Inhibition of P-fimbriated *Escherichia coli* adhesion by multivalent galabiose derivatives studied by a live-bacteria application of surface plasmon resonance

Annika Salminen¹, Vuokko Loimaranta¹, John A. F. Joosten², A. Salam Khan³, Jörg Hacker³, Roland J. Pieters² and Jukka Finne¹*

¹Department of Medical Biochemistry and Molecular Biology, University of Turku, FI-20520 Turku, Finland; ²Department of Medicinal Chemistry, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, PO Box 80082, NL-3508 TB Utrecht, The Netherlands; ³Institute for Molecular Biology of Infectious Diseases, University of Würzburg, D-97070 Würzburg, Germany

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**Objectives:** Uropathogenic P-fimbriated *Escherichia coli* adheres to host cells by specific adhesins recognizing galabiose (Galα1-4Gal)-containing structures on cell surfaces. In search of agents inhibiting this first step of infection, the inhibition potency of a set of synthetic mono- and multivalent galabiose compounds was evaluated. In order to mimic the flow conditions of natural infections, a live-bacteria application of surface plasmon resonance (SPR) was established.

**Methods and results:** For the measurement of the binding of *E. coli* to a surface containing galabiose, live bacteria were injected over the flow cell, and the inhibition of adhesion caused by the galabiose inhibitors was recorded. Quantitative binding data were recorded in real-time for each inhibitor. The results were compared with those of conventional static haemagglutination and ELISA-based cell adhesion assays. Compared with the Gram-positive *Streptococcus suis* bacteria, which also bind to galabiose and whose binding inhibition is strongly dependent on the multivalency of the inhibitor, *E. coli* inhibition was only moderately affected by the valency. However, a novel octavalent compound was found to be the most effective inhibitor of *E. coli* PapG J96 adhesion, with an IC₅₀ value of 2 μM.

**Conclusions:** Measurement of bacterial adhesion by SPR is an efficient way to characterize the adhesion of whole bacterial cells and allows the characterization of the inhibitory potency of adhesion inhibitors under dynamic flow conditions. Under these conditions, multivalency increases the anti-adhesion potency of galabiose-based inhibitors of P-fimbriated *E. coli* adhesion and provides a promising approach for the design of high-affinity anti-adhesion agents.

Keywords: anti-adhesion, glycodendrimers, oligovalent inhibitors, *Streptococcus suis*

**Introduction**

In most infectious diseases, the initial event is the adherence of pathogenic organisms to the host.⁴ Bacterial engagement of host receptors can target a pathogen to a particular niche, capture underlying signalling pathways, establish persistent infections and induce invasion.⁵ In many instances, the host receptor for bacterial binding has been identified to be the carbohydrate portion of glycolipids or glycoproteins.⁶ The bacterial interaction with target cells can be blocked by receptor-structure mimicking soluble carbohydrates or analogues. Anti-adhesion is a highly promising approach to fight against pathogens in the era of increasing antibiotic resistance. Carbohydrates are unlikely to be...
toxic or immunogenic, especially because many of them are natural constituents of cell surfaces and body fluids. Furthermore, it is improbable that resistance would give the bacteria the capability to overcome the inhibitory effect of the anti-adhesive drug without impairing their ability to adhere to the host cell. The strength of intercellular adhesions mediated by non-covalent protein–carbohydrate pairs derives from the large number of pairs involved. Polyvalent oligosaccharides can be orders of magnitude better adhesion inhibitors than their monoclonal counterparts.

Adhesins are often assembled into hair-like appendages called pili or fimbriae. P-fimbriae containing a tip-associated adhesin that recognizes the galactosyl-α1-4-galactose (galabiose) moiety of the globoseries of glycolipids are important virulence factors for the establishment of Escherichia coli urinary tract infections. A galabiose-specific adhesion activity has also been discovered in Streptococcus suis. This is a zoonotic bacteria causing a wide range of clinical diseases in young pigs. In humans, S. suis type 2 can cause meningitis, septicaemia and endocarditis. Both galabiose-binding pathogens, E. coli and S. suis, represent potential targets for anti-adhesion therapy.

Surface plasmon resonance (SPR) is an optical biosensor technique that detects molecular interactions on a metal chip surface by measuring changes in the refractive index. It has been widely used for the quantification and kinetic analysis of receptor–ligand interactions, but so far SPR has not been in much use for the characterization of the bacterial cell interactions. SPR has been applied to the study of the interaction of staphylococci and streptococci with fibronectin and salivary. For E. coli, it has been shown that it is possible to record bacterial binding to a globoside surface. Besides being a quantitative method, SPR measures interactions dynamically as a function of time in a continuous flow system. The SPR assay may mimic the natural conditions better than a classical static adhesion assay, since the main purpose of adhesion is to prevent bacteria from detaching from their target surface under vigorous shear stress caused by body fluids such as urine or blood.

In this study, we have determined the inhibitory potency of multivalent galabiose derivatives as model compounds for anti-adhesion therapy and furthermore investigated the use of SPR for the characterization of the binding of E. coli to galabiose-coated surfaces. Comparison of the binding inhibition to that of S. suis revealed differences in the influence of valency on the inhibition between the bacteria recognizing the same carbohydrate structure.

Materials and methods

Bacterial strains

E. coli strain HB101 expressing PapGJ96 (class I), cloning vector pBR322 (control), PapGAD110 (class II) or PrsGJ96 (class III) were cultivated overnight at 37°C on Luria–Bertani agar plates supplemented with ampicillin (50 mg/L), chloramphenicol (20 mg/L) or tetracycline (10 mg/L). The galabiose-binding S. suis strain 628 was cultivated in Todd–Hewitt broth supplemented with 5% (w/v) yeast extract at 37°C.

Synthesis of the galabiose-BSA molecule and the galabiose derivatives

The mono- and multivalent galabiose derivatives (Figure 1) were prepared as described by Joosten et al. Galabiose was conjugated to BSA by a coupling reaction between the lysines in BSA and the galabiose derivative (Figure 1) outfitted with a carboxylic acid moiety. These two were coupled by the peptide coupling reagent TSTU (N, N', N'-tetramethyl-O-(N-succinimidyl)uronium hexafluorophosphate) for 3 h in a dioxane/borate buffer mixture at pH 8.5. The product was purified by dialysis, lyophilized and analysed by MALDI-TOF analysis. Each BSA-galabiose conjugate contained on average 20 galabiose moieties per BSA.

Bacterial whole-cell SPR assay

For experiments with E. coli, a flow cell of the sensor chip CM3 (Biacore AB, Uppsala, Sweden) was coated with galabiose-BSA conjugate in the coating buffer (10 mM sodium acetate, pH 5.0) using the amine coupling kit according to manufacturer’s instructions. BSA (Sigma) was used as a reference-coated to the reference cell of the chip. Alternative pHs 4.0, 4.5 and 5.0 of the coating buffer were tested to find the best conditions for coating of the conjugate molecule. On average surfaces with 970 or 1490 resonance units (RU) were obtained for galabiose-BSA and BSA, respectively.

The change in RUs caused by bacterial cell binding to the galabiose-BSA on the chip was measured with Biacore X (Biacore AB). All binding experiments were run at 25°C. The overnight grown bacteria were washed twice with HBS-P buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl and 0.005% (v/v) polysorbate 20) and re-suspended in 2 mL of HBS-P. The optical density (OD600) was adjusted using HBS-P. To find an optimal flow rate for E. coli inhibition assays, 100 μL of the bacteria with an OD600 of 0.6 was injected over the sensor chip at flow rates between 5 and 40 μL/min. The flow rate 20 μL/min was chosen for the inhibition measurements. Bacterial dilutions with OD600 values between 0.1 and 1.0 were tested and a dilution with an OD600 of 0.8 was used in the inhibition measurements. In assays with S. suis, a pigeon ovomucoid-coated sensor chip was used. The S. suis cells were adjusted in HBS-P to an OD600 of 0.3 and the flow rate of 10 μL/min was applied in all assays.

The inhibitory galabiose compounds were diluted in HBS-P and incubated with 5 min before injection over the sensor chip. The bacteria were allowed to bind for 5 min. At the end of each binding cycle the sensor chip surface was regenerated consecutively with 4 M MgCl₂ and 50 mM EDTA in E. coli, or 10 mM NaOH in S. suis experiments. The background signal from the BSA coated reference cell was subtracted from the galabiose-BSA conjugate’s signal. Mannose (Sigma) was used as a negative control for binding. The BIAevaluation program (version 3.0, Biacore) was utilized in the inhibition curve calculations and in curve normalization from 0 to 100.

Haemagglutination assay

Equal volumes (50 μL) of bacteria expressing PapGJ96 or PapGAD110 and 1% sialidase-treated human or sheep (PrsGJ96) erythrocytes were mixed and haemagglutination was visually recorded after incubation for 1 h on ice. For inhibition assays, 2-fold dilutions of the galabiose compounds (25 μL) were mixed with bacteria (25 μL). After 5 min incubation at room temperature (21–23°C), 50 μL of the erythrocytes were added and the MIC values were recorded.

Cell adhesion assay

The cell adhesion assay was performed essentially as described before. A cell line (T24) of human origin with epithelial-like
morphology was used as a model for eukaryotic cells. After harvesting cells from confluent monolayers, epithelial cells were seeded in 96-well flat-bottomed cell culture plates (Greiner) in 200 μL aliquots for 24–30 h. The culture medium was removed, the monolayers were washed once with sterile PBS and the monolayers were fixed by incubation with 100 μL of glutaraldehyde (1.25% in PBS) at room temperature for 30 min. After washing three times with PBS, the monolayers were blocked with 3% (w/v) BSA-PBS (pH 7.4) for 2 h at 37°C. Serial dilutions of E. coli HB101 expressing PapGJ96 or pBR322 (a control) were first made to determine the numbers of bacteria required to obtain 50% binding. The number of bacteria was determined by measuring optical density (OD) at 550 nm, with a standardized chart correlating OD with viable counts. After removal of blocking solution and washing, 100 μL of the serially diluted bacterial suspensions (3 × 10^8 bacteria in the first well) in 1% BSA-PBS was added to each well, and plates were incubated for 2.5 h at 37°C. To determine the binding of bacteria to the monolayers, wells were washed as above and incubated with rabbit anti-E. coli polyclonal antibody (Biodesign International, Kennebunk, Maine) in 1% BSA-PBS (1:2000) was added and incubated for 1.5 h at 37°C (100 μL/well). Following another washing step, peroxidase-conjugated goat anti-rabbit immunoglobulin G (Dako) in 1% BSA-PBS (1:2000) was added and incubated for 1.5 h at 37°C (100 μL/well). Finally, the wells were washed and the bound enzyme was detected by the addition of 100 μL of substrate (Pierce ImmunoPure TMB substrate Kit) for 5–30 min.

To stop the reaction, 100 μL of 1 M HSO₄ was added and the absorbance measured at 450 nm with an ELISA reader. Control wells were treated in the same manner except that the wells had no bacteria.

To test the inhibitory potency of the galabiose derivatives, the binding inhibition assay was performed essentially as described before. Briefly, P-fimbriated and non-fimbriated bacteria at the number 1 × 10^8/well, which gave 50% binding, were preincubated with 2-fold serially diluted galabiose derivatives for 15 min at room temperature. After preincubation, the whole mixture was transformed to the fixed monolayers of T24 cell line. The IC50 values were defined as described. Control studies to identify the effect of spacer arms and polymer carrying no sugar moieties were also performed. The bacterial strain was tested in three independent experiments, and in each experiment, three determinations of bacterial adherence were performed in parallel.

Figure 1. The multivalent galabiose derivatives used in this study.

Results

The whole bacterial cell SPR assay

PapGJ96 (class I) is the most specific PapG adhesin binding to the terminal Galα1-4Gal structure. In haemagglutination assay (see below), only the recombinant class I E. coli strain HB101 expressing PapGJ96 was inhibited by the galabiose derivatives and was therefore selected for the SPR studies. Various flow rates and bacterial densities were tested to find suitable conditions for an SPR assay measuring the E. coli PapGJ96 binding (Figure 2). Since 100 μL is the maximum volume of the bacterial dilution that can be loaded in the injection loop, the duration of the runs varied with different flow rates (Figure 2a). The somewhat irregular output of the sensorgrams was probably caused by bacterial aggregates. The sensorgram responses were dependent on the dilution of the bacteria (Figure 2b). After the background signal from the BSA-coated reference cell was subtracted, a difference of ~240 RU was reached using a bacterial dilution with an OD₆₀₀ of 1.0. The binding was reversible as bacteria mostly dissociated after the injection of the bacterial cells was discontinued. A flow rate of 20 μL/min (Figure 2a) and a bacterial dilution with an OD₆₀₀ of 0.8 (Figure 2b) were chosen for the inhibition measurements. The chip could be regenerated for repeated analysis and the sensor chip was stable for several days. However, in some cases a significant drop in the signal could not be assigned to any known cause.

The galabiose derivatives inhibited bacterial adhesion to the coated chip surfaces in a dose-dependent manner (Figure 3). The adhesion could be almost totally inhibited with all the galabiose compounds tested, when a sufficient concentration of the compounds was used. Mannose was used as a negative control and it caused no inhibition even at 10 mM concentration (data not shown).

Relative adherence inhibition curves were calculated and plotted for each measured galabiose derivative and the curves for mono-, tetra- and octavalent derivatives are shown in Figure 4. The inhibition of E. coli PapGJ96 adhesion required higher concentrations of the compounds than was needed for S. suis.
inhibition. In the case of *E. coli* PapGJ96 adhesion inhibition, the octavalent galabiose compound was superior to the tetravalent derivative, which in turn was a better inhibitor than monovalent galabiose. In the case of *S. suis*, when valency was taken into account the tetravalent galabiose compound was the most effective inhibitor and was clearly better than the octavalent inhibitor.

**Figure 2.** Effect of flow rate (a) and bacterial density (b) on the adhesion of *E. coli* PapGJ96 cells to galabiose-BSA-coated chip in SPR assay. To find optimal conditions for *E. coli* inhibition assays, bacterial cells were injected over the sensor chip at the flow rates and the OD$_{600}$ values indicated.

**Figure 3.** Inhibition of *E. coli* PapGJ96 adhesion to galabiose-BSA-coated chip by various concentrations of galabiose inhibitors. The bacteria were mixed with monovalent (a), divalent short arms (b), divalent long arms (c), tetravalent (d), octavalent (e), octavalent PAMAM dendrimer (f) and allowed to adhere to the galabiose-BSA chip.
were normalized to a scale of 0–100 for each compound (NRU).

Table 1. Relative inhibitory potency [IC50 (monovalent compound)/IC50 (multivalent compound)] of galabiose dendrimers in SPR adhesion assay with E. coli and S. suis. For the calculation of the potency per sugar unit, the relative potency was divided by the valency of the compound.

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<th>E. coli</th>
<th>S. suis</th>
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<td></td>
<td>relative potency per sugar</td>
<td>relative potency per sugar</td>
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<tr>
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<td>Octavalent</td>
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<tr>
<td>PAMAM-8</td>
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<td>0.76</td>
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\*Joosten et al.21

To better compare the inhibition strengths of the multivalent compounds, the IC50 values were determined and the relative inhibitory potency per sugar was calculated for each inhibitor (Table 1). The most effective inhibitor for E. coli PapGJ96 adhesion was the octavalent compound, which had a 5-fold higher inhibitory potency per sugar compared with the monovalent inhibitor. This indicated that multivalency moderately increases the effect of E. coli PapGJ96 inhibitors. The effect of multivalency was more pronounced in S. suis. The tetravalent compound had inhibitory potency per sugar over 60 times higher than the monovalent compound as expected.21 In contrast to the results with E. coli, the inhibitory potency per sugar was not further increased when the valency was amplified (Table 1).

Haemagglutination and cell adhesion assays

Human erythrocytes express glycolipids with the Galα1-4Gal disaccharide on their surface and therefore haemagglutination assay can be utilized in the adhesion inhibition studies of bacteria binding to galabiose.28 For PapGJ96, the MIC of the poly(aminodioamine) (PAMAM-8) dendrimer was as low as 7 μM, which was a 46 times lower concentration than the MIC of the monovalent compound (Table 2). Taking valency into consideration, the PAMAM-8 compound was six times as potent an inhibitor as the monovalent compound. On the whole, when the valency of the inhibitor was increased, the relative potency per Galα1-4Gal disaccharide molecule was also slightly increased. The derivatives were not inhibitory for PapGAD110 (class II) and PrsG96 (class III) within the concentration range used (up to 2.5 mM). This is in line with previously published results showing that binding of PrsG96 prefers a terminal GalNAc residue attached to galabiose, and that galabiose-O-Me has an inhibitory concentration much higher for PapGAD110 than for PapGJ96 (7.4 mM29 as compared to 0.2 mM30).

The inhibitory potency of the galabiose derivatives was also investigated by measuring E. coli PapGJ96 adhesion to cells with an epithelial-like morphology. The IC50 values determined with the cell adhesion assay are depicted in Table 2. The octavalent PAMAM-8 molecule was found to be the most potent inhibitor with an IC50 value of 22 μM. Essentially no multivalency effect could be seen in these assays.

Comparison of the assay results

The inhibition results from the SPR assay correlated rather well with the results of the two conventional assays (Table 2). The inhibitory concentrations of the compounds were slightly different as they were determined with the different assays, but the IC50 or MIC values decreased in all the assays when the valency of the compound was increased. In SPR and haemagglutination assays, the inhibitory potency per sugar was higher in the compounds with a higher valency. This was, however, not seen in the cell adhesion assay. In all the assays, the divalent compound with a long spacer arm showed slightly better inhibition than the divalent compound with a short spacer, but the difference was small.

Discussion

We set up an SPR assay that measures the binding of live uropathogenic E. coli to a galabiose surface constructed from galabiose-BSA. With the assay a series of mono- and multivalent galabiose derivatives were evaluated for their effectiveness to inhibit the binding of the pathogen. For comparison, the inhibitory concentrations of the compounds were also determined with the more conventional haemagglutination and ELISA-based cell adhesion assays.

None of the assays utilized requires ligand or receptor labeling, which could interfere with the binding interaction. The adhesins are in their natural environment on the bacterial cell...
surfaces. Compared with the classical adhesion assays, the SPR assay has some advantages. First, it detects the interactions of the intact bacteria in real-time. Second, the SPR runs require substantially less ligand and receptor than the conventional methods. Third, the binding surface can be easily varied with a specific target using amine, thiol, aldehyde or biotin coupling. Fourth, the SPR method may also be utilized when the adhesin does not cause haemagglutination or there is no suitable cell line available. Fifth and most importantly, as a continuous flow system the SPR assay simulates the dynamic flow of physiological conditions. It has been suggested that in some cases bacterial adhesion is enhanced by the shear stress caused by body fluids.31 Shear-activated adhesion could provide bacteria a mechanism to resist, at least to a certain extent, soluble inhibitors that would otherwise be effective in blocking adhesion. Thus, the SPR assay employing whole-cell bacterial binding may offer an appropriate method for the evaluation of new anti-adhesive agents under dynamic flow conditions using defined binding targets. In the present study, the SPR assay resembled the haemagglutination assay, whereas the cell adhesion assay gave slightly different results in terms of inhibitory activity per sugar residue.

Polyvalent macromolecules can potentially engage several adhesin molecules on the bacterial cell surface at the same time.6 Based on the haemagglutination and SPR assay results, multivalency had a moderate but clear effect on E. coli PapGJ96 inhibition. In the SPR assay, for instance, the octavalent derivative had five times the relative inhibitory potency per sugar compared with the monovalent inhibitor. A similar moderate multivalency effect has been recently reported for F1C-fimbriated E. coli, which was inhibited with multivalent GalNAcβ1-4Gal ligands.32 On the other hand, a multivalency effect was observed when type-1-fimbriated S. suis, the inhibitory potency was not further increased in S. suis when the valency was increased from tetravalent to octavalent.

The divergence of the inhibition results between E. coli and S. suis is not surprising in view of the finding that the adhesins are quite distinct from each other although they both bind to galabiose. A model of the binding epitope of galabiose-containing saccharides has been designed for both pathogens.10,35,36 As the PapGJ96 adhesin is fimbrial tip-associated, it is likely that the multivalent inhibitors do not reach multiple adhesin molecules as effectively as in the case of S. suis,34 and on the other hand the spacing of the binding sites within the molecule may be different. Although the difference was quite small, in all the three different assays the divalent inhibitor with longer spacer arms appeared to be a more potent inhibitor than the divalent inhibitor with shorter arms, which suggests that the distance between the galabiose units in a multivalent molecule could affect the inhibition potency.

The oligovalent ligands studied represent a novel type of inhibitor of P-fimbriated E. coli. The octavalent compound was found to be the most effective inhibitor for E. coli PapGJ96 adhesion with an IC50 value of 2 μM, which is comparable with previously described monovalent inhibitors having aromatic substituents at the O-3′ and the O-1 positions of the galabiose molecule.37 It remains to be seen whether even more potent anti-adhesive agents might be constructed using aromatic substituents in a multivalent presentation.

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Transparency declarations

None to declare.

References


SPR of live E. coli adhesion inhibited by multivalent galabiose


