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Cholera Toxin Inhibitors Studied with High-Performance Liquid Affinity Chromatography: A Robust Method to Evaluate Receptor–Ligand Interactions

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Abstract

Anti-adhesion drugs may be an alternative to antibiotics to control infection of micro-organisms. The well-characterized interaction between cholera toxin and the cellular glycolipid GM1 makes it an attractive model for inhibition studies in general. In this report, we demonstrate a high-performance liquid affinity chromatography approach called weak affinity chromatography to evaluate cholera toxin inhibitors. The cholera toxin B-subunit was covalently coupled to porous silica and a (weak) affinity column was produced. The K_D values of galactose and meta-nitrophenyl α -D-galactoside were determined with weak affinity chromatography to be 52 and 1 mM, respectively, which agree well with IC_{50} values previously reported. To increase inhibition potency multivalent inhibitors have been developed and the interaction with multivalent glycopolypeptides was also evaluated. The affinity of these compounds was found to correlate with the galactoside content but K_D values were not obtained because of the inhomogeneous response and slow off-rate from multivalent interactions. Despite the limitations in obtaining direct K_D values of the multivalent galactopolypeptides, weak affinity chromatography represents an additional and valuable tool in the evaluation of monovalent as well as multivalent cholera toxin inhibitors. It offers multiple advantages, such as a low sample consumption, high reproducibility and short analysis time, which are often not observed in other methods of analysis.

Keywords

affinity constant; carbohydrate interactions; cholera toxin; high-performance liquid chromatography; receptor; ligand interactions; weak affinity chromatography

Carbohydrate structures on mammalian epithelial cells are targeted by micro-organisms for specific attachment and initiation of infection or toxic effect. A specific inhibition of this initial interaction is an attractive strategy to control viral and bacterial infections (1). Anti-adhesion drugs could, for example, be of outmost importance in defeating cholera and traveller's diarrhea (2). The causative agents in these diseases are the toxins produced by *Vibrio cholerae* and *Escherichia coli*, respectively. The two toxins, cholera toxin (CT) and the heat-labile enterotoxin (LT), have a homology of about 80% and are essentially identical in binding and toxic effect, even though LT causes less severe symptoms than CT (3). They belong to the

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heterohexameric AB₅ bacterial toxins which all have an organization comprising a toxic A-subunit and an independent homopentamer of B-subunits, responsible for the specific recognition of glycosphingolipids on epithelial cells. The natural target in the case of CT and LT is the ganglioside GM1 (Gal β 1-3GalNAc β 1-4[NeuAc α 2-3]Gal β 1-4Glc β 1,1-ceramide). The binding to ganglioside GD1b, which is identical to GM1 except for a second NeuAc, is about 1000 times less efficient and other gangliosides are not known to interact (3). The binding between the B-subunits of CT (CTB) and the GM1-oligosaccharide (GM1os, Figure 1D) has been studied in great detail (4). It is clear from these studies that it is the terminal Gal β 1-3GalNAc β 1 and the NeuAc that interact with CTB, and the affinity between CTB and GM1os is one of the highest known for a protein–carbohydrate interaction, with K_D values determined via isothermal calorimetry to be 43 nM at 25 °C (5). The organization of the B-subunits into a regular pentamer indicates that a multivalent interaction between CTB₅ and the cell surface is possible and the affinity towards phospholipid membranes was found to be about 10 nM (6).

The detailed contribution of the monosaccharides in GM1os to the CTB binding has been evaluated (5) and the terminal Gal and NeuAc were found to be the primary donors to the intrinsic binding energy (54% and 44%, respectively). The contribution of the terminal Gal on the CTB–GM1os affinity arises both from the stacking with a tryptophan residue (Trp 88) in the CTB-binding pocket and from several hydrogen bonds between the terminal Gal and the protein and bridging water molecules. The binding of Gal is regarded as the ‘anchor’ of the interaction but the contribution of NeuAc is important to both total affinity and selectivity. The affinity towards different sections of the GM1os has been determined and Gal- β OMe, Gal β 1-3GalNAc β OMe and Neu5Ac α OMe had K_D values of 15, 8 and 210 mM, respectively (5). The substantially higher affinity of GM1os compared with these saccharides originates from the relatively rigid and preorganized conformation of GM1os that fits perfectly into the binding pocket of CTB.

The well-characterized interaction between CTB and GM1 makes it an attractive model for inhibition studies. It has been shown that an oligosaccharide mimicking the ‘forefinger and thumb grip’ with Gal β 1-3GalNAc β 1 and NeuAc attached to cyclohexanediol (instead of Gal) has an affinity that is comparable with GM1os (7). Simplified structures, where NeuAc was replaced by different α -hydroxyacids, were shown to conserve most of the binding energy and a K_D of 0.10 mM was achieved for the best inhibitor (8). Another study showed that several commercial galactose derivatives interact with CTB (9) and the most efficient inhibitor among the tested compounds was *meta*-nitrophenyl α -D-galactoside (MNPG; Figure 1A), which was shown to be about 50–100 times more efficient than Gal. Different multivalent scaffolds have been used in combination with galactose or other inhibitors to increase the total affinity (avidity) (2). The most efficient of these constructs have an inhibition in the same range as the monovalent GM1os, a fact that reflects the difficulties in competing with nature’s own design.

Most of the CTB inhibitors, monovalent as well as multivalent, have been evaluated with direct enzyme-linked assay (DELA), which is similar to an inhibition ELISA (9). This type of assay is excellent for detecting the inhibition potency of a potential inhibitor but the response may not be identical over time and between laboratories. Other methods to evaluate CTB interactions have also been employed such as isothermal titration calorimetry (5), surface plasmon resonance (10) and nuclear magnetic resonance (NMR) (11). These techniques have the capacity to produce high quality data when operated/interpreted correctly but they require relatively large amounts of material, especially when low affinity interactions are studied. Affinity is determined from measuring the response at several concentrations, which is labor-intensive and time-consuming even if automated procedures have been developed.

To systematically and efficiently evaluate small differences in binding of different inhibitor variants a robust analytical method is needed which has a reproducible and high precision response. High-performance liquid affinity chromatography (HPLAC) (12–14), and especially the variant called weak affinity chromatography (WAC) (15,16), should be well suited to meet these criteria. The principle of WAC is to immobilize a high amount of an (weak) affinity binder (CTB in this case) onto an efficient support to construct a HPLC column. The affinity of an interacting compound (inhibitor) is directly related to the retardation volume under standardized conditions, and the affinity constant of the interaction can be calculated if the number of binding sites on the column is known from frontal chromatography analysis or other techniques (14,16,17). A number of HPLAC/WAC studies with different affinity ligands such as antibodies, albumin and lectins have been performed (16–20) demonstrating the applicability of the technique.

A great advantage with HPLAC/WAC compared with other techniques aiming to determine the affinity of an interaction is that purity and concentration of the compound to be tested are relatively unimportant parameters as long as the concentration is sufficiently low to permit separation under linear conditions. In screening procedures, several compounds might even be mixed and evaluated concomitantly, to speed up the analysis time. Less than 1 μg of sample is normally enough for evaluation. Inhibition studies may also be performed in the HPLAC/WAC format by adding one compound to the mobile phase and study the retardation of another compound (14,17,20). All these separation modes are performed with standard analytical liquid chromatography instrumentation.

In this report, we demonstrate the performance of a CTB WAC column in the characterization of some known monovalent and multivalent inhibitors.

Materials and Methods

Synthesis of multivalent CTB inhibitors (glycopolypeptides)

The glycopolypeptides used in the experiments were produced by a combination of recombinant expression and chemical modification, as reported previously (21). The architecture of the glycopolypeptides was controlled on the basis of the selective placement of chemically reactive glutamic acid residues in an otherwise chemically unreactive polypeptide (22,23). The distance between the glutamic acid residues commensurate with the known distances between binding pockets on the CTB5 subunit, as indicated in energy minimization calculations. The polypeptides were derivatized with *N*-(ϵ -aminocaproyl)- β -galactosylamine (CapGal; Figure 1B) by coupling with the carboxylic acid groups of the glutamic acid residues to produce three different galactopolypeptides designated 17-H-6/CapGal₁₂, 17-H-6/CapGal₅ and 35-H-6/CapGal₆ (24). The 6-carbon linker arm of the galactopyranoside ligand was chosen based on previous studies of the contribution of the hydrophobic linker arms to the binding of CTB (25). As a negative control, a multivalent glucopolypeptide 17-H-6/Glc₁₂ was synthesized via attachment of glucosylamine to the 17-H-6 polypeptide scaffold; the glucopyranoside is known to not bind to CTB and therefore served as an appropriate negative control even in the absence of any linker arm. The polypeptide sequences and pendant saccharide ligands are listed in Table 1 for all four glycopolypeptides tested in this study.

All coupling reactions were carried out in dimethyl sulfoxide in the presence of *O*-benzotriazole-*N,N,N',N'*-tetramethyl-uranium-hexafluoro-phosphate (HBTU) and diisopropylethylamine (DIEA). The stoichiometry of saccharide ligands, HBTU and DIEA were used to produce glycopolypeptides with either complete (near 100%) or partial (near 50%) modification of the carboxylic acid groups; a schematic of the resulting architectures is given in Figure 2. For the completely functionalized glycopolypeptides 17-H-6/CapGal₁₂ and 35-H-6/CapGal₆, the controlled placement of the glutamic acid residues also directs the placement

of the galactopyranoside. In the partially modified glycopolypeptide 17-H-6/CapGal₅, ethanolamine was used to quench the unfunctionalized carboxylic acid groups. The degree of functionality of these glycopolypeptides was determined via ¹H NMR and the average numbers of pendant saccharide ligands are shown in Table 1. The secondary structure was determined via circular dichroism (CD) spectroscopy; the observation of two minima at 208 and 222 nm in the CD spectrum suggested their helical conformation. The binding of these glycopolypeptides to CTB was determined by DELA, and their inhibition enhancements (approximately 100- to 400-fold) over the monovalent galactose were reported previously (24).

Preparation of affinity column

Spherical and porous Nucleosilsilica[®] (Macherey-Nagel, Düren, Germany) with a particle diameter of 10 μm and a pore size of 300 Å was derivatized with (3-glycidyloxypropyl)-trimethoxysilane (Hüls, Marl, Germany) to obtain a matrix suitable for protein immobilization (Figure 3). The procedure was a modification of previously published methods (26). The pure silica was mixed with 5.5 M hydrochloric acid and conditioned by boiling for 4 h. After rinsing with Milli-Q water (Millipore, Billerica, MA, USA), the silica was refluxed with a 10% aqueous solution of the silane at neutral pH during 24 h. The oxirane groups were immediately hydrolyzed to diols by refluxing for 4 h under acidic conditions. The produced diol silica was rinsed with acetone and diethyl ether and dried. A small amount (110 mg) of dry diol silica was mixed with 1 mL of Milli-Q water and the diol groups were oxidized to aldehydes with by adding 100 mg periodic acid. The aldehyde silica was then washed by repeated centrifugations and mixing with 0.1 M sodium phosphate, pH 7.0. Finally, 8 mg of recombinant CTB (SBL vaccines, Stockholm, Sweden) dissolved in 1 mL 0.1 M sodium phosphate, pH 7, was added to the silica. Immobilization was performed by reductive amination (27) utilizing aldehyde groups on the silica matrix and primary amines present in lysine residues and the N-terminus of the CTB protein (Figure 3). Sodium cyanoborohydride (5 mg/mL) was used in the coupling reaction to reduce the Schiff base intermediate to a stable secondary amine. Immobilization was allowed to proceed for 48 h at 4 °C on a rotating machine. The immobilized silica (CTB-silica) was washed by repeated centrifugations and mixing with sodium phosphate buffer as above. Immobilization efficiency was determined indirectly from the absorbance at 280 nm in the supernatant before and after immobilization (including the washing buffer). A slurry of CTB-silica in 0.1 M sodium phosphate, pH 7.0, was packed in a 50 × 2.1 mm stainless steel column (Hichrom, Berkshire, UK) with an air-driven liquid pump (Haskel, Burbank, CA, USA) at 300 bar. About 75% of the total amount of immobilized silica was used to fill the column.

Frontal chromatography

Frontal chromatography was performed essentially as has been described by Kasai *et al.* (28). A Varian HPLC system (Walnut Creek, CA, USA) equipped with a 5012 pump, a Rheodyne manual injector with a 0.5-mL injection loop and a 9050 single wavelength detector, was used in the experiment. Mobile phase was 10 mM phosphate buffer, 150 mM NaCl, pH 7.0 (PBS), which was filtered through a 0.45-μm membrane (Whatman, Springfield Mill, UK) before use. The flow rate was 0.1 mL/min and the temperature was 22 ± 1 °C. MNPG (Figure 1A) was dissolved in the mobile phase, 0.5 mL was injected and the break-through curve (front) was detected at 350 nm (Figure 4A). The MNPG concentrations were 0.18, 0.35, 0.70, 1.41 and 2.42 mM. The front of sodium azide (10 μg/mL) was detected at 210 nm and used to determine the void volume of the column. Chromatography data were evaluated with EZChrom software version 6.8 (Scientific Software, San Ramon, CA, USA) and the midpoint of each front was determined with from the apex of the first derivative of the front (Figure 4B). A value of Δ*v* (retardation volume subtracted with void volume) was calculated for each MNPG concentration, and the number of moles that saturated the column at each concentration (Δ*v* ×

[MNPG]) was used to construct a one-site binding hyperbola according to eqn 1 (Figure 4C) using GraphPad Prism 4 (San-Diego, CA, USA). The number of binding sites ($B_{\max}$) on the column and the dissociation constant (K_D) of MNPG were obtained via non-linear regression analysis.

$$\Delta v \times [\text{MNPG}] = \frac{B_{\max} \times [\text{MNPG}]}{K_D + [\text{MNPG}]} \quad (1)$$

A Scatchard plot was also constructed to confirm that the interaction was homogenous (inset in Figure 4C).

Zonal chromatography of saccharides

The zonal chromatography experiments were performed on an Agilent 1100 series HPLC system, equipped with a multiple wavelength detector (Agilent Technologies, Santa Clara, CA, USA). Collected signals were analyzed with the Agilent ChemStation chromatography data system. The injection volume was 5 μL , the sample concentration was 10 $\mu\text{g/mL}$ (about 30 μM for all compounds) and detection was performed at 214 nm. All other conditions were identical to those in the frontal chromatography experiments.

When the concentration of the injected analyte is low compared with the K_D of the interaction, eqn 1 is simplified to $\Delta v = B_{\max}/K_D$; a linear isotherm. $B_{\max}$ is known from the frontal chromatography evaluation and K_D of any analyte is therefore easily obtained from the retention on the column, expressed as retarded volume (Δv). A number of nitrophenyl-galactosides (Sigma-Aldrich, St. Louis, MO, USA) was analyzed on the CTB column: 2-nitrophenyl β -D-glucopyranoside (onp- β -Glc), 4-nitrophenyl α -D-glucopyranoside (pnp- α -Glc), 4-nitrophenyl β -D-galactopyranoside (pnp- β -Gal), 4-nitrophenyl α -D-galactopyranoside (pnp- α -Gal), 3-nitrophenyl α -D-galactopyranoside (MNPG, Figure 1A), 2-nitrophenyl β -D-galactopyranoside (ONPG, Figure 1C) and phenyl- β -D-galactopyranoside (PG). CapGal (Figure 1B) was synthesized as described previously (24) and analyzed under identical conditions.

Inhibition chromatography

Inhibition chromatography was performed under identical chromatography conditions as the zonal chromatography except for detection, which was monitored at 350 nm. An additional mobile phase was prepared with 0.50 M galactose dissolved in the PBS buffer. The two mobile phases were connected to separate inlets of the HPLC pump and the CTB column was successively equilibrated with different concentrations of galactose (25, 50, 100, 200, 300 and 400 mM) by adjusting the proportions of the two mobile phases. Three zonal injections of MNPG (10 $\mu\text{g/mL}$ dissolved in mobile phase without galactose) were performed at each galactose concentration and the retention times of MNPG were recorded. MNPG was injected under linear conditions and the retention is therefore directly correlated to the number of non-occupied binding sites in equilibrium with different galactose concentrations. The K_D of galactose was determined from eqn 2 with non-linear regression analysis, where Δt (Figure 5) represents the shift in retention time of MNPG when changing the galactose concentration in the mobile phase. Total inhibition implicates that the MNPG peak elutes at the void position ($\Delta t_{\max}$). A Scatchard plot was also constructed to confirm that the inhibition agreed with a single site competition model.

$$\Delta t = \frac{\Delta t_{\max} \times [\text{galactose}]}{K_D + [\text{galactose}]} \quad (2)$$

Analysis of glycopolypeptides

Zonal chromatography experiments of the four polyvalent glycopolypeptides (Table 1 and Figure 2) were performed on the Agilent 1100 series HPLC system, equipped as above. The mobile phase was 20 mM sodium phosphate, 0.8 M sodium chloride, pH 7.0 and polypeptide concentration was 1 mg/mL (about 70 μ M). Other chromatography conditions were identical to those in the zonal chromatography experiments. In one experiment, the CTB column was equilibrated with a mobile phase supplemented with 56 mM galactose before the glycopolypeptides were injected.

Results and Discussion

Characterization of CTB column

Chromatography using unmodified silica suffers from severe loss of resolution as a result of ionic interactions of the analyte with the negatively charged silanol groups on the surface. Organic silanes, such as (3-glycidioxypropyl)-trimethoxysilane, have been demonstrated to be versatile coating agents (26) to obtain bio-compatible silica materials with different chemical properties. The covalent coupling procedure described in this paper (Figure 3) has been found to be effective and reproducible, even though tri-methoxy-silanes may generate polymers in water. In our experience, this is a minor problem, possibly because of the low concentration of silane in the coating procedure (10%). The produced diol silica maintained a high porosity and a chromatography column with the material did not show any interaction with a selection of proteins (data not shown). The diol silica is stable and possible to store for several years and we usually oxidize the diol silica to aldehyde silica immediately before immobilization. Protein immobilization was performed by reductive amination (Figure 3), which has proven to have high yield and immobilization capacity and further to be selective and stable (12,13,27). The CTB protein behaved normally and about 95% of the protein was coupled to the silica, determined from the absorbance of the supernatant and washing buffer after protein immobilization. The concentration of CTB on the silica was estimated to 73 mg protein/g silica, which probably is close to the maximum immobilization capacity (29). The CTB column was evaluated with MNPG via frontal chromatography (Figure 4) and the number of binding sites ($B_{\max}$) was determined to 261 ± 7 nmol with nonlinear regression analysis assuming a one-site binding isotherm ($r^2 = 0.998$). The straight line of the Scatchard plot indicated that the binding were homogenous in the concentration range studied. The calculated number of binding sites corresponds to an active fraction of 50%. Similar results have been obtained with other immobilized proteins in WAC (19,29).

Monovalent CTB inhibitors (saccharides)

The retention volume (Δv) in zonal chromatography of an inhibitor can be predicted if a rough value of the affinity is known from other studies, using the relationship valid for linear isotherms ($\Delta v = B_{\max}/K_D$). Galactose has been reported to have an IC_{50} of about 50 mM (9, 24), which probably is close to the K_D of the interaction. $B_{\max}$ is known from the frontal analysis and Δv can be estimated to approximately 0.005 mL (a retardation of 3 s with a flow rate of 0.1 mL/min). The resolution must be extremely high to detect such infinite retardation and therefore the affinity of CTB towards galactose was determined with inhibition chromatography in which the retardation of a reporter molecule (MNPG) was recorded when the column was equilibrated with various concentrations of galactose (25–400 mM) (Figure 5A). The maximum retardation of MNPG ($\Delta t_{\max}$) was obtained when a mobile phase without

galactose was used (indicated with a thick solid line in Figure 5A). The shift in MNPG retardation (Δt) with increasing concentrations of galactose was used to calculate the K_D of galactose according to eqn 2 and a value of 52 ± 1 mM was determined with non-linear regression analysis of the inhibition hyperbola (Figure 5B), which agrees with previously reported IC_{50} values (9,24). The calculated value of Δt_{max} was 2.19 min which corresponds to a theoretical void time (total inhibition) of 1.79 min. The experimental value of the void was 1.59 min and the difference (about 10%) might represent MNPG binding which not is inhibited by galactose. The inhibition study does, however, confirm that galactose and MNPG in principal interact with the same site and the linearity of the Scatchard plot (Figure 5C) indicates the homogeneity of the interaction.

MNPG (Figure 1A) has been reported to have an IC_{50} of approximately 1 mM (9,11) and therefore it is expected to be retarded on the CTB column to a greater extent than galactose, which was the rational behind using this compound a reporter molecule in the inhibition assay described in the preceding paragraph. The affinity of CTB towards MNPG was evaluated with both frontal (Figure 4) and zonal chromatography (Figure 6). The position of the break-through curve in the frontal analysis at different MNPG concentrations (0.18–2.42 mM) (Figure 4A) was calculated from the first derivative of the detector signal (Figure 4B) and the number of moles of MNPG which was required to saturate the CTB column was used to construct a binding hyperbola (Figure 4C). K_D and B_{max} were determined to be 1.3 ± 0.1 mM and 261 ± 7 nmol, respectively. In zonal chromatography, the simplified form of eqn 1 ($\Delta v = B_{max}/K_D$) was applied. The retardation volume (Δv) of MNPG was obtained from the flow rate (0.1 mL/min) and the difference in retention time of MNPG (3.98 min) and the void (1.59 min) (Figure 6) which generated a K_D of 1.1 mM, in good agreement with the frontal chromatography results. The other phenyl-galactosides (pnp- β -Gal, pnp- α -Gal, ONPG and PG) were analyzed only with zonal chromatography and found to elute approximately in the same position as ONPG (Figure 6). The ONPG peak is close to the void and the K_D is estimated to be approximately 10 mM. It might be suspected that the retardation of these compounds is influenced by some non-specific interactions from the hydrophobic phenyl group, as all have a similar retardation. This conclusion was also supported by the elution position of the two phenyl-glucoside controls (onp- β -Glc and pnp- α -Glc) which were slightly retarded and eluted approximately in the same position as ONPG. IC_{50} values have not been determined for these weakly interacting compounds, but semi-quantitative values in the range of 10–15 mM have been reported for pnp- α -Gal and PG (9). The caproyl-linked aminogalactoside (CapGal), which was used in the preparation of the galactopolypeptides, was more significantly retarded (T_r was 2.11 min, Figure 6) and a K_D of 5.1 mM was determined from the zonal elution; the reported IC_{50} value is 3.2 mM (9).

The reported IC_{50} values of galactose and MNPG varies in different DELA studies but values in the same range as the K_D determined with zonal chromatography have frequently been reported (9,11,24). Chromatography analysis is generally more reproducible compared with the ELISA (DELA) approach and more straightforward to perform in the case of large sample numbers. Conditions such as temperature, buffer composition and pH can easily be varied and this technique could be very valuable in the evaluation of novel monovalent inhibitors.

Multivalent CTB inhibitors (glycopolypeptides)

Four different polyvalent glycopolypeptides (Table 1 and Figure 2) were analyzed with the CTB column; 17-H-6/CapGal₁₂, 17-H-6/CapGal₅, 35-H-6/CapGal₆ and 17-H-6/Glc₁₂ (Figure 7). The initial peak close to the void of the glycopolypeptides probably represents the fraction which, for statistical reasons, has interacted monovalently in travelling through the column. The ionic strength of the mobile phase was increased to avoid non-specific interactions. It was found that the three galactose-containing polypeptides (17-H-6/CapGal₁₂, 35-H-6/CapGal₆

and 17-H-6/CapGal₅) were retarded on the column while the control glycopolypeptide (17-H-6/Glc₁₂) eluted in principal at the void position when a mobile phase without galactose was used (solid lines in Figure 7). Upon injection of the three galactopolypeptides in the presence of 56 mM galactose (dissolved in the mobile phase), their interaction was almost completely inhibited (dotted lines in Figure 7A–C), while the profile of the control glucopolypeptide (17-H-6/Glc₁₂) was not influenced by galactose in the mobile phase (Figure 7D). The inhibition by galactose indicates that the interaction with CTB relies on the pendant galactosides on the polypeptide chains of 17-H-6/CapGal₁₂, 17-H-6/CapGal₅ and 35-H-6/CapGal₆. The galactose concentration in the mobile phase was close to the K_D of galactose and in theory this would induce a change in Δv of 50% for the galactopolypeptides (for a monovalent interaction). The degree of competition in the experiment was difficult to determine, because of the broad elution peaks of the galactopolypeptides, but the rather dramatic change in the elution profile suggests that multivalent interactions are involved.

From the elution profiles of the three galactopolypeptides, it was evident that 17-H-6/CapGal₁₂ interacted more efficiently with the CTB column compared with 17-H-6/CapGal₅ and 35-H-6/CapGal₆ (Figure 7), most likely because of the higher saccharide content which increases the probability of both monovalent and multivalent interactions with the polypeptides (Figure 1). A value of the affinity (avidity) is difficult to obtain from the wide peaks in the chromatogram but the midpoint of each elution curve corresponds to apparent K_D values of about 1 mM for 17-H-6/CapGal₁₂ (Figure 7A) and 10 mM for 17-H-6/CapGal₅ (Figure 7B) and 35-H-6/CapGal₆ (Figure 7C). Comparing and ranking multivalent inhibitors from the elution profiles in WAC may, however, be more useful than calculating apparent K_D values because the degree of multivalency is highly dependent on the analysis conditions and obtained values are difficult to compare with values found with other systems. The similarity in the elution profiles of 35-H-6/CapGal₆ and 17-H-6/CapGal₅ supports the finding that the galactose content seems to determine the interaction in WAC. The results in DELA of these galactopolypeptides are somewhat contradictory as 35-H-6/CapGal₆ was identified as a more potent inhibitor compared with 17-H-6/CapGal₁₂ (24). The result of 17-H-6/CapGal₅ also differs as no inhibition was detected in DELA (data not shown) while 17-H-6/CapGal₅ exhibited an interaction with WAC (Figure 7B). The control glycopolypeptide, 17-H-6/Glc₁₂, exhibited no detectable affinity in either of the assays.

The differences in the inhibition/binding results obtained for the glycopolypeptides may result from several factors. The most important is probably that inhibition in DELA is performed under static conditions (30 min incubation time), which are more advantageous for slow interaction processes and the development of multivalent interactions, compared with the mobile flow conditions in the WAC analysis. For this reason, multivalent inhibitors might be expected to show a greater apparent affinity/inhibition in DELA than that indicated by WAC. Multivalent interactions are sensitive to the correct organization of the interacting entities and as multivalent interactions probably are more dominant in DELA compared with WAC this might explain why the galactopolypeptides interacted differently in the two assays. Another circumstance, which might be of importance, is the immobilization of CTB in WAC. The interaction of the glycopolypeptides with immobilized CTB could be hindered for steric reasons, even if the pores of the silica material are large (300 Å before derivatization) and there are no signs of exclusion of the glycopolypeptides in the chromatography analysis.

Although the DELA and WAC results are not in perfect agreement, the overall result is consistent; namely that the galactose content and distribution determine the inhibition potency of the galactopolypeptides. The two techniques complement each other as they determine the interaction under different conditions. Even if DELA may better capture the multivalent interactions of polyvalent inhibitors, the simplicity of the HPLAC/WAC experiment, its utility in evaluating interactions under flow conditions and the reasonable agreement of binding with

inhibition suggested in this report, recommends it as a useful tool in the evaluation of multivalent inhibitors.

Conclusions

Cholera toxin and the very similar thermo-labile toxin is the target of several newly developed monovalent and multivalent inhibitors. The HPLAC/WAC technique was studied as an option in evaluating structure–activity relationships of different CT inhibitors, monovalent as well as polyvalent. The K_D values of galactose and MNPG were determined with WAC to be 52 and 1 mM, respectively, which agree well with previously reported IC_{50} values. The elution profiles of three multivalent galactopolypeptides were found to be closely correlated with the galactoside content, but the ranking of the galactopolypeptides differed from previous IC_{50} measurements. We therefore conclude that WAC offers an additional alternative in the evaluation of CT inhibitors which might give complementary information of a specific inhibitor. The advantages of the chromatography technique are several; the sample consumption is low (in the range of 1 μ g), the reproducibility is high, the analysis is fast and a high number of samples might be analyzed automatically and compared under identical conditions.

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Abbreviations

CapGal	<i>N</i> -(ϵ -aminocaproyl)- β -galactosylamine
CD	circular dichroism
CT	cholera toxin
CTB	B-subunits of cholera toxin
DELA	direct enzyme-linked assay
DIEA	diisopropylethylamine
GM1os	GM1-oligosaccharide
HBUTU	<i>O</i> -benzotriazole- <i>N</i> , <i>N</i> , <i>N'</i> , <i>N'</i> -tetramethyl-uranium-hexafluoro-phosphate
HPLAC	High-performance liquid affinity chromatography
LT	heat-labile enterotoxin
MNPG	

3-nitrophenyl α -D-galactopyranoside

NMR

nuclear magnetic resonance

ONPG

2-nitrophenyl β -D-galactopyranoside

onp- β -Glc

2-nitrophenyl β -D-glucopyranoside

PG

phenyl- β -D-galactopyranoside

pnp- α -Gal

4-nitrophenyl α -D-galactopyranoside

pnp- α -Glc

4-nitrophenyl α -D-glucopyranoside

pnp- β -Gal

4-nitrophenyl β -D-galactopyranoside

WAC

weak affinity chromatography

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Figure 1. Structures of monovalent inhibitors as well as the natural ligand GM1os. (A) 3-nitrophenyl α -D-galactopyranoside (MNPG). (B) *N*-(ϵ -aminocaproyl)- β -galactosylamine (CapGal). (C) 2-nitrophenyl β -D-galactopyranoside (ONPG). (D) Gal β 1-3GalNAc β 1-4[NeuAc α 2-3]Gal β 1-4Glc (GM1os).

Figure 2.
The schemes of multivalent CTB inhibitors with different density of pendant saccharide ligands.

Figure 3.
The reaction scheme of the coating of silica with (3-glycidyloxypropyl)-trimethoxysilane and the immobilization with reductive amination.

Figure 4.

Evaluation of the MNPG-CTB interaction with frontal chromatography at pH 7.0 and 22 °C. (A) Break-through curves (fronts) from the injection of 2.82, 1.41 and 0.35 mM MNPG (0.70 and 0.18 mM fronts are not shown). (B) First derivative of the fronts; the peak apex was used to determine the midpoint of each front. (C) One-site binding isotherm based on the frontal chromatography results; K_D (1.3 mM) and B_{max} (260 nmol) were determined from the non-linear regression. The corresponding Scatchard plot is shown as an inset.

Figure 5. Evaluation of the galactose–CTB interaction with inhibition chromatography at pH 7.0 and 22 °C. (A) Retardation of MNPG (injection volume: 5 μ L, concentration 33 μ M) with increasing galactose concentration in mobile phase (25, 50, 100 and 200 mM; 300 and 400 mM are not shown). (B) One-site binding isotherm based on the inhibition chromatography results; K_D (52 mM) was determined from the non-linear regression. (C) Scatchard plot of the inhibition chromatography experiment.

Figure 6.

Zonal chromatography under linear conditions of MNPG, CapGal and ONPG at pH 7.0 and 22 °C. Injection volume was 5 μ L and sample concentrations were 10 μ g/mL (about 30 μ M for all compounds).

Figure 7.

Zonal chromatography of the four glycopolypeptides (solid lines) at pH 7.0 and 22 °C. The interaction with CTB was partly inhibited with 56 mM galactose in the mobile phase (dotted lines). (A) 17-H-6/CapGal₁₂; (B) 17-H-6/CapGal₅; (C) 35-H-6/CapGal₆; (D) 17-H-6/Glc₁₂.

Table 1

Sequences of polypeptide repeat units, pendant saccharide ligands and the average number of saccharides on the polypeptides as determined via ^1H NMR

Glycopolypeptide	Polypeptide sequence	Pendant saccharide	Number of saccharides/polypeptide
17-H-6/CapGal ₁₂ ^a	[AAAEAAAAQAAQAEAAQAAQ] ₆	Caproyl galactosylamine	12
17-H-6/CapGal ₅	[AAAEAAAAQAAQAEAAQAAQ] ₆	Caproyl galactosylamine	5
17-H-6/Glc ₁₂	[AAAEAAAAQAAQAEAAQAAQ] ₆	Glucosylamine	12
35-H-6/CapGal ₆	[AAQAAQAQAAAEAAQAAQAAQ] ₆	Caproyl galactosylamine	6

^a $x\text{-H-}y/\text{Sn}$: x represents the approximate distance (Å) between the adjacent glutamic acids as determined via molecular modeling simulations, H indicates the helical conformation of the polypeptide and y represents the number of repeats of the monomeric peptide sequence. S represents the type of pendant saccharide ligand and n represents the average number of saccharides coupled on the polypeptide backbones.