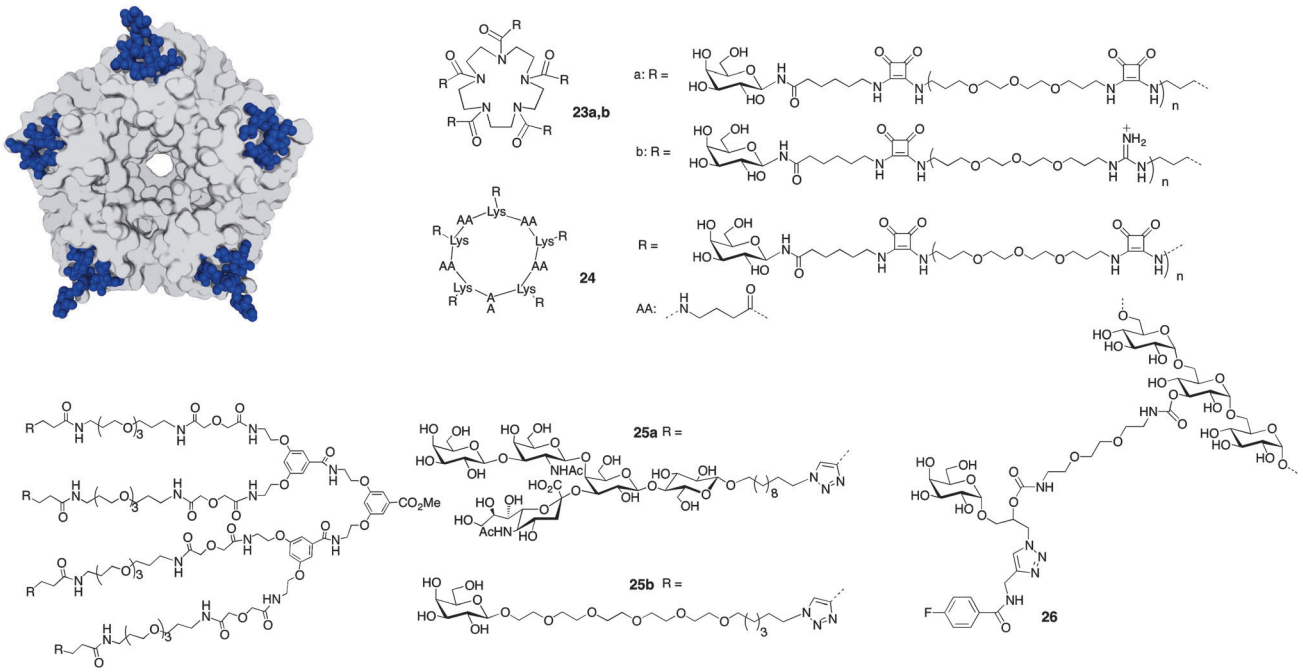

Fig. 4 X-ray structure of cholera toxin bound to GM1os and inhibitors of CT and the heat-labile enterotoxin of *E. coli* (LT).

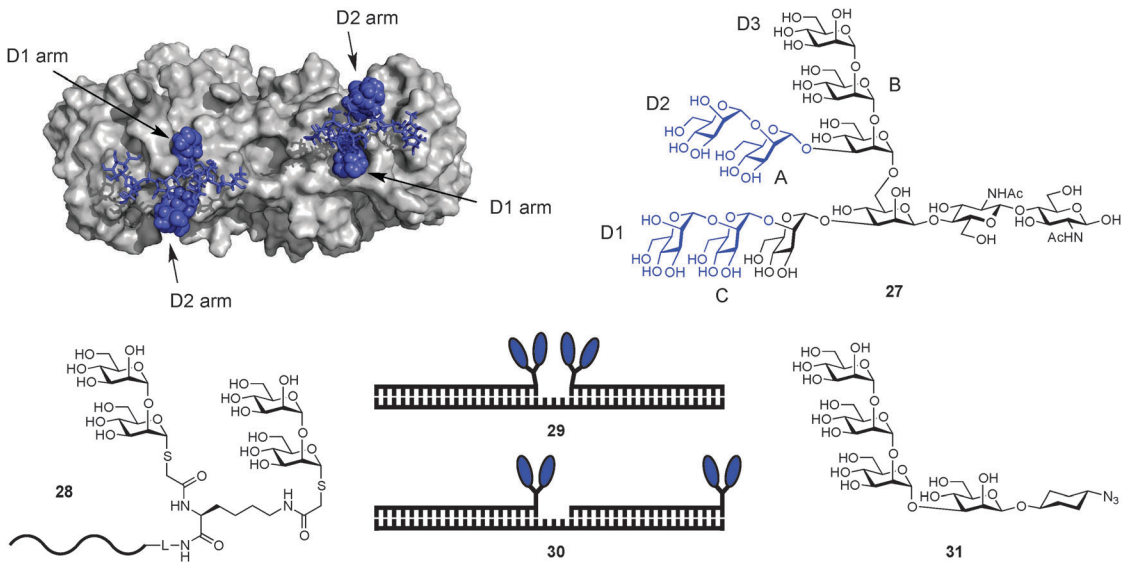

Fig. 5 X-ray structure of dimeric Fab 2G12 in complex with two molecules of **27** (ref. 71) (D1 and D2 arms colored blue in the formula are shown with space filling models in the structure) and synthetic ligands.

binding sites at the V_H/V_H' interface (Fig. 5).[71,72] This extraordinary structure provides the multivalent surface necessary for multiple interactions with the gp120 oligomannose residues. According to the crystal structure, the distance between the two primary binding sites of the antibody is 31 Å.[73] Each of the two oligosaccharides **27** found in the complex bind with the D1 arm to one of the primary binding sites with the majority of contacts with the terminal Man(α1-2)Man disaccharide (sugars C and D1). The D2 arms provide additional contacts with the secondary binding sites mainly with mannose A. The distance between the anomeric oxygens of the terminal Man(α1-2)Man disaccharides of the D1 and D2 branches (mannose residues A and C) is 9.5 Å.

Whereas several groups positioned oligosaccharide **27** or substructures thereof on different scaffold molecules, such as cyclic peptides,[74,75] or as part of dendrons[76] leading to varyingly increased binding affinities, Winssinger *et al.* systematically screened glycosylated PNA–DNA hybrids with different sugar spacings.[85] Two Man(α1-2)Man disaccharides were connected by a linker *via* S-glycosidic bonds mimicking the substructure

of oligosaccharide **27** mostly responsible for antibody binding and conjugated to either the C- or N-terminus of a PNA molecule. Such glycosylated PNAs were then hybridized onto different DNA templates resulting in varying distances between the oligosaccharide mimics. The obtained glycoclusters were then tested for binding to 2G12 by SPR. It turned out that both the spacing between the covalently linked Man(α1-2)Man disaccharides as well as the topology of the supramolecular architecture are important for high-affinity binding. Only oligosaccharide mimics of type **28** with 11 atoms between the anomeric sulfur atoms bound to 2G12. This is the same spacing as in **27**. Considering the distance between the oligosaccharide mimics, a complex of type **29** (maximum distance between the branching points joining the disaccharides is 38.5 Å) was the best binder (K_d = 4.2 μM). Longer distances as in **30** diminished binding affinity in line with the required distance of approx. 31 Å.

Whereas Winssinger *et al.* investigated 29 individually synthesized glycoclusters as 2G12 ligands, the group of Krauss and Hedstrom employed a SELEX-type technique to screen approx. 2×10^{13} different glycoclusters.[77] In their approach named SELMA (selection with modified aptamers), single-stranded DNA functions as a scaffold for positioning glycans. However, the DNA molecules themselves might also contain additional binding elements for interaction with the target. SELMA is a multistep procedure that allows to screen and amplify modified DNA. Employed was an alkyne-modified deoxyuridine triphosphate that is accepted by the polymerase used for the library preparation. In a click reaction the library of alkyne-DNA was then reacted with azide-substituted oligomannoside **31** representing the D1 arm of 2G12 ligand **27**. The glyco-DNA library was then screened for 2G12 binding and subsequently amplified making use of a coding DNA strand. In this way, the group was able to identify a series of DNA glycoclusters containing 7–14 glycosylation sites with K_d values down to 220 nM. Mutation experiments revealed that the affinity of the selected ligands is sequence-dependent and not simply the result of high valency. Furthermore, it was shown that binding is dependent on the tertiary structure of the ligands. Obviously, not all glycans contained in the ligands were involved in direct sugar binding. However, their removal resulted in reduced affinity suggesting that they are important for maintaining the correct tertiary structure. The long-term goal of such studies is the development of antigens for use as vaccines against HIV.

7. Bridging longer distances

Moving to larger distances is a great challenge. Doing this effectively with spacers based on PEG is not likely to succeed. According to the Flory radius[62] of PEG-based systems, it takes *ca.* 27 PEG units to bridge just 25 Å and *ca.* 85 units to bridge 50 Å. These spacers become impractically long and will also likely not allow the creation of selective systems. Therefore researchers have turned to other types of spacers to bridge longer distances. DNA has been used in a few cases as a spacer. Naturally the length of DNA-based spacers can be tuned very well and also the valency. Recently such a system was described for the inhibition of concanavalin A (ConA). This lectin binds to both α-linked glucosides and mannosides with a preference for the latter. It contains four tetrahedrally oriented subunits that are spaced *ca.* 72 Å apart.[78] A trigonal DNA–carbohydrate conjugate consisting of a three way junction piece of DNA was designed to contain multiple copies of maltose.[79] Notably those systems that contained sugars in all three arms bound most strongly to ConA, presumably by bridging to three of its four binding sites. The affinities were determined in a fluorescence assay. The most potent compound was **32**, which contained 18 sugars and showed a K_d of 1 μM, which was 760-fold more potent (42-fold per sugar) than monovalent maltose (Fig. 6). With ConA it is clear that the chelation mechanism is only possible for large systems. These systems should also be capable of the statistical rebinding mechanism, as was shown by Mangold and Cloninger.[80] The fact that the rebinding mechanism can be very efficient is illustrated by compound **33**, which is both relatively small and of relatively low valency, yet exhibited a very large potency enhancement of 3750-fold per sugar in a hemagglutination inhibition assay in comparison with Me-α-D-glucopyranoside.[81] The results of the three way junction DNA with ConA can also be due to the rebinding mechanism but this seems less likely.

Potent binding in this system is the result of a proper (trigonal) orientation rather than being directly proportional to the valency. The three way junction pieces of DNA were also conjugated to lactose moieties and applied in the binding of the lectin RCA_{120}, a galactose specific lectin with two binding sites separated by *ca.* 100 Å. In this case the presence of sugars in two of the three arms was sufficient to achieve optimal binding (K_d 1 μM), in line with a chelation mechanism

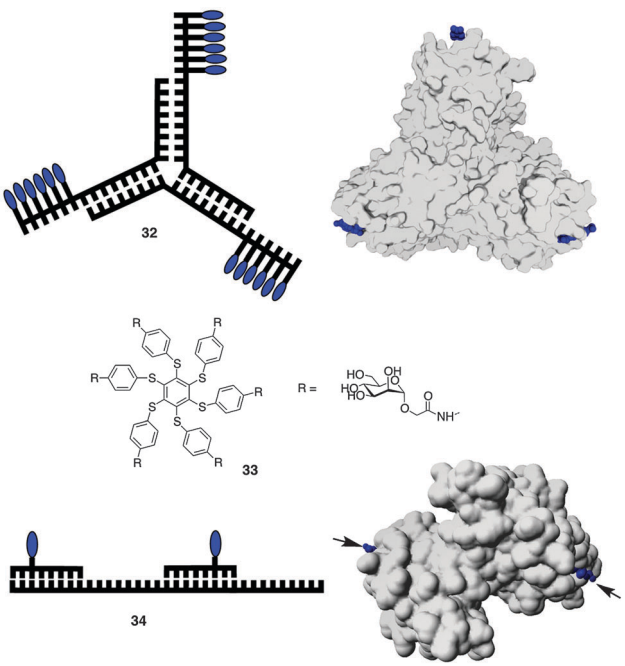

Fig. 6 X-ray structures of ConA[78] and of the ECL lectin[87] and their inhibitors/ligands.

involving two RCA_{120} binding sites. The possibility for long range chelation type multivalency effects is also supported by the work of Seitz and coworkers on PNA–DNA hybrids,[82,83] a method pioneered also by the groups of Kobayashi[84] and Winssinger.[85] Seitz *et al.* hybridized several sections of PNA containing a LacNAc ligand onto a DNA template thus creating a series of low valent systems (valency 1–4) with different distances between the ligands. An example is the divalent **34**. The dimeric *Erythrina cristagalli* lectin (ECL) target contains two binding sites spaced *ca.* 100 Å apart, when taking the curvature of the protein into consideration. In SPR binding studies the best divalent complex **34** bound the lectin with a K_d of *ca.* 10 μM, which represents an 80-fold potency increase (40-fold per sugar), an impressive result for such a long distance with such a low valency. The distance between the sugars was found to be optimal at *ca.* 104 Å, but the system was relatively forgiving in this respect. Increasing the flexibility of the systems was actually beneficial for the binding. Due to the lectin surface curvature the DNA–PNA scaffold complex needs to be able to bend somewhat to reach both binding sites. Recently Seeberger and coworkers used a DNA double helix to position two copies of the Lewis X saccharide at a fixed distance (34 Å).[86] This presentation enabled binding to mMGL1-Fc, a mouse C-type lectin on antigen presenting cells, although no quantification of the binding effects were reported. It is clear that longer distances can be bridged by molecules with the right dimensions and rigidity. DNA and DNA–PNA hybrids are particularly useful because their architecture allows relatively precise placement of the sugars, although naturally not all distances can be generated. The latter issue can be overcome with a flexible link between the DNA or PNA scaffold and the carbohydrate ligand. Potency enhancements reported so far are more moderate than those covering shorter distances. There should be other types of molecules with a fixed conformation and sufficient solubility to explore these challenges further.

8. Conclusions

Over the years many advances have been reported with respect to the potency enhancements that were achievable with multivalent carbohydrates. We here mostly wrote on the chelation based advances but one can never do this exclusively as rebinding phenomena can also occur and additional aggregation phenomena may complicate matters. Nevertheless chelation is a clear goal to aim for as was shown in many cases. In fact with the advent of the X-ray structures and the EPR-based analysis performed for WGA there should not be any doubt that it was operational for this lectin. Architectures and spacers play an important role. The spacers bridging the binding sites have historically been very long and flexible and their lengths were ideally in line with the Flory radius. Since this puts a limitation on achieving larger systems, more rigid well-defined systems have appeared based on glucose–triazole alternations for the medium size ranged or those based on nucleic acids (incl. PNA) to bridge distances of around 100 Å. In other words well-defined spacers are needed to achieve longer distances and do so with the highest potency enhancements. As far as the architecture is concerned, a system containing residues capable of acting as hinges is clearly capable of adjusting its size and can therefore be a potent inhibitor, however, when we are aiming at *in vivo* applications, selectivity is a very important aspect, and more well-defined systems need to be developed to bridge specific distances. To some extent this has been shown, but a lot more is possible. There is no limit to the imagination of the synthetic chemist so thrilling future examples may be anticipated.

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