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Towards bacterial adhesion-based therapeutics and detection methods

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Núria Parera Pera,[a] and Roland J. Pieters[a*]

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Bacterial adhesion is an important first step towards bacterial infection and plays a role in colonization, invasion and biofilm formation. Interference with this process is an intriguing approach to fight or prevent bacterial infections, that should lead to less resistance, as selection mechanisms are not triggered. The adhesion process involves in many cases lectin-like adhesion proteins on the bacteria with binding specificity for carbohydrates on the tissue surfaces. Here progress is reported on the development of new carbohydrate-based adhesion inhibitors of the human pathogens uropathogenic *E. coli* and *Pseudomonas aeruginosa* and the pig and zoonotic pathogen *Streprococcus suis*. Both monovalent as well as multivalent carbohydrate ligands have been explored for this purpose. The best systems have been applied *in vivo* with several promising results. The recognition process responsible for adhesion has also been used for the detection and removal of bacteria by large multivalent molecules or nanoparticles.

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Introduction

Bacterial adhesion is the attachment process of a pathogen to a tissue cell leading to an infection. The adhesion process is often a required prelude to colonization, to invasion and also to biofilm formation. It is an important determinant of the infectivity of pathogens for certain species and also for certain tissues, called tissue tropism. The adhesion process is often mediated by carbohydrate-protein interactions,[1] involving bacterial proteins and tissue carbohydrates. The bacterial proteins are called adhesins and they are either located on the bacterial surface or are part of bacterial appendages such as pili or fimbriae. The discovery of carbohydrate binding specificities of bacteria and also of bacterial toxins has opened a new area for the design and synthesis of new bacterial inhibitors. This approach aims to interfere with the very early stages of an infection, avoiding strategies that kill the pathogens and lead to selection pressure and antibiotic resistance.[2] Both the use as a stand-alone therapy approach or usage in combination with an antibiotic can be foreseen. Furthermore the use as a prophylactic may also be useful in specific situations. The protein carbohydrate interactions that are involved in the infection can also be used for developing bacterial detection tools. As such, one would potentially be able to selectively detect harmful bacteria, since their binding specificity is linked to their virulence. Besides detection, mild bacterial removal may also be achievable in specific industrial contexts. To this end glyconanoparticles are starting to play important roles. Glyconanoparticles contain a large surface area whose carbohydrates binds tightly to the bacterial adhesins, making them particularly suitable for the detection strategy.

This review will give an overview of important carbohydrate binding specificities of relevant pathogens. Furthermore, the latest developments in the use of carbohydrates as part of designed monovalent inhibitors and multivalent inhibitors and their use in *in vivo* experiments will be discussed, followed by detection studies. The focus is on three well-studied, but very different pathogens: 1) the fimbriated uropathogenic Gram negative *E. coli*, 2) the Gram negative pathogen and biofilm forming *Pseudomonas aeruginosa*, and 3) *Streptococcus suis*, a non-fimbriated, Gram positive pig and zoonotic pathogen. Conclusions will be drawn with respect to the feasibility of an anti-adhesion therapy for each of the pathogens and the feasibility of detection systems based on bacterial adhesion.

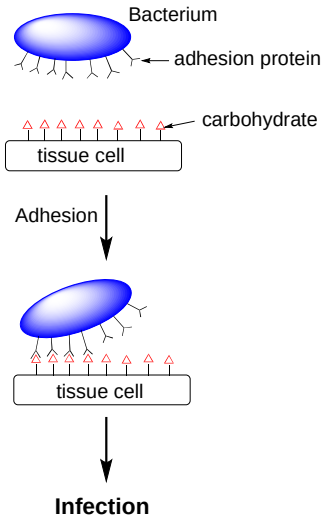

Fig. 1. The bacterial adhesion principle towards infections.

Bacterial adhesion and carbohydrate recognition

The most widely studied adhesins are those from uropathogenic *E. coli* (UPEC). This bacterium has the ability to adhere to urothelial cells and to ascend the urinary tract. In order to do so *E. coli* strains exist that contain type 1 pili and there are those that contain P-pili. The mannose-specific type 1 pili are responsible for the initial colonization of the bladder and posterior cystitis, whereas the galabiose specific P-pili account for infection of the kidneys. Another case represents the streptococcus bacteria. Different adhesion specificities from different species have been observed, the most common ones being sialic acid, and galactose specific (Fig. 2).[3] In addition to host tissues, bacteria adhere to other microbial cells and to various surfaces. This kind of adhesion is a prerequisite for the formation of bacterial communities or biofilms. Opportunistic pathogens like *P. aeruginosa*, *B. cenocepacia*, *H. influenza*, *S. aureus*, and *S. pneumoni*, have carbohydrate binding adhesins that have been studied (*vide infra*). The opportunistic pathogen *B. cenocepacia* produces four soluble carbohydrate-binding proteins and they each contain at least one domain related to the fucose-binding lectin of *Pseudomonas aeruginosa*, LecB (PA-IIL, *vide infra*). The lectins are BC2L-A, -B, -C and -D. BC2L-A is a dimer with mannose specificity, while the other three have additional N-terminal domains. Particularly interesting is BC2L-C whose N-terminal domain binds fucosides.[4] This protein therefore binds two types of carbohydrates. The mannose recognition is responsible for displaying the lectin on the bacterial surfaces where its flexible hexameric structure is ideally positioned for a role of linking bacterial and epithelial cells.

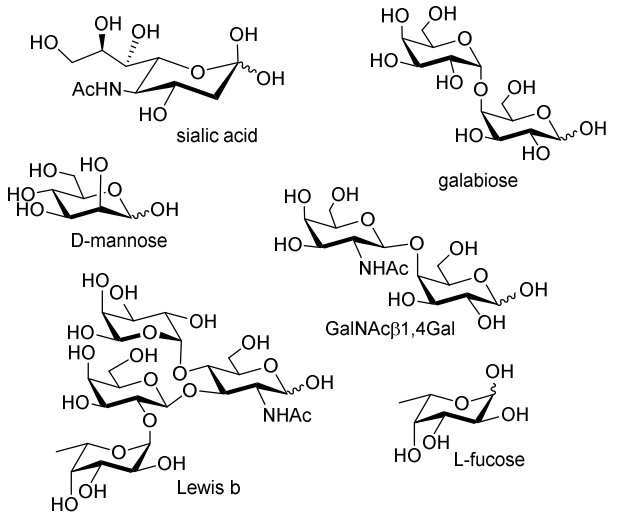

Fig. 2. Common carbohydrate structures to which bacterial pathogens bind.

Haemophilus influenza, *Staphylococcus aureus*, *Streptococcus pneumonia* are opportunistic pathogens of the upper airways and also a common cause of ear infections. These pulmonary pathogens bind to GalNAcβ1-4Gal structures (Fig.2) as their minimal adhesion sequence as part of the gangliosides GM_1 and GM_2.[5] *H. pylori* is a Gram-negative bacterium and the causative agent of chronic gastritis. *H. pylori* infection is associated with the development of gastric and duodenal ulcer disease and gastric carcinoma. For the adhesion of *H. pylori* to host gastric epithelium, different adhesins have been identified; the Lewis-b (Fig. 2) binding BabA, and the two sialic acid binding proteins SabA and HP0721.

In the review a few cases are selected, i.e uropathogenic *E. coli* with a focus on FimH, *Pseudomonas aeruginoisa* with a focus on lectin LecA and the pig pathogen *Streptococcus suis* with a focus on its adhesin SadP. For each of these pathogens achieving complete bacterial adhesion inhibition require relatively high concentrations i.e. typically in the micro- to milimolar range. To improve the situation chemical modifications of the sugars can and have been made, in some cases guided by available X-ray structures. Another approach that was used was the design and synthesis of multivalent inhibitors. Such a design can lead to a major potency enhancement depending on the orientation of the glycans of the inhibitor. It also depends on the spatial arrangement of the binding sites within the adhesin or of the relative arrangement of the adhesins themselves on the pathogen.[6] The ultimate goal of all the efforts to create potent inhibitors is the development of anti-adhesive drugs. An important step towards this is the *in vivo* evaluation of the most potent compounds. Studies that reported on this are discussed. Besides synthetic multivalent scaffold molecules, new multivalent platforms like nanoparticles have also been reported that were used mostly for detection purposes, i.e. to ultimately provide a rapid detection method as an alternative to time consuming culturing.

Uropathogenic *E. coli* (UPEC) with type I fimbriae displaying the FimH adhesin.

Monovalent inhibitors. A bacterial pathogen whose adhesion properties are widely studied is type 1 fimbriated *E. coli*. It contains fimbriae or pili, which in uropathogenic *E. coli* (UPEC) are assembled from 4 subunits: FimA, FimF, FimG, and FimH. The FimH adhesin is present at the tip of the fimbria and possibly at 100-150 nm intervals[7] along the shaft. It is formed by two domains that contain a single carbohydrate-recognition domain (CRD). The adhesin is responsible for the recognition of mannose derivatives of the glycoprotein uroplakin Ia (UPIa), which is located on the urinary bladder mucosa.[8] Monovalent mannosides were found to inhibit the FimH adhesin albeit with low potency.[9] New and greatly improved monovalent inhibitors were designed in part based on structural information from the crystal structure of FimH co-crystallized with a mannoside.[10] With the X-ray structure new and valuable information about a hydrophobic ridge outside the CRD was found, explaining the previously observed potency enhancement with hydrophobic aglycons. The compounds with *p*-nitrophenyl (**2**) and the umbeliferyl (**3**) aglycons were significantly more potent than the corresponding methyl isomer (**1**) (Fig. 3).[11] As a consequence, more hydrophobic mannose conjugates were synthesized. Studies showed that a simple heptyl chain (as in **4**) would bring the potency into the same range as **2** and **3**, with a 70-140 fold K_d lowering in comparison to **1**.[12] Further optimizations led to compound **5** (R= OEt), with a 6900-fold enhancement over the methyl analog in an ELISA assay. Additional elaboration of the system by displacing the ethoxy group proved counterproductive.[13] However also the nitrogen analog (R= NHEt) lacking the Cl proved to be a very potent adhesion inhibitor of UPEC adhesion to human cells.[14,15] Ernst and coworkers produced a series of indolylphenyl and indolinylphenyl α-D-mannopyranosides.[16] These were evaluated first in a cell-free binding assay. An ortho-chloro substituent on the phenyl ring adjacent to the anomeric oxygen and an electron-withdrawing substituent on the indole/indoline moiety led to the highest affinities as seen for **6** which was ca. 30 times more potent than **4** in this assay. This is likely due to the π−π stacking with one of the electron rich tyrosine residues of the hydrophobic ridge or tyrosine gate. Similarly, a large series of biphenyl derivatives was investigated resulting in **8** with a similar affinity as **6**.[17,18] Related optimization studies with the biphenyl based inhibitors led to compounds **9**-**11**.[19,20] While the potency is hard to compare to other known compounds and the used assays were different, it is notable that **9** was 1000-fold more potent in a hemaglutination inhibiton assay than the corresponding non-substituted biphenyl analog. Compounds **6**-**10** were all studied *in vivo* (*vide infra*).

Compound **12** resulted from an optimization study of a series of mannose derivatives with N-linked heterocyclic aglycons.[21] These were evaluated to inhibit the FimH displaying adherent-invasive *Escherichia coli* (AIEC) that induce gut inflammation in patients with Crohn's disease. Significant potencies were obtained, especially for **12,** and solubility issues, often a problem for lipophilic mannose derivatives are less prominent when using heterocycles.

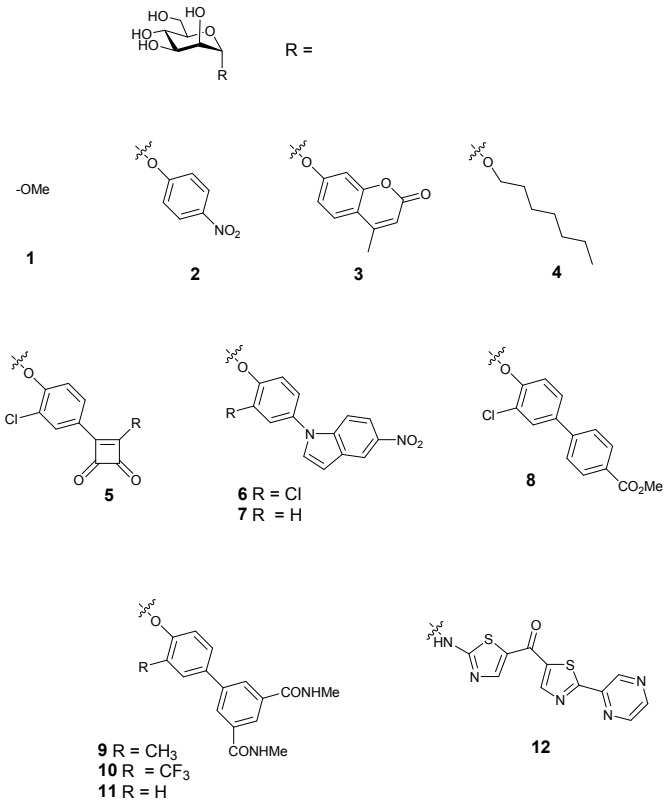

Fig. 3. Structures of increasingly potent monovalent FimH inhibitors.

Multivalent inhibitors Besides the optimization of monovalent inhibitors a major effort has been directed towards multivalent inhibitors. The distances between the mannose binding sites are large, as only a single FimH molecule, containing a single binding site, resides at the tip of each of the fimbriae. Multiple systems have been evaluated over the years as has been summarized previously.[22] Considering that the distances between the binding sites are very large, a chelation type multivalency effect is unlikely for synthesized inhibitors.[22] Nevertheless major effects due to other mechanisms, such as statistical rebinding, are possible and have in fact been observed.[23,24] It should be kept in mind that the nature of the reference compound in this case is very important considering the extreme sensitivity of FimH binding to the type of the aglycon, as illustrated above. Enhancements might otherwise be deceptively large compared to α-methyl mannoside. Heptavalent **13,** whose scaffold originated from a β-cyclodextrin, was the most potent hemaglutination inhibitor of a study being 9-fold per sugar more potent than α–heptyl mannosidesub.[25] Similarly, heptavalent **14** which contains a β-cyclodextrin scaffold, showed nanomolar affinity, while the scaffold containing only a single ligand arm bound in the low micromolar range.[26] In general the potency increases due to multivalency are modest as also observed for functionalized dendrimers[27,28] and fullerenes.[29]

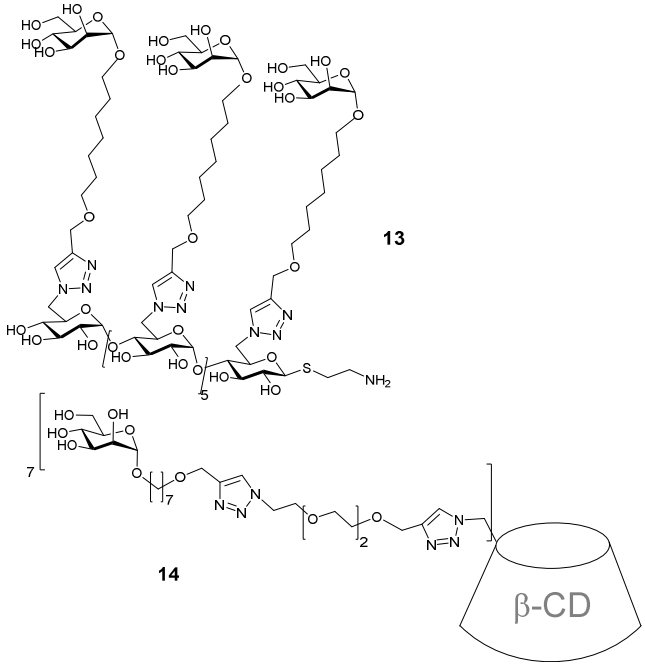

Fig. 4. Multivalent UPEC inhibitors.

In vivo studies The first *in vivo* study was carried out by Svanborg Edén *et al*, in the 1980s, where they introduced monovalent α-D-mannopyranoside in combination with bacteria and in high concentrations into the bladder of mice.[30] The results obtained showed that the low affinity of the monovalent mannoside required high mannose concentrations for inhibition. More recently, new initiatives have been undertaken. For an orally active therapeutic two major barriers need to be passed. The first is the uptake into the blood form the intestines. The second is the secretion into the urine, since the UPEC resides in the bladder. The compound has to arrive unmodified in the bladder or at least in a form that is still biologically active. Ernst *et al*. have systematically studied the relevant properties for their inhibitors, e.g. compound **6**.[16] They recommend a log D for the compounds in the range of 1 to 2. This results in good absorption in the intestines. Furthermore, this range leads to optimal, i.e. slow, clearance into the urine, which is useful to keep the therapeutic dose present in the bladder for a longer period of time. Compound **6** had a logP of 1.9 and a minimal inhibitory concentration of adhesion ($MIC_{adhesion}$) of 0.14 μg/ml. Intravenous administration of **6** at a dose of only 0.05 mg/kg in a mouse lead to an 8 h time period in which the concentration of **6** was above 0.14 μg/ml. For compound **7** (log D = 1.9, $MIC_{adhesion}$ = 0.49 μg/ml), the availability in the urine extended well beyond 8 h, at a higher dose (1 mg/kg) due to better solubility. Compound **7** was subsequently used intravenously in a treatment study at 1 mg/kg in a mouse infected with UPEC. After 3 h bacterial counts in the bladder tissue were reduced by an impressive 3.7 log units, a result similar to an sc dose of 8 mg/kg of ciprofloxacin, a standard antibiotic treatment. In a separate study, compound **9** was given orally to a mouse (50 mg/kg), resulting in a 6 h time span in which the urine level was over 10 μM, i.e. far above the predicted minimum effective concentration. The related compound **11** performed similarly in this study with respect to urine levels, albeit at twice the dosage. Compound **11** had previously been evaluated in a treatment and infection model using oral administration.[31]

First, it was shown that intraperitoneal administration (10 mg/kg) resulted in effective compound levels in the urine until at least 8 h afterwards. Oral administration (100 mg/kg) yielded three times the intraperitoneal level after 8 h. Of the administered drug over 95% reached the bladder unchanged while the remainder was hydrolysed at the glycosidic bond. In a chronic mouse cystitis model, a single oral dose (100 mg/kg) resulted in a drop of bacterial levels of 3 log units, a bigger drop than was obtained with the administration of the antibiotics trimethoprim-sulfamethoxazole. The first *in vivo* studies with multivalent mannosides were recently reported with compound **14**.[32] The compound (10 μM) was brought into the bladder by catheder along with bacteria. After 6 hours the bacterial levels in the bladder tissue were reduced by ca. 1 log unit in comparison to the control. The same result required ca. 1 mM of a monovalent mannoside, indicating a modest multivalency effect. A distribution study of a radioactive relative of **14** was also performed. Intravenous injection of 60 μg into a mouse led to a rapid accumulation of *ca.* 20 % in the bladder followed by a slow but steady subsequent excretion, likely maintaining therapeutic levels in the bladder for 24 h.

Detection Multivalency has played an important role in the development of UPEC detection devices. Disney *et al*, synthesized a fluorescent poly(p-phenylene ethynylene) polymer with appending mannose units which was not only able to bind to bacteria but was used for detection. Using this method it was possible to detect 10^4 bacteria per mL by fluorescence microscopy.[33] The same group also used microarray technology to detect bacteria by displaying various monosaccharides on the surface of the array. The bacteria were then made visible using a fluorescent dye staining the bacterial DNA. Only the mannose containing spots on the array were visible and with a detection limit of around 10^5 bacteria per mL.[34]

A related approach was reported by the group of Huang, who used a multivalent presentation of mannosides on the surface of magnetic nanoparticles.[35] The use of magnetic particles has the distinct advantage that the bacteria bound to the particle can be separated from unbound matter with a simple magnet. It proved possible to capture up to 88% of the bacteria present in solution, clearly showing the potential use for decontamination. The new glyconanoparticles had an average diameter of 10 nm, and were therefore much smaller than the bacteria (a rod-shaped *E. coli* is ca. 2000 nm long), leading to the attachment of numerous particles per bacterium. Fluorescence was used as a visualization technique with a detection limit of 10^4 per mL. The non-magnetic nanodiamonds also showed decontamination potential.[36] Bacterial agglutinates are formed in the presence of the nanodiamonds that can be filtered off.

More groups have been interested in the use of magnetic particles for detection of bacteria. Iyer *et al*, used the much larger magnetic microparticles conjugated to mannose derivatives via a streptavidin-biotin linkage.[37] The method was shown to be more sensitive in comparison with the same particles displaying specific antibodies.

Other techniques based on a multivalent presentation of mannose for the binding to FimH have been reported based on Quarz Crystal Microbalance (QCM)[38] or quantum dots (QDs).[39] The Man-QDs had a diameter of ca. 15 nm and were able to aggregate the bacteria. Centrifugation was used to isolate the aggregates and fluorescence to quantify the bacteria. This way the method was shown to be able to detect as few as 10^4 bacteria per mL. Control experiments with an *E. coli* strain defective in the FimH or using galactose-coated QDs, were both negative.

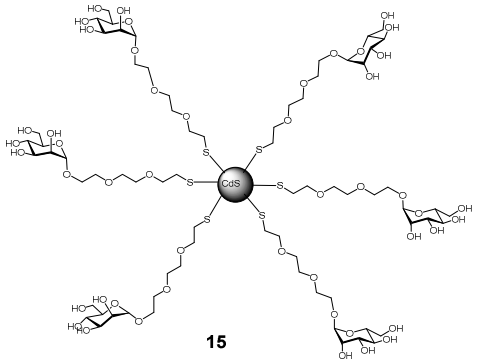

Fig. 5. Schematic representation of Man-QDs.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic pathogen causing lethal airway infections in cystic fibrosis (CF) and immuno-compromised patients. It binds to the GalNAcβ1-4Gal epitope using an adhesin that is part of its type IV pili.[40] Besides this *P. aeruginosa* synthesizes two soluble lectins, LecA (or PA-IL) and LecB (or PA-IIL) with binding specificities for galactosides and fucosides respectively. These soluble lectins are tetrameric and seem to mediate adhesion and are responsible for biofilm formation. They attach themselves to the bacterial surface through binding to glycoproteins and to components of the airway mucosa.

Monovalent inhibitors The GalNAcβ1-4Gal epitope has been modified in order to optimize potency.[41] Introduction of a propyloxy group at C(2) yielded a ten-fold potency enhancement. Far more optimizations have taken place for galactosyl ligands for LecA. This resulted in the observation that the p-nitrophenyl aglycon as in **16** (K_d 14.1 μM), led to a 6-fold potency increase relative to free D-galactose (K_d 87.5 μM) and even a 21-fold increase for **17** (K_d 4.2 μM), both based on ITC measurements.[42,43] X-ray structures of both these compounds in complex with LecA identified a T-stack or edge to face interaction between the aryl part of the aglycone and His50 as the likely cause of the potency enhancement. Similar observations about aromatic aglycons being beneficial for binding were also reported by Vidal *et al.*[44] Compound **18** bound LecA with a K_d of 5.8 μM, which is 12-fold more potent than β-GalOMe. Reymond *et al.* studied the stabilization in more detail and found further confirmations of the T-shaped stacking motif in other derivatives such as **19** (K_d 4.2 μM), although no additional affinity enhancements were obtained.[45] The results were largely confirmed in a recent report describing the thioglycoside version of **19** (K_d 6.3 μM).[46]

The close relative of LecA on *P. aeruginosa*, LecB, binds strongly to L-fucose (K_d 3 μM). The protein binds to fucosides, but a microarray study showed the Lewis a sequence was the strongest binder, and its disaccharide substructure L-Fucβ1-

4GlcNAc was identified as a lead structure for further optimizations.[47] Compound **20** emerged as the strongest binder (K_d 290 nM) with a similar potency as Lewis a (K_d 210 nM). Recently the cross reactivity of this type of inhibitors with DC-SIGN was identified as a potential problem.[48] Starting from the observation that LecB binds to mannosides (K_d α-methyl mannoside 71 μM), new ligands were developed. By elaborating the C6 carbon, new contacts to the protein were introduced leading to **21** and **22** with K_d's of 3.3 and 18.5 μM respectively. The new contacts may lead to improved selectivity relative to other fucoside-specific lectins, an important feature for therapeutic application.

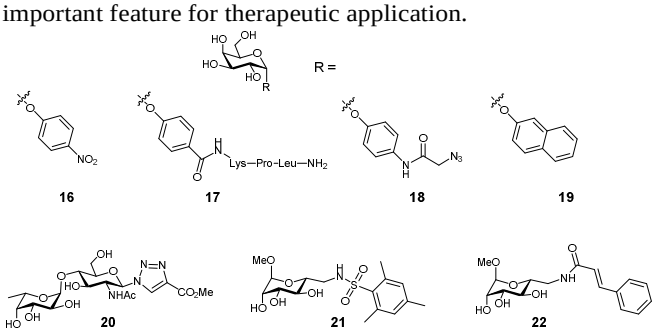

Fig. 6. Monovalent Inhibitors of LecA (**16**-**19** and LecB (**20**-**22**).

Multivalent inhibitors Multivalent inhibitors were synthesized based on the GalNAcβ1,4Gal sequence yielding good inhibition but modest multivalency enhancements.[49] Multivalency played an important role in the binding of LecA to galactosyl conjugates as for example has been demonstrated by microarray studies.[50] The reason for this is that two of the four binding sites of the tetramer are spaced closely together, i.e. the distance is 26 Å, making chelation possible.[6] A large number of glycoconjugates have been synthesized using multivalency as the design principle and major potency enhancements have been observed. Here only a small selection will be shown, other recent summaries are available.[2,6]

A calixarene scaffold proved to be suitable for bridging the binding sites. Compound **23** (K_d = 90 nM) with a relative potency of 1138 (285-fold per sugar) when compared to just the monovalent 'arm' molecule.[44] Linking 12 galactosides to a fullerene (**24**) resulted in very high potencies, with the most striking result being an IC_{50} of 40 nM in an ELLA experiment, representing a relative potency per sugar of 458-fold. The cyclic tetraglucosamine core scaffold **25** containing four attached galactoside ligands was recently reported as an effective LecA inhibitor. It combines a potent aromatic aglycon moiety in the spacer arm with a suitable scaffold structure. The result is a K_d of 79 nM. In an attempt to design a divalent system specifically to bridge the 26 Å gap between the binding sites, we explore a well-defined more rigid spacer composed of alternating glucose and triazole units.[51,52] This design was intended to be a straight bridge between the binding sites. The best potency was obtained with compound **27** which showed an IC_{50} in an ELISA type assay of 2.7 nM, a potency enhancement of ca. 7500 fold, while it's K_d was determined to be 28 nM. The shorter (**26**) and longer (**28**) analogs were far less potent with IC_{50}'s of 3.5 μM and 0.84 μM respectively, supporting the notion that optimizing a design is possible with the glucose-triazole units. Molecular modeling confirmed both the 'effective' spacer lengths of members of this spacer family and also indicated a good spatial match between divalent ligand and protein. The study underscores the potential for selective multivalent inhibitors and its low valency also likely does not lead to counterproductive bacterial aggregation, which systems of higher valency are more likely to induce.

In vivo studies The number of *in vivo* studies is very limited so far even though the arsenal of potent inhibitors, for both LecA and LecB has recently become large. An important *in vivo* study was undertaken to study both the effects of LecA and LecB on *P. aeruginosa* pathogenicity and the effect of LecA and LecB inhibitors.[53] Bacterial counts of the lungs were ca. 4 log units lower when mutants not expressing LecA or LecB were compared with the wild type. A situation that could be recreated by taking wild type bacteria and co-administrating it with Me-α-Gal and Me-α Fuc (15 mM each). Similarly, bacterial dissemination was also reduced due to the inhibitors. However, the pathogenicity of *P. aeruginosa* is multifactorial, and LecA and LecB only affect the early stages. This is also indicated by the result that no significant difference was observed in survival in a 7 day murine model using wild type strains or LecA and LecB deletion mutants. Nevertheless the early stages, combined with effects of inihibors on biofilm formation does indicate that LecA and LecA can be valuable therapeutic targets. Both LecB[54,55] and lecA[42] inhibitors were shown to block formation of *P. aeruginosa* biofilms or facilitate their dispersion.

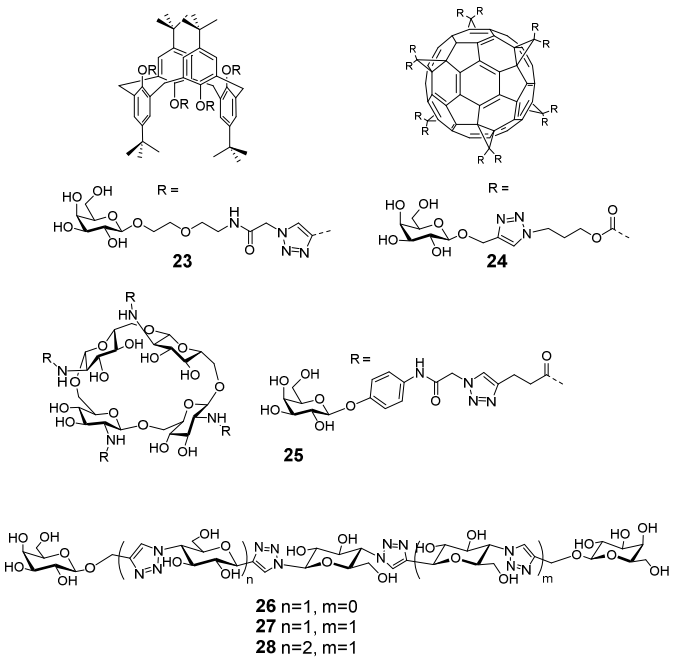

Fig. 7. Ligands of *P. aeruginosa* lectin LecA

Detection *Pseudomonas aeruginosa* detection experiments were undertaken by immobilizing glycans on a surface by photolitography and microcontact printing.[56] Exposure of the surfaces to the bacteria resulted in patterns that were best detected by dark field microscopy using a Fourier image transformation. Two glycans were used: GalNAcβ1,4Galβ1,4Glc presumably binding to the lectin present of the type IV pili,[40] and Fucα1,2Galβ1,4Glc, presumably binding to LecB. Detection with the former sequence was more reliable and led to a detection limit of 10^3

cfu/mL, whereas the lecB based detection was more sensitive to the conditions. It was suggested that the quorum sensing machinery is responsible for variable lecB expression and subsequent detection.

Streptococcus suis

Streptococcus suis is Gram positive bacterium and an important pathogen that can cause meningitis, septicemia, and pneumonia in pigs, but it is also zoonotic, i.e. capable of causing human diseases.[57] Human *S. suis* infections are quite uncommon but potentially life-threatening and the pathogen is an emerging public health concern. Of the numerous serotypes that exist, *S. suis* serotype 2 is considered the most virulent, also in zoonosis. Only recently the adhesion protein responsible for the galabiose specificity has been identified and characterized as SadP.[58] The adhesin is a ca. 200 kDa protein with specificity for only Galα1,4Gal, and not for related sequences such as lactose. The adhesion sequence contains and LPXTG motif for anchoring the protein to the cell surface.

Monovalent inhibitors Inhibition studies have found the disaccharide Galabiose (Galα1-4Gal) to be a very specific inhibitor for the bacterium. Deleting of individual OH's of galabiose and evaluating the compounds as inhibitors revealed that the 2-, 3-, 4'-, and 6'-hydroxyxls were essential in the recognition.[59] Interestingly, this is a different subset than is recognized by *E. coli*'s PapG adhesin, also galabiose specific. Nilsson and co-workers have since then created a large variety of derivatives, that indeed can benefit from substitution on C(2') and C(3').[60] This resulted in inhibitors **29** and **30** (Fig. 8), with IC_{50}'s for a hemagglutination inhibition assay of 30 and 50 nM, respectively, ca. one order of magnitude improvement over de parent p-methoxyphenyl galabioside (IC_{50} 310 nM).

Multivalency has also been a benefit for the inhibition and detection of this bacterium. Already in 1997 Magnusson and coworkers showed the benefits of multivalency by making a series of multivalent compounds. The best of these compounds was **31**, with an IC_{50} for a hemaglutination inhibition assay for two strains of 2 nM, considerably lower than monovalent reference compounds with IC_{50}'s of 300-1300 nM.[61] We explored the limits of the effect by making a larger series of compounds.[62,63] We noticed that a valency of 4 was sufficient, as shown by a surface plasmon resonance assay, indicating **32** as the most potent compound per sugar (64-fold). An octavalent PAMAM derivative was the most potent in a hemaglutiunation inhibition assay (IC_{50} = 0.3 nM). Varying the linker but keeping the length the same resulted in a compound **32** with similar potency to **31**.[64]

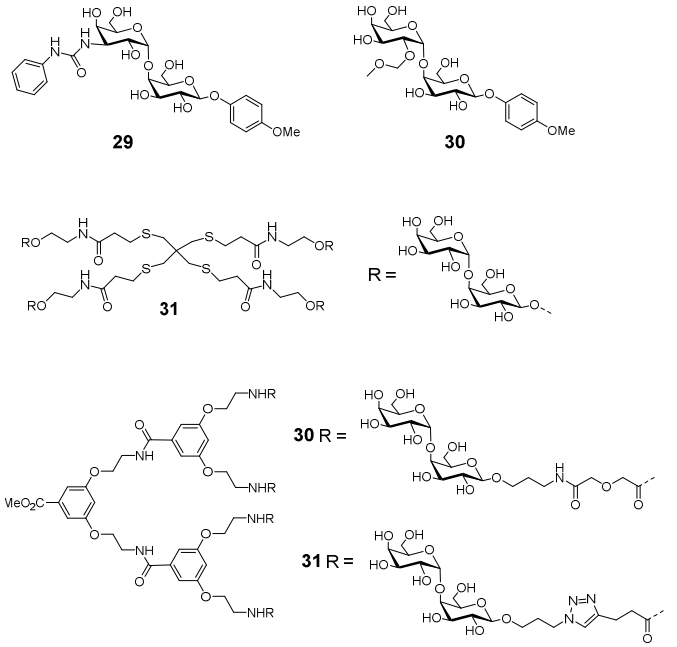

Fig. 8. Inhibitors of *Streprococcus suis*

In vivo Compound **31** was tested in an *in vivo* model.[65] The study involved a peritonitis mouse model. Although not overall significant, notable effects were observed with the bacterial levels in the liver, lungs and spleen despite the low dose (ca. 7.5 mg/kg). The variable results between experiments were attributed to varying capsule expression or the relatively long time between dosing and bacterial evaluation.

Detection *Streptococcus suis* detection was explored using magnetic particles with a diameter of 250 nm that were decorated with streptavidin units. The particles were decorated with biotin-linked galabiose, and incubated with varying amounts of *S. suis*. Magnetic capture and quantification by determining ATP-linked luminescence gave a detection limit of 10^4 bacteria/mL.[66]

Conclusions

The specific adhesive capabilities of bacterial pathogens have certainly inspired a lot of scientists among which are increasing numbers of carbohydrate chemists to design and synthesize inhibitory molecules. The appeal is strong to block the pathogenicity rather than kill the pathogen and face the inevitable resistance build-up. If the pathogen mutates, it should no longer be infective as the same recognition process is the foundation for both processes, i.e the adhesion proteins are immutable. While potentially applicable to numerous pathogens, here has been a distinct focus on a select number of bacteria. The focus has been on UPEC, *P. aeruginosa* and *S. suis*. For each of these the recognition process has distinct features. For UPEC the monovalent recognition is well understood and highly potent ligands have been made and studied both *in vitro* and *in vivo*. For UPEC major advancements have been made in recent years. Not only in achieving very strong *in vitro* inhibitors but also in gaining insights into the absorption and excretion process these compounds need to go through in order to be therapeutic. Therapeutic *in vivo* results are promising, although the results

of different labs cannot always be directly compared due to different approaches, such as oral, IP, or intravenous administration of the compounds. Nevertheless in all cases reduction of bacteria either in the urine or in the bladder tissue have been determined. The results indicate that a therapeutic is a possibility. The situation for multivalent compounds is less far advanced. In part this is due to the relatively small affinity benefits that are obtained by this and furthermore the increased complexity for *in vivo* applications, e.g. with respect to pharmacokinetics. Nevertheless also here positive results indicate that there is potential there. For the detection, multivalency is a necessary feature to bind and/or aggregate bacteria to enable detection. Several methods are reported, but the use of magnetic particles seem to have the most potential for easy application. For *P. aeruginosa* most of the recent activity has been on the inhibition of both LecA and LecB. For LecA a distinct T-shaped aryl-CH interaction has been identified, enhancing monovalent affinities. However the most striking advances have been made in the design and synthesis of numerous multivalent systems. Affinities in the low nanomolar range have now been reported. The challenge now lies in translating these results to the *in vivo* situation, although peptidodendrimers were already shown to block or destroy biofilms induced by LecA and LecB. For LecB, the most advances were made in the monovalent optimization.

The pig and zoonotic pathogen *S. suis* has seen constructive monovalent ligand optimization and also proved subject to major multivalent enhancement by low multivalency systems. The *in vivo* results were inconclusive but do offer hope, while detection based on *S suis* adhesion has clearly shown promise.

Based on the covered literature is not possible to give a general answer with regards to the feasibility of anti-adhesive therapy or detection methods. For each pathogen the situation needs to be evaluated. Clearly monovalent ligand optimization is always possible, especially when X-ray structures are around. This can be a good step to *in vivo* studies if potencies are sufficient. If they are not, multivalency can help tremendously if multiple binding sites are present. However multivalent ligands are rarely drug-like structures, so additional hurdles towards application have to be overcome. It is clear that more scientists are willing to go this route and depending on the type and area of application this has distinct possibilities for success. General challenges for therapeutic applications include phase variation, i.e. the variability of expression of the adhesion proteins as a function of the circumstances. Perhaps this is no problem for UPEC as these bacteria will always need the ability to adhere to survive. However, for others the adhesion abilities may vary with numerous factors, possibly limiting therapeutic possibilities. Biofilm blocking or destruction seems a very promising application, where multivalency seems to be an important feature of effective compounds. For detection purposes, the demands are less strict than for therapeutic applications and clearly detection systems can be developed. However PCR-based methods may be hard to beat unless the adhesion-based methods provide additional virulence-based information. The adhesion-based method, besides being very fast, has the potential to detect only the virulent pathogens. This, has yet to be demonstrated, but is a distinct possibility. Using bacterial adhesion properties for selective bacterial removal is also a distinct possibility, yet this will have to be demonstrated by a stirring practical application.

[a] Department of Medicinal Chemistry and Chemical Biology. Utrecht Institute for Pharmaceutical Sciences, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, The Netherlands, Fax: (+) 31-30-2536655, Tel: +31 620293387; E-mail: R.J.Pieters@uu.nl.

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