Nanomolar cholera toxin inhibitors based on symmetrical pentavalent ganglioside GM1os-sym-corannulenes†

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Eight symmetric and pentavalent corannulene derivatives were functionalized with galactose and the ganglioside GM1-oligosaccharide (GM1os) via copper-catalyzed alkyne-azide cycloaddition (CuAAC) reactions. The compounds were evaluated for their ability to inhibit the binding of the pentavalent cholera toxin to its natural ligand, ganglioside GM1. In this assay, all ganglioside GM1os-sym-corannulenes proved to be highly potent nanomolar inhibitors of cholera toxin.

1. Introduction

The World Health Organization (WHO) estimates that annually 3–5 million people worldwide are infected with cholera, resulting in over a hundred thousand fatalities.1 The responsible pathogen, the Vibrio cholerae bacterium, produces the cholera toxin (CT) protein that is responsible for the severe clinical symptoms. CT belongs to the protein family of AB5 bacterial toxins.2 The structure and activity of CT and other AB5 toxins have been investigated in detail.3 These proteins consist of two distinct domains with different roles. The A-subunit is an enzyme that – when delivered inside a host cell – is toxic and responsible for the subsequent disease symptoms. The cholera toxin B-subunit (CTB) is a lectin and plays a crucial role in the recognition and interaction with its natural ligand, ganglioside GM1 (Fig. 1a), on the periphery of intestinal cells. The crystal structure of CT4 shows that the protein complex consists of five identical monomeric CTB subunits, arranged in a pentagonal symmetry, and each of these subunits can bind the ganglioside GM1 in a one-to-one stoichiometry. Detailed calorimetric studies revealed that CT exhibits allosteric cooperativity,5 which contributes to increasingly higher binding affinities to CT when more ligands are bound.6

One of the possible approaches for the design of CT inhibitors aims to prevent the receptor-recognition process.7 Two major routes can be discerned in the literature to achieve this goal. The first strategy, monovalent receptor-binding approach, focuses on strong binding interactions, and it is based on the design and synthesis of ligands that closely mimic the natural ligand on the cell surface5 in order to obtain a strong interaction with the CTB receptor. The second approach, multivalent receptor-binding,8 exploiting chelate cooperativity, takes advantage of the pentavalent character of the ligand-binding sites of CT. This approach is based on the synthesis of a functionalized branched system, in which each branch carries a single-site inhibitor, like galactose10,11 or lactose,12 leading to a compound that has an overall stronger interaction with the toxin than the sum of the independent inhibitors. The synthesis of dendritic multivalent inhibitors, functionalized with the GM1os, has been reported;13 these displayed unprecedented high inhibitory potencies for CTB, in the picomolar range.

An important improvement in the design of multivalent binders was achieved with symmetrical pentameric molecules based on the concept of “finger-linker-core” systems:12 the pentavalent “core” is connected by flexible “linkers” to “fingers” that include the monovalent receptor-binding ligand. Pentavalent CT inhibitors were synthesized using various “cores”: acylated pentacyclen,12a a large cyclic peptide12e and calix[5]arene.14

This paper details a study that combines the two strategies to obtain an optimal binding by the design and synthesis of pentavalent GM1os-presenting compounds based on a 5-fold symmetrical sym-substituted15 corannulene scaffold (Fig. 1b). Binding assays allowed the determination of the interaction with CT-B5 (the protein complex without the toxic A part) for
these and analogous pentavalent galactose corannulenes. This revealed a high inhibitory potency towards CTB by this new class of inhibitors.

2. Results and discussion

Apart from its obvious 5-fold symmetry, the corannulene scaffold is a good candidate to function as the “core” of pentavalent receptor-binding inhibitors for AB5 toxins on the basis of the following observations: the recent development of a kilogram-scale synthesis of corannulene (1)\(^1\), its further functionalization to sym-pentachloro-corannulene (2),\(^1\) and the growing number of robust and flexible procedures for the preparation of molecular pentapods displaying functional groups and bioconjugated moieties, by iron-catalyzed\(^1\) cross-coupling and copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction.

The synthetic pathway towards PEG-corannulene systems starts from the conjugation of the terminal acetylene \(3\)\(^1\) by an efficient copper nanoparticle-catalyzed CuAAC reaction with \(n\)-mers of \(\alpha\)-azidoethyl-\(\omega\)-propargyl diglyme \((n = 1, 2, 4)\) (Fig. S1 in the ESI\(^*\)\(^1\)). This route gave satisfactory results for the preparation of other sym-bioconjugated corannulenes, and was used here to prepare the five-fold symmetric compounds 5, 6 and 7 in good yield and purity. The PEG arms function as linkers of different lengths and improve water solubility and flexibility of the inhibitor. The terminal TIPS-protected acetylenes were deprotected by reaction with TBAF\(^1\) yielding the alkyne-terminated PEG-corannulenes 8, 9 and 10 (Scheme 1).

After preparing the three PEG-corannulenes the focus shifted to attaching the (oligo)saccharides. In this work, two different CTB binders were used: galactose, an easily obtainable but quite poor binder of CTB, and the oligosaccharide of ganglioside GM1 (GM1os), the natural ligand of CTB.

In order to introduce the galactose-based “finger” to the terminal acetylens 3, 8, 9 and 10, galactoside 11 was functionalized at the anomeric position with a short PEG chain that bears an azido group (compound 12, Scheme 2). An analogous compound without ethylene oxide moieties belonging to this inhibitor family was previously synthesized\(^1\) by CuAAC reaction on the terminal acetylene 3 and 2-azidoethyl-\(\beta\)-D-galactopyranoside.\(^20,21\) The synthetic pathway for the synthesis of azido-PEG-galactoside 14 starts with a Koenigs–Knorr-type glycosylation of azido-PEG hydroxide 12\(^22,23\) with peracetylated galactosyl bromide 11 to afford \(\beta\)-galactoside 13. The deprotected compound 14 was then obtained by treatment of 13 with sodium methoxide. The pentavalent Gal-functionalized sym-corannulene inhibitor was prepared in good yield by employing the microwave-assisted CuAAC reaction on the

![Fig. 1](image-url) (a) Structure of CTB’s natural ligand, ganglioside GM1; (b) structure of the pentavalent GM1os-functionalized sym-corannulene, a nanomolar inhibitor of cholera toxin.
terminal acetylenes 3, 8, 9 and 10 with the azido galactose derivative 14 affording the four galactose-based CTB inhibitors 15–18 (Scheme 3).

The second family of pentavalent inhibitors displays five GM1os moieties (Scheme 4). The chemo-enzymatic synthesis from lactose of the azide-terminated GM1os oligosaccharide was previously reported.24 The pentavalent GM1os inhibitors 20, 21 and 22 were synthesized, in the presence of copper nanoparticles, by microwave-assisted CuAAC reaction of azido-pentasaccharide 19 with the terminal acetylenes 8, 9 and 10. Because of the low solubility of the GM1 analog in DMF, the reactions were performed in water, allowing a complete dissolution of 19 in the reaction media.

The inhibitory efficiency of the synthesized five-fold symmetric pentavalent CT ligands (15–18 and 20–22) was evaluated by an ELISA-type assay on 96-well plates.25,26 Solubility issues at concentrations >1 mM limited the experiments intended to find an IC_{50} value for galactose-based compounds
When compared to previously reported IC\textsubscript{50} values for multivalent ligands functionalized with galactose\textsuperscript{10,11}, these concentrations should not have been limiting. One hypothesis to account for this observation is that the supramolecular aggregation competes against binding to the pentad-complex.

Further investigations by $^1$H-NMR, UV-Vis and fluorescence spectroscopy on the properties of $C_5$-symmetric galactose conjugated corannulene in aqueous solution suggest the formation of supramolecular aggregates of this amphiphilic molecule in water. If the formation of the supramolecular
combining the natural valency with the natural ganglioside to reach optimal blocking of multivalent lectins. Design efforts to create systems that optimize spacer geometry and avoid self-complexation will likely lead to new derivatives with inhibitory concentrations more favorably comparable to other previously reported GM1os-based inhibitors.

3. Conclusions

This manuscript reports a powerful method for the synthesis of a new class of cholera toxin inhibitors with a design based on a pentavalent sym-substituted corannulene as the core unit and equipped with the galactose and the GM1os as CTB binders. Microwave-assisted CuAAC reactions, catalyzed by copper nanoparticles, were employed for the conjugation of the monovalent CTB ligands (galactose and GM1os) to the corannulene core via azide-presenting PEG-linkers of various lengths. The potent CTB inhibition of 25, 5.0, and 7.3 nM observed for the penta-GM1os corannulenes, 20, 21, and 22, respectively, prove that multivalent systems functionalized with strong CTB binders represent a solid strategic approach for the synthesis of CT inhibitors with high potency in comparison with previously reported monovalent inhibitors.

The developed method allows for the use of sym-substituted corannulenes as a possible core unit for the development of new multivalent binders of cholera toxin or other possible biological targets that rely on multivalent binding of their target ligand.

4. Representative experimental procedures

**Compound 6**

A mixture of 3 (9.4 mg, 18 μmol), the proper azide-functionalized PEG (83.0 mg, 0.14 mmol) and copper nanoparticles (11.7 mg, 0.18 mmol) in DMF (1.0 mL) was loaded in a microwave vessel and was heated at 80 °C in a microwave reactor for 2 h. The reaction mixture was filtered over celite and the solvent evaporated. The crude mixture was purified by column chromatography on silica gel (from DCM–MeOH 94 : 6 to DCM–MeOH 9 : 1) to yield a reddish oil (49 mg, 79%).

**Compound 9**

A solution of TBAF in THF (1 M, 1.0 mL) was added to a solution of 6 (103 mg, 30.1 μmol) in THF (1.0 mL) and the reaction mixture was stirred at r.t. for 2 h. The solution was then diluted with a saturated aqueous solution of NH₄Cl and...
extracted with ethyl acetate. The collected organic phases were dried over Na2SO4 and evaporated to yield the crude product. The crude was then heated at 75 °C under high vacuum for 18 h. The product was then purified by column chromatography on silica gel (DCM–MeOH 9:1) to yield a reddish oil (24 mg, 30%). 1H-NMR (500 MHz, CDCl3): δ 7.70 (s, 5H), 7.64 (s, 5H), 7.53 (s, 5H), 4.62 (s, 10H), 4.50-4.46 (m, 20 H), 4.16 (d, J = 2.5 Hz, 10H), 3.82 (t, J = 5.0 Hz, 20H), 3.64-3.51 (m, 90H), 3.23 (t, J = 8.0 Hz, 10H), 2.44 (d, J = 2.5 Hz, 5H). 13C-NMR (125 MHz, CDCl3): δ 147.11, 144.83, 140.47, 138.49, 131.89, 127.96, 123.97, 123.07, 122.47, 79.70, 74.86, 70.57, 70.55, 70.47, 69.65, 69.52, 69.14, 64.72, 58.48, 50.26, 50.20, 33.39, 28.52. HRMS (ESI) m/z: found 529.2767 (M + 5H); calc. (C130H185N30O30) 529.2769.

Compound 17
A mixture of 9 (8.5 mg, 3.2 μmol) and copper nanoparticles (1.5 mg, 23.6 μmol) in DMF (300 μL) was loaded into a microwave vessel and was heated at 80 °C in a microwave reactor for 2 h. The mixture was filtrated over celite and the solid was washed with water. The crude was lyophilized and purified by size exclusion chromatography (Sephadex® G-25) to yield a reddish solid (9.1 mg, 57%). 1H-NMR (500 MHz, D2O/d4-MeOD): δ 8.01 (s, 5H), 7.88 (s, 5H), 7.68 (s, 5H), 7.60 (s, 5H), 4.58-4.39 (m, 50H), 4.08-4.06 (m, 5H), 3.98–3.32 (m, 255H). 13C-NMR (125 MHz, D2O/d4-MeOD): δ 147.90, 144.84, 141.31, 134.78, 130.36, 126.25, 126.05, 124.76, 124.23, 103.86, 76.12, 73.69, 71.73, 70.69, 70.57, 70.52, 70.42, 70.41, 70.35, 70.27, 70.24, 69.86, 69.79, 69.73, 69.65, 69.61, 69.54, 63.99, 61.93, 50.90, 50.77, 49.52, 33.97, 28.11. HRMS (ESI) m/z: found 854.2378 (M + 6Na); calc. (C220H355N45Na6O85) 854.2366.

Compound 21
A mixture of 9 (2.88 mg, 1.09 μmol), 19 (11.1 mg, 9.3 μmol) and copper nanoparticles (0.76 12.0 μmol) in water (300 μL) was loaded into a microwave vessel and was heated at 80 °C in a microwave reactor for 2 h. The mixture was filtrated over celite and the solid was washed with water. The crude was lyophilized and purified by size exclusion chromatography (Sephadex® G-25, water) to yield a colorless solid (4.8 mg, 51%). 1H-NMR (500 MHz, D2O/d4-MeOD): δ 7.96–7.75 (m, 20H), 4.57–4.54 (m, 70H), 4.18–4.05 (m, 45H), 3.94–3.34 (m, 286H), 2.70 (d, J = 10.0 Hz, 5H), 2.06 (d, J = 15.0 Hz, 35H), 1.98–1.94 (t, J = 10.0 Hz, 5H), 1.62–1.45 (m br, 25H), 1.28–1.25 (m br, 20H), 1.01–0.90 (m br, 75H). 13C-NMR (125 MHz, D2O/d4-MeOD): δ 175.96, 175.06, 105.70, 103.61, 103.48, 103.18, 102.70, 75.83, 75.32, 73.47, 71.66, 70.66, 70.64, 70.62, 70.52, 70.50, 70.48, 70.47, 70.45, 69.59, 69.57, 61.90, 56.12, 52.12, 51.13, 51.11, 29.73, 26.58, 26.23, 23.58, 22.99. HRMS (ESI) m/z: found 1721.1778 (M + 5H); calc. (C370H590N35O175) 1720.7797.

ELISA assays
Each well of a 96-well microtiter plate was coated with a 100 μL native GM1 solution (1.3 μM in ethanol) after which the solvent was evaporated. Unattached GM1 was removed by washing with PBS (3 × 450 μL), and the remaining free binding sites were blocked by incubation with 100 μL of a 1% (w/v) BSA solution in PBS for 30 min at 37 °C. Subsequently, the wells were incubated with PBS (3 × 450 μL). In separate vials, a logarithmic serial dilution, starting from 2.0 mM, of 150 μL saccharide-corannulenes in 0.1% BSA, 0.05% Tween-20 in PBS, mixed with 150 μL of a 50 ng mL−1 CTB–HRP solution in the same buffer, were incubated. This gave an initial inhibitor concentration of 1.0 mM. In the case of potent inhibitors, based on the logarithmic experiments, a more accurate, serial dilution of a factor of two was performed around the expected IC50 temperature for 2 h and then transferred to the coated wells. After 30 min of incubation at room temperature, unbound CTB–HRP–corannulene complexes were removed from the wells by washing with 0.1% BSA, 0.05% Tween-20 in PBS (3 × 500 μL). 100 μL of a freshly prepared OPD solution (25 mg OPD-2HCl, 7.5 mL 0.1 M citric acid, 7.5 mL 0.1 M sodium citrate and 6 μL of a 30% H2O2 solution, pH was adjusted to 6.0 with NaOH) was added to each well and allowed to react with HRP in the absence of light, at room temperature, for 15 min. The oxidation reaction was quenched by the addition of 50 μL 1 M H2SO4. Within 5 min, the absorbance was measured at 490 nm.

Acknowledgements
Funding from the Swiss National Foundation (SNF) and INTERREG IV a Germany–Netherlands programme of a) the European Regional Development Fund (ERDF); b) the Ministry for Economic Affairs, Energy, Building, Housing and Transport of the State of North-Rhine Westphalia; and, c) the Dutch Ministry of Economic Affairs and the Province of Gelderland.

Notes and references


Prefix sym is used here to indicate the C5-symmetric-substituted corannulene corresponding to 1,3,5,7,9-pentasubstitution.