Carbohydrate Research 343 (2008) 2589–2594

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Sialyloligosaccharides inhibit cholera toxin binding to the GM1 receptor

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ARTICLE INFO

Article history:
Received 19 May 2008
Received in revised form 10 July 2008
Accepted 12 July 2008
Available online 19 July 2008

Keywords:
Antiadhesive
Sialyloligosaccharides
Biantennary
Vibrio cholerae toxin
Receptor-analogue
Glycomimetics

ABSTRACT

It is recognised that cholera toxin (Ctx) is a significant cause of gastrointestinal disease globally, particularly in developing countries where access to uncontaminated drinking water is at a premium. Ctx vaccines are prohibitively expensive and only give short-term protection. Consequently, there is scope for the development of alternative control strategies or prophylactics. This may include the use of oligosaccharides as functional mimics for the cell-surface toxin receptor (GM1). Furthermore, the sialic acid component of epithelial receptors has already been shown to contribute significantly to the adhesion and pathogenesis of Ctx. Here, we demonstrate the total inhibition of Ctx using GM1-competitive ELISA with 25 mg mL^{-1} of a commercial preparation of sialyloligosaccharides (SOS). The IC_{50} value was calculated as 5.21 mg mL^{-1}. One-hundred percent inhibition was also observed at all concentrations of Ctx–HRP tested with 500 ng mL^{-1} GM1–OS. Whilst SOS has much lower affinity for Ctx than GM1–OS, the commercial preparation is impure containing only 33.6% carbohydrate; however, the biantennary nature of SOS appears to give a significant increase in potency over constituent monosaccahride residues. It is proposed that SOS could be used as a conventional food additive, such as in emulsifiers, stabilisers or sweeteners, and are classified as nondigestible oligosaccharides that pass into the small intestine, which is the site of Ctx pathogenesis.

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1. Introduction

Intestinal infection with *Vibrio cholerae* toxin has afflicted the developing world for almost 200 years.[1] Outbreaks of this acute intestinal infection occur primarily in developing countries, where water supplies, sanitation, food safety and hygiene practices are inadequate.[2] Overcrowded communities with poor sanitation and unsafe supplies of drinking water are most frequently affected. In addition to the human suffering caused by cholera, outbreaks cause panic, disrupt the social and economic structure, and can impede development in affected communities.[3] The World Health Organisation (WHO) epidemiological records showed that in 2005, 52 countries reported 131,943 cases and 2272 deaths, with Africa accounting for around 95% of all cases in this global total.[4] The global case fatality rate (CFR) of 1.72% is low compared to the CFR of 15.56% and 15.38% in high risk areas such as Chad and the Ivory Coast, respectively.[4] Such figures illustrate the scale of cholera amongst malnourished populations that also do not have access to clean drinking water.

The causative agent of cholera is a pathologically active protein secreted by *Vibrio cholerae*, which is a member of the AB_5 toxins, so called because of their pentameric structure.[5] The natural biological receptor for cholera toxin has been identified as an ubiquitously expressed glycosphingolipid–ganglioside–GM1.[6–8] There is currently no prophylactic agent against cholera toxin, while without treatment fatality rates may reach 30–50%.[4] Limited success has been demonstrated by two types of Oral Cholera Vaccines (OCVs) although they are ineffective against *Vibrio cholerae* serotype O139 which has a peculiar emergence pattern.[9,10] Both OCVs provide between only 50% and 90% efficacy and must remain stored at 4 °C to maintain activity, which is a limitation in hot climates. Furthermore, both offer effective protection 8–10 days following full immunisation.[11–13]

Another associated problem is that antimicrobial resistance is evident in many clinically isolated and environmental strains of *V. cholerae*, and this has been demonstrated in many developing countries.[14–17] Such resistance is likely to increase if *V. cholerae* continues to be exposed to widely used antibiotics such as tetracycline, chloramphenicol and nalidixic acid.[18]

Antiadhesive agents, or receptor analogues, have been shown to be effective in a number of in vitro studies and offer a promising alternative to conventional treatment strategies.[19–27] With cholera toxin (Ctx), this could take the form of a glycomimetic, since it is known that Ctx binds to a multitude of ganglioside receptors on the host epithelium.[28–30] Of these, Ctx has higher affinity with

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doi:10.1016/j.carres.2008.07.008

GM1,[29] and it has been shown that the contribution of the terminal galactose, lactose and sialic acid in GM1 is most significant.[24,31,32] An example of this was shown by Minke et al.,[33] where 'receptor antagonists' were designed based on the terminal sugar of GM1: galactose. In this study, sialyloligosaccharides (SOS) were chosen since they have previously been shown to prevent binding of pathogenic bacteria and viruses.[32,34–36] Significantly, the GM1 oligosaccharide is known to contain sialosyl residues.[7,37]

Several acidic oligosaccharides have also been characterised in both bovine and human milk or colostrums.[38] The SOS content of human colostrum and milk has been extensively studied, where SOSs have been shown to be important biological components with respect to be source of brain gangliosides in infants.[39] There is also a strong link between complex human milk oligosaccharides and protection against infections and inflammation, particularly in infants from developing countries.[40,41] It is the observation that these oligosaccharides are structurally related to naturally occurring cell-surface glycoconjugates and immunomodulatory components that facilitates efficacy.[42–44] With this in mind, functional foods have received much attention in recent years. In particular, by virtue of the presence of physiologically active components, the egg has become a functional food, with studies into compounds which could be extracted from eggs for use in food sciences, pharmacology and clinical sciences intensifying.[45] One such constituent compound in eggs is *N*-acetylneuraminic acid, which is the only sialic acid found in egg yolk.[46] Additionally, the sialyloligosaccharide moieties of the water-soluble fraction of delipidated egg yolk have been characterised.[47]

SOS are inexpensive and are already approved for human consumption.[38,48] They could be used to fortify conventional food additives by way of emulsifiers, sweeteners and/or preservatives. Pharmaceutical compositions and dietary supplements may also be provided in the form of powders, syrups and/or liquid suspensions. The hypothesis is that SOS could be a promising natural anti-adhesive glycomimetic against Ctx.

2. Materials and methods

2.1. Chemicals

Analytical grade (+)-D-galactose monohydrate, lactose, monosialoganglioside-GM1 isolated from bovine brain (GM1–OS), TWEEN® 20, bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO) and sulfuric acid (H_2SO_4) were supplied by Sigma–Aldrich (Gillingham, Dorset, UK). Phosphate-buffered saline (PBS) was purchased from Oxoid Ltd (Basingstoke, Hants, UK) in tablet form without calcium and magnesium (pH 7.2). *Vibrio cholera* toxin B subunit conjugated to horseradish peroxidase (Ctx–HRP) was purchased from Quadratech Ltd, Surrey, UK (manufactured by List Biologicals, CA, USA). The commercially available sialyloligosaccharide (SOS) mixture Sunsial E® from hen egg yolk, which is not a pure preparation, containing 33.6% SOS and 66.4% protein, was used without further purification (Taiyo Kagaku Co; Ltd, Japan).[47] The sialyloligosaccharide fraction contains mono (SI-1 and SI-2) and disialylated (SII-1) oligosaccharides in the ratio 9.6%, 6.5% and 17.5%, respectively.[47] All solutions were prepared using ultrapure MilliQ distilled water.

2.2. Inhibitory GM1-linked ELISA

Microtitre plates (F96 Maxisorp; Fisher Scientific, Loughborough, UK) were incubated at room temperature overnight with 500 ng mL^{-1} of ganglioside GM1 dissolved per well in phosphate-buffered saline (pH 7.2) containing 160 mM NaCl and 9 mM potassium phosphate (PBS). Unattached ganglioside was removed by washing the wells three times with PBS containing 0.1% (w/v) Tween 20. Additional binding sites on the plate surface were blocked by incubating the wells with 2% (w/v) bovine serum albumin (BSA)-PBS solution overnight at room temperature, followed by washing with 0.1% (w/v) Tween 20-PBS three times.[33]

Table 1
Concentrations of each of the carbohydrate inhibitors tested

Carbohydrate	Concentration series
Galactose	100–3.125 mg mL^{-1} (554.9–17.3 μM)
Lactose	100–3.125 mg mL^{-1} (292.1–9.1 μM)
GM1–OS	500–0.05 ng mL^{-1} (324.7–0.033 pM)
SOS mixture	25–0.1 mg mL^{-1}

It was necessary to test the ability of each carbohydrate to inhibit Ctx using different concentrations because of differences in the observed affinity for Ctx. Table 1 summarises these solutions.

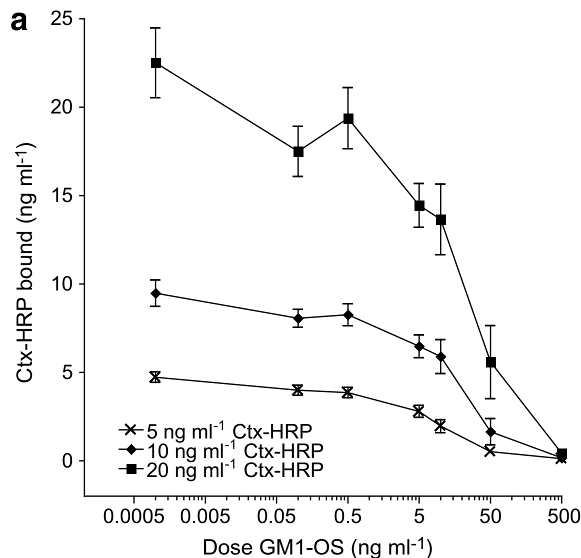
Each carbohydrate was prepared in 0.1% (w/v) BSA-PBS and incubated with either 5, 10, 15, 20 or 45 ng mL^{-1} of Ctx–HRP for 2 h at room temperature. Following addition to the plate, a further 2-h incubation period ensured that immobilised GM1–OS could bind to any uninhibited Ctx–HRP. Inhibited Ctx was removed by washing three times with 0.1% (w/v) Tween 20-PBS. This was followed by Ctx–HRP development by incubation with freshly made 3,3′,5,5′-tetramethylbenzidine (TMB) solution (1 mg of TMB in 500 μL of DMSO, 50 mL of 0.1 M potassium citrate buffer, and 5 μL of 30% hydrogen peroxide) for 15 min at room temperature. TMB produced a soluble end product that was pale blue in colour and read spectrophotometrically (Genios, Tecan UK Ltd, Thatcham, UK) after stopping with 2 M H_2SO_4 (resulting in a yellow colour) at 450 nm.

Each experiment was carried out in triplicate and validated against a standard curve of 0, 0.97, 1.95, 3.90, 7.81, 15.62, 31.25 and 62.5 ng mL^{-1} Ctx–HRP. A control solution of 30 ng mL^{-1} Ctx–HRP was also used throughout every plate as a control to measure the intraassay coefficient of variation or absorbance drift and assay stability. Unknown absorbance readings and subsequent EC_{50} values were calculated from the standard curve and compared with PRISM v. 4.0 software (GRAPHPAD® Software Inc, CA, USA). ANOVA was calculated using MINITAB® v. 14 (Mintab Ltd, Coventry, UK).

3. Results

3.1. Inhibition of Ctx–HRP binding to GM1-ELISA using GM1–OS, SOS, lactose, and galactose

Soluble GM1–OS inhibition of Ctx–HRP binding to immobilised GM1–OS was used as a positive control throughout this study. Two-way ANOVA between values of Ctx–HRP bound to immobilised GM1–OS and dose of soluble GM1–OS confirmed a dose-dependent effect ($P < 0.001$, Fig. 1a). PRISM® v. 4 software (GRAPHPAD Software Inc, CA, USA) was used to transform Ctx–HRP concentration from ng mL^{-1} to percentage inhibition and also normalise inhibitor values onto a log scale. The software was also used to fit a sigmoidal dose–response curve or three-parameter logistic curve to the experimental data (Fig. 1b). Comparing each IC_{50} curve at increasing Ctx–HRP concentration reveals no statistical difference, where 100% inhibition was observed at all concentrations of Ctx–HRP with 500 ng mL^{-1} soluble GM1–OS and global IC_{50} value equal to 1.104 ng mL^{-1} (Fig. 1b). On a molar basis, the ratio between Ctx–HRP and GM1–OS was confirmed as 1:5, based on the molecular weights of GM1 and Ctx–HRP as 1.54 kDa and 98 kDa, respectively.



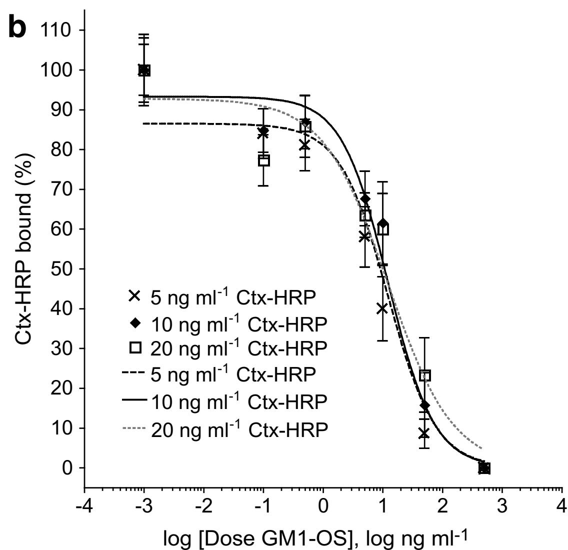
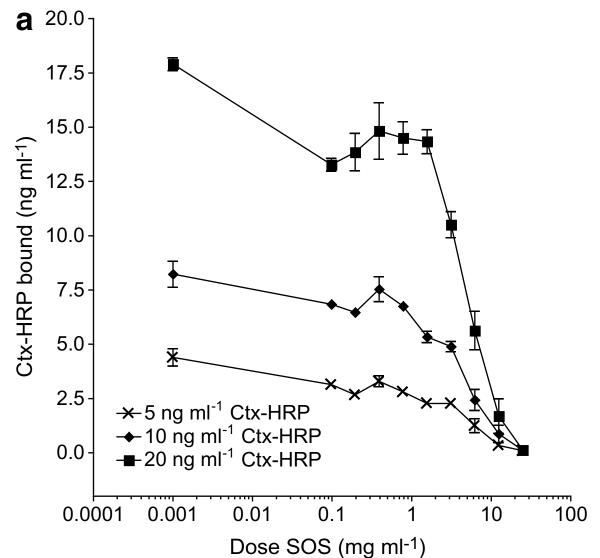


Figure 1. (a) GM1–OS inhibition of Ctx–HRP after a 2-h pre-incubation and 2-h incubation in contact with immobilised GM1–OS ($n = 6$, $P < 0.001$, $F = 86.71$). Error bars represent one standard error from the mean. (b) The sigmoidal dose–response curve fitted by PRISM® software confirms that each dilution of soluble GM1–OS is equally effective at inhibiting Ctx–HRP binding to immobilised GM1–OS irrespective of the concentration of Ctx–HRP ($P = 0.0583$, $F = 2.904$, global GM1–OS $IC_{50} = 1.104$ ng mL^{-1}).



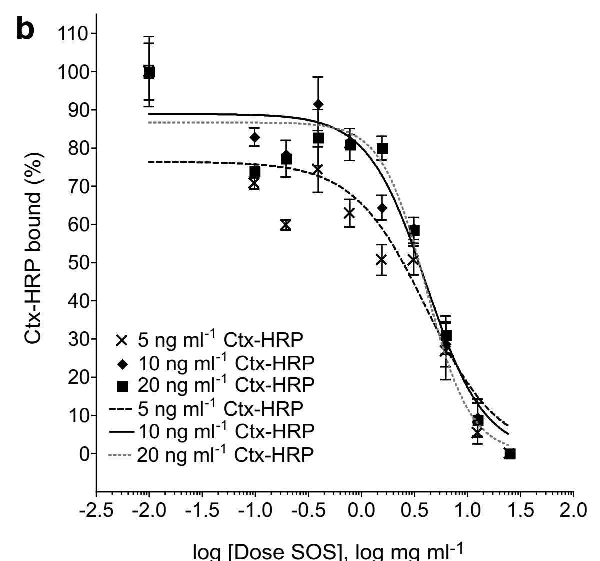


Figure 2. (a) SOS inhibition of Ctx–HRP after a 2-h pre-incubation and 2-h incubation in contact with immobilised GM1–OS ($n = 6$, $P < 0.001$, $F = 236.38$). Error bars represent one standard error from the mean. (b) The sigmoidal dose–response curve fitted by PRISM® software confirms that each dilution of soluble SOS is equally effective at inhibiting Ctx–HRP binding to immobilised GM1–OS irrespective of the concentration of Ctx–HRP ($P = 0.1070$, $F = 2.804$, global SOS $IC_{50} = 5.206$ mg mL^{-1}).

SOS were demonstrated to inhibit Ctx–HRP binding to GM1–OS using competitive ELISA (Fig. 2a). One hundred percent inhibition was observed at all concentrations of Ctx–HRP tested at 25 mg mL^{-1} SOS (Fig. 2b). Two-way ANOVA on log transformed Ctx–HRP bound values and sigmoidal dose–response curves confirm that inhibition was not dependant on the concentration of Ctx–HRP, and IC_{50} was calculated to be 5.206 mg mL^{-1} ($P = 0.1070$, F, 2.295, (Fig. 2b).

Figure 3 shows the effect on inhibition of Ctx with galactose and lactose. It was not possible to calculate an IC_{50} value for these carbohydrates as total inhibition was not observed. However, 5, 15 and 45 ng mL^{-1} Ctx were inhibited with 100 mg mL^{-1} (292.1 M) lactose by 34.3%, 53.2% and 52.7%, respectively (Fig. 3a). Galactose showed higher affinity for Ctx, where 5, 10 and 20 ng mL^{-1} Ctx were inhibited with 100 mg mL^{-1} (554.9 M) galactose by 55.1%, 61.4% and 64.9%, respectively (Fig. 3b).

4. Discussion

In this work, the ability of 'food-grade' SOS to inhibit Ctx binding to GM1–OS was measured using inhibitory GM1–ELISA. The natural receptor was used as a model sugar since it has been shown to have greatest efficacy for Ctx.

Following characterisation of the GM1–OS ELISA by Dawson,[49] a number of modifications were made to the experimental design, including the rate of change of absorbance with cholera toxin concentration, which was found to be a critical variable in measuring the inhibition consistently. The relationship between Ctx–HRP concentration and peroxide-induced colour formation is sigmoidal, a result in agreement with other studies.[49,50] Furthermore, the concentration of Ctx–HRP corresponding to half-maximal binding was estimated to be 15 ng mL^{-1} or 0.153 nM.

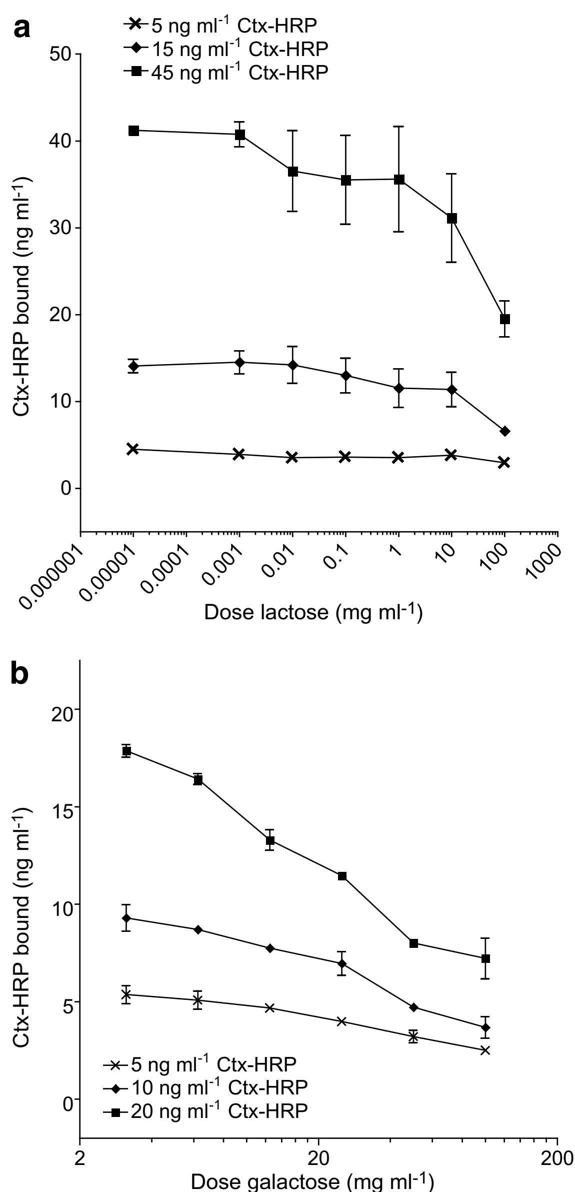


Figure 3. (a) Lactose inhibition of Ctx–HRP after a 2-h pre-incubation and 2-h incubation in contact with immobilised GM1–OS ($n = 3$, $P = 0.083$, $F = 1.63$). Error bars represent one standard error from the mean. (b) Galactose inhibition of Ctx–HRP after a 2-h pre-incubation and 2-h incubation in contact with immobilised GM1–OS ($n = 3$, $P < 0.000$, $F = 3.97$). Error bars represent one standard error from the mean.

Other important developments include the incubation period between potential inhibitors and Ctx, which was set at 2 h before addition to the plate to maximise interactions between sugar and toxin. A further 2-h incubation 'on plate' was chosen because time-dependency experiments have shown that the maximum binding of Ctx to GM1 is reached in 90 min.[49]

Bound/uninhibited Ctx–HRP was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) rather than *o*-phenylenediamine free base as the peroxidase substrate because of its sensitivity in detecting low concentrations of Ctx–HRP. The statistical mean of each set of absorbance readings was used to calculate the concentration of Ctx–HRP that was not inhibited, and consequently able to bind to the immobilised GM1 surface. Higher absorbance readings were indicative of greater concentrations of uninhibited Ctx–HRP and/or poor inhibitor performance. All ELISA experiments were performed in triplicate on different dates using various batches of reagent, and therefore, error bars reflect the standard error of the mean of the actual uninhibited Ctx–HRP from independent replicate experiments.

Ctx–HRP concentrations above 70 ng mL^{-1} were found to increase the rate of colour formation such that development times were as short as 5 min. This was undesirable, because differences between wells treated with lower Ctx–HRP concentrations were undetectable. A broad standard curve of 0, 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, and 62.5 ng mL^{-1} was used on every plate to normalise readings between experiments. Therefore, concentrations on either side of half-maximal that bind Ctx–HRP to immobilised GM1–OS (5, 10 and 20 ng mL^{-1} or 0.051, 0.102 and 0.204 nM, respectively) were chosen to compete with SOS. Such low values reflect the high affinity between Ctx and GM1–OS and are consistent with an earlier study, where radiolabelled Ctx binding to mucosa cells from the small intestine gave a K_d value of 1 nM.[51] Dissociation constant (K_d) values of 0.7–7 nM have also been recorded and were dependent on the concentration of GM1–OS.[51]

The ELISA showed 0.974 pM soluble GM1–OS inhibits 0.204 pM Ctx–HRP and confirmed the findings of Merritt et al.,[52] where the crystal structure of cholera toxin B-pentamer bound to GM1–OS was also found to interact in a 1:5 ratio. Importantly, GM1 will bind to the complete AB5 assembly and also to the B-pentamer, but not to monomeric B-subunits, indicating that binding between GM1 and the B-subunits was cooperative.[53,54] When comparing GM1–OS and SOS IC_{50} values of 1.104 ng mL^{-1} and 5.206 mg mL^{-1}, respectively, GM1–OS is 5×10^6 times more effective at inhibiting Ctx. However, this is expected because GM1 is the receptor associated with highest affinity for Ctx. Furthermore, the SOS used in this study contains only 33.6% carbohydrate of which there are three constituent structures. These take the form of mono- (SI-1 and SI-2) and di-sialylated (SII-1) oligosaccharides in the ratio 9.6%, 6.5% and 17.5%, respectively (Fig. 4). One or all of these individual structures are potential inhibitors of Ctx–HRP.

Nondigestible sialyloligosaccharides (SOS) that are structurally suitable candidates for antiadhesives against Ctx as they pass through the small intestine intact, are found naturally in egg yolk, and have been isolated so that individual structures could be determined.[55] These biantenally branched oligosaccharides that contain *N*-acetyllactosamine have already been shown to play important roles in animals and humans by serving as receptors for bacteria, viruses, and toxins.[34,48,56–58] There are a few examples of the attainability of mg concentrations of SOS inhibiting pathogenesis in vivo. In one study, SOS and their derivatives from chicken egg yolk inhibited bacterial adhesion and *Salmonella enteritidis* infection in BALB/c mice. Mice given water containing 1 μM biantenally branched di-sialyloligosaccharides prophylactically two days before infection survived in greater numbers compared to placebo-controlled mice (100% survival vs 80% mortality).[34] In another example, suckling mice were inoculated with rotavirus SA-11, followed by treatment with 2.5 mg of a SOS fraction and studied for five days. At days 3 and 5, the incidence of diarrhoea was significantly reduced by 24% and 43%, respectively, suggesting that the sialic acid moiety of the oligosaccharides plays an important role in the inhibition.[46] *N*-acetylneuraminic acid is the starting material in the synthesis of 4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid, which has been shown to exhibit strong inhibitory activity against human influenza virus sialidase in mice and ferrets.[59,60]

It is well known that the majority of interactions between the receptor and the toxin involve two terminal sugars of GM1, galactose and *N*-acetylneuraminic acid (NeuAc), with a smaller contribution from the *N*-acetyl galactosamine residue.[61] Moreover, removal of either terminal galactose or NeuAc confers loss of binding as revealed by crystallography data between GM1 and Ctx.[52] We

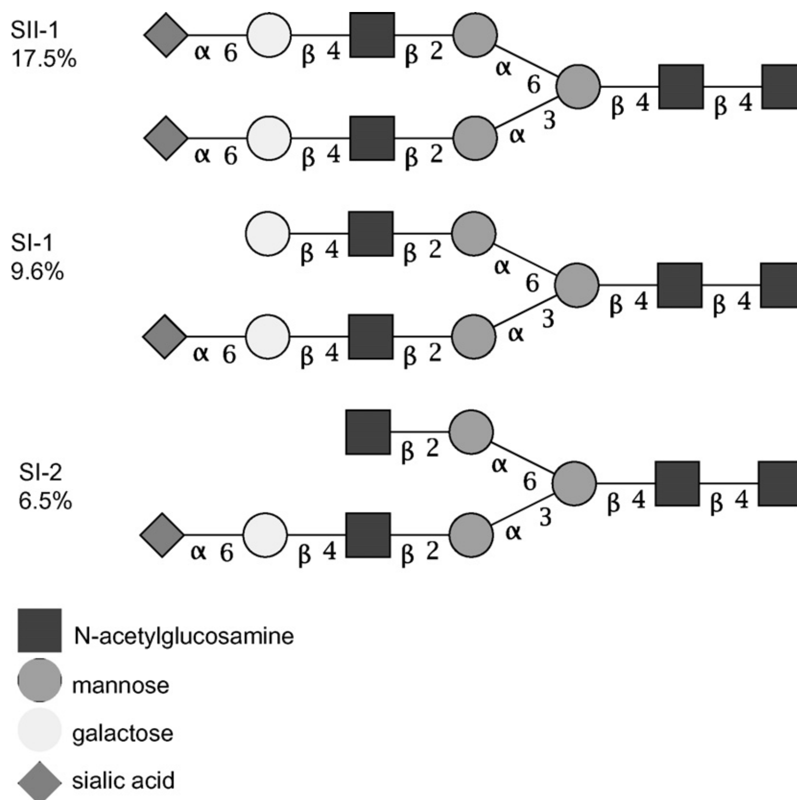


Figure 4. The glycan structures present in sialyloligosaccharide mixture Sunsial E®. These contain mono (SI-1 and SI-2) and disialylated (SII-1) oligosaccharides in the ratio of 9.6%, 6.5%, and 17.5%.

proposed that the biantennary NeuAc residues in SOS could contribute greatly to potency.

We did not observe total inhibition of Ctx with lactose and galactose, contrary to the study by Minke et al.[33] However, this was to be expected since our GM1–OS-linked ELISA was chosen over GD1b because the ability for SOS to compete against a natural receptor, with affinity as high as GM1, was an important objective. Minke et al.[33] chose GD1b over GM1, because it has an affinity of over one order of magnitude lower for Ctx. This meant that inhibitors with weaker affinities for Ctx could be tested; consequently, it is not surprising those monosaccharides that are normally poor inhibitors such as GalNAc, lactose and galactose were effective in the GD1b ELISA but not in our GM1 ELISA.[54,62] Furthermore, an earlier study found that galactose reduced the extent of binding to GM1 on liver membranes by only 20% at 200 mM (or 36.04 mg mL^{-1}). GalNAc, glucose and sialic acid have also been found to be ineffective on inhibiting Ctx binding to GM1 at concentrations of up to 40 mM.[63]

Whilst the individual contribution of each of the constituent sugars has low affinity for Cholera toxin, GM1–OS multivalency increases this affinity significantly. For example, K_d values of 210 mM, 14.8 mM and 7.6 mM for sialic acid, galactose and *N*-acetyllactosamine, respectively, are weak compared to 4.3×10^{-5} mM for GM1–OS.[64] It is therefore clear that whilst simple sugars have poor affinities in the mM range, weakly binding sugars as part of a larger molecule exhibit cooperative effects.[54] Furthermore, the binding between Ctx and GM1–OS resembles a two-fingered pinch, where sialic acid is the 'thumb' and galactose the 'finger'.[65] It is possible that the biantennary nature of the glycan chains in SOS contributes to their potency, whilst as in GM1–OS, the individual monosaccharide residues are not inherently strong inhibitors.

A number of research groups have investigated protein–carbohydrate binding using multivalent synthetic ligands to increase efficacy. In one example, Kitov et al. synthesised dimers of the trisaccharide P^k with the linker designed to span the 10 Å required to bind both binding sites of *E. coli* shiga-like toxin .[66] This design was further enhanced by attaching the dimers to a pentavalent scaffold with linkers of sufficient length to span the 30-Å distance from the centre of the toxin to each B-subunit.[66] With IC_{50} values of 4×10^{-10} and 6×10^{-9} M for SLT-I and SLT-II, respectively, the decavalent inhibitor was 10^6 times more potent against SL-I than P^k alone.[66]

Simultaneously, Fan et al. synthesised pentavalent molecules to span nonadjacent binding sites on the toxin of 45 Å. Initially they used galactose as the terminal residue, although this was substituted with a nitrophenyl sugar that had been identified to be a 100-fold more potent inhibitor of *E. coli* HLT than galactose alone.[33] The resultant pentavalent *m*-nitrophenyl α-D-galactoside was shown to be a further 100 times more potent than the pentavalent galactose-based inhibitor with IC_{50} values of 0.72 mM and 0.6 mM, respectively.[67,68] It is important to note that the Ctx and *E. coli* HLT-inhibitory ELISA datasets correlated with an R^2 value of 0.9[33]. Furthermore, Brandhorst et al.[69,70] recently reported that GM1–OS-conjugated dendrimers based on 'click' conjugation showed increased affinity of up to 380,000-fold compared to GM1–OS alone. Since complex bioactive oligosaccharides are not often readily available, Bernardi et al. have been producing multivalent displays of synthetically more accessible sugar mimics.[71] Using a pseudo-GM1 tetrasaccharide on a polyvalent 3,5-di-(2-aminoethoxy)benzoic acid branched scaffold, a highly active Ctx ligand was produced with inhibitory ELISA establishing this structure as a strong artificial monovalent Ctx binder, where >200 g mL^{-1} inhibited 100% of 1.75 g mL^{-1} Ctx–HRP.[72]

Despite this success, such synthesised inhibitors are expensive and are not food grade molecules, and would therefore require extensive clinical trials in animal models before their consideration

as prophylactics in humans. Sialyloligosaccharides appear to have an advantage over simple sugars whose potency and suitability as a glycomimetic could improve further if incorporated into a glycodendritic structure.

Acknowledgement

Financial support was provided for this study by Royal Friesland Foods BV.

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