Interaction of the verotoxin 1B subunit with soluble aminodeoxy analogues of globotriaosyl ceramides¹

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Specific hydroxy groups of the terminal disaccharide unit of globotriaosyl ceramide (Gb₃Cer) were identified from binding studies with deoxyGb₃Cer and verotoxins (VTs) [Nyholm, Magnusson, Zheng, Norel, Binnington-Boyd and Lingwood (1996) *Chem. Biol.* 3, 263–275]. Four such hydroxy groups (2'', 4'', 6'' and 6') were each substituted with an amino group and the corresponding deoxyamino globotrioses were conjugated to a ceramide-like aglycone which contained an adamantyl group instead of an acyl chain. Such aglycone modification significantly enhanced the water-solubility of the glycoconjugates [Mylvaganam and Lingwood (1999) *Biochem. Biophys. Res. Commun.* 257, 391–394]. The inhibitory potential of these soluble aminodeoxy conjugates on the binding of VT₁ to Gb₃Cer immobilized on an ELISA plate was evaluated. Only the 2'' and the 6' deoxyamino conjugates were effective inhibitors (IC₅₀ 10 µM); the 4'' and 6'' conjugates were ineffective up to 10 mM. To evaluate the importance of incorporating a rigid adamantyl

hydrocarbon group into the ceramide aglycone, globotriaose was conjugated to a *t*-butylacetamido or an adamantaneacetamido aglycone. By similar ELISAs, only the adamantaneacetamido conjugate inhibited the binding of VT₁ to Gb₃Cer. When deoxyamino conjugates were adsorbed to silica on TLC plates, only the 2'' and 6'' conjugates bound VT₁ and VT₂. By a similar TLC assay, acetamido derivatives of 2'' and 6' deoxyamino conjugates showed less binding to VT₁ and VT₂. Neither the crystallographically determined structure of the VT₁-globotriaose complex nor modelling studies fully explain the binding patterns shown by these deoxyamino glycoconjugates. Enhanced solvation of the ammonium group of the deoxyamino conjugate could enforce greater constraints in the binding interactions.

Key words: glycolipid receptor, haemolytic uraemic syndrome, soluble glycolipid mimic.

INTRODUCTION

Gastrointestinal infection with verotoxin (VT)-producing *Escherichia coli* is the cause of the vasculopathy haemorrhagic colitis, as well as the more severe haemolytic uraemic syndrome that may subsequently develop, particularly in children. VTs form a family of related AB₅ subunit toxins, of which VT₁ and VT₂ are primarily involved in human disease [1]. Cytotoxicity is a consequence of the initial cell surface targeting by the receptor-binding pentameric B subunit via its interaction with the glycosphingolipid globotriaosyl ceramide (Gb₃Cer) on sensitive cells *in vitro* [2], and probably *in vivo* too [3]. The holotoxin is then internalized by receptor-mediated endocytosis via clathrin-coated pits [4] to undergo retrograde transport to the Golgi and endoplasmic reticulum [5]. Inhibition of protein synthesis by the A subunit is then observed, and is followed by necrosis and/or apoptosis [6,7]. Solid-phase binding studies using Gb₃Cer homologues, in which the ceramide consisted of only one type of acyl chain, established the important role played by the lipid moiety [8]. Also, the composition of acyl chains associated with the phospholipids surrounding Gb₃Cer influenced the affinity of Gb₃Cer binding [8].

From the VT₁B crystal structure and consideration of conserved residues between the different VTs, it was first proposed that the Gb₃Cer binding site lay in the cleft between the monomers [9]. Molecular modelling studies using the VT₁B co-ordinates

and the minimum-energy conformation of Gb₃Cer identified a site (Site 1) adjacent to phenylalanine-30 [10]. Also, a second site (Site 2) on the opposite side of Phe-30 was suggested. The subsequent X-ray structure of the complex between VT₁B and 8-(methoxycarbonyl)octyl globotriaoside (Gb₃P^K) identified three binding sites [11]. Site 1 of the structure showed many similarities to the Site 1 identified by modelling, but the occupancy of Site 2 by Gb₃P^K in the structure was different from that predicted by modelling. Also, the structure identified a third site (Site 3) centred around the tryptophan-34 residue. The structure showed full occupancy of Site 2 for all subunits of the pentamer, which was therefore assigned as the preferred site for Gb₃Cer binding. NMR studies also identified Site 2 as the preferred site for Gb₃P^K ligand [12]. However, site-specific mutational studies on VT₁B have shown the importance of residues from both Sites 1 and 2 [13]. Also, evidence for two distinct binding sites has been provided from binding studies using the surface plasmon resonance technique [13,14].

Our approach has been to design water-soluble univalent glycoconjugates that mimic the functional properties of natural glycolipids. We observed that when the acyl chain of Gb₃Cer was replaced by a rigid hydrocarbon, the resulting Gb₃Cer analogue was significantly more water-soluble than the natural Gb₃Cer [15]. This adamantyl ceramide-based globotriaoside (AdaGb₃) partitioned into the aqueous phase (> 99%) of a chloroform/water mixture, whereas natural Gb₃Cer partitioned into the

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic) diammonium salt; AdaGb₃, adamantyl ceramide-based globotriaoside; Gb₃Cer, globotriaosyl ceramide; Gb₃P^K, 8-(methoxycarbonyl)octyl globotriaoside; VT, verotoxin; VT₁B, B subunit of VT.

¹ This paper is dedicated to the memory of Professor Göran Magnusson, collaborator and friend.

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organic layer (> 95%). Also, this AdaGb₃ conjugate inhibited the binding of VT₁ to Gb₃Cer with an IC₅₀ of 10 µM [15]. This approach is applicable to other glycolipids, where water-soluble adamantyl glycoconjugates have been shown to be effective inhibitors [16].

Previous binding studies with deoxyGb₃Cer and VT₁ [10] showed that the 2'', 4'', 6'' and 6' hydroxy groups of Gb₃Cer were important for binding. These hydroxy groups were substituted with amino groups and conjugated to an adamantyl ceramide aglycone [17]. The binding results obtained for these deoxyamino AdaGb₃ derivatives are presented and discussed in relation to the structures of the different Gb₃Cer binding sites proposed for the VT₁B pentamer.

EXPERIMENTAL

Materials

Galabiosyl and globotriaosyl glycoconjugates were synthesized as previously described [17–21]. The 2'' and 6' deoxyamino AdaGb₃ conjugates were transformed into the acetamido conjugates by treatment with a mixture of pyridine/acetic anhydride (1:1, v/v) for 2 h at room temperature. The reaction mixture was then dried and treated with triethylamine/methanol/water (1:1:1, by vol.) overnight at room temperature to ensure the hydrolysis of ester groups. Dried material was purified on a mini silica column and characterized by MS. Electrospray MS (ion, %): C-14, 961.9 (*M*+H, 100), 983.8 (*M*+Na, 15); acetamido derivative of C-14, 1003.9 (*M*+H, 100), 1020.8 (*M*+NH₄⁺, 10), 1025.8 (*M*+Na, 15); F-14, 961.9 (*M*+H, 100), 984.0 (*M*+Na, 20); acetamido derivative of F-14, 1003.9 (*M*+H, 100).

TLC overlay

Portions of 0.5 µg of glycolipids were separated by TLC on plastic-backed polygram SIL/G TLC plates (Macherey-Nagel). The plates were blocked with 0.6% (w/v) gelatin overnight and, after washing, were incubated with 0.1 µg/ml VT₁ or 0.5 µg/ml VT₂ for 1 h at room temp. Bound toxin was visualized immunologically as described in [2].

Glycolipid receptor ELISA

Human renal Gb₃Cer was coated on microtitre plate wells by evaporation from an ethanolic solution. Glycolipid aliquots were measured from stock solutions in dichloromethane/methanol (2:1, v/v) into screw-capped glass tubes, and the solvent was removed under a stream of nitrogen. Ethanol was added to the lipid residue, and a uniform glycolipid solution was prepared by immersing the tube for 30 s in a bath sonicator followed by 1 min of vortexing. A 100 µl aliquot of ethanolic Gb₃Cer (0.5 µg/ml) was added per microtitre plate well in triplicate and the ethanol was allowed to evaporate at room temperature overnight. Once dry, the plates were stored in a dessicator at 4 °C. Each well was blocked with 200 µl of 0.2% (w/v) BSA in 10 mM sodium phosphate/150 mM NaCl, pH 7.4 (BSA-PBS) for 1–2 h, then the wells were washed twice with 150 µl/well 0.2% BSA-PBS. All subsequent incubations were carried out at room temperature with gentle agitation. Dilutions of VT were prepared in BSA-PBS to give 4 ng/50 µl per well. The solution was dispensed into the wells and incubated for 1 h. For inhibition studies, 50 µl containing 8 ng of VT₁ was preincubated with an equal volume of soluble Gb₃Cer derivative at 2× the final concentration in PBS for 20 min at room temperature prior to the transfer of 50 µl aliquots to the wells.

The wells were emptied and washed three times (1 min each) with 200 µl of 0.2% BSA-PBS. Then 100 µl of 1 µg/ml PH1 (monoclonal anti-VT₁ antibody) in 0.2% BSA-PBS was added for 1 h. The wells were washed as before and a 1:2000 dilution of goat anti-(mouse IgG)–horseradish peroxidase conjugate in 0.2% BSA-PBS was added to the wells for 1 h. The substrate, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic) diammonium salt (ABTS) was dissolved in 0.08 M citric acid/0.1 M disodium phosphate, pH 7.4, at 0.5 mg/ml. A volume of 3 µl of 30% H₂O₂ was added per 10 ml of solution. The wells were emptied and washed three times with 200 µl of 0.2% BSA-PBS for 3 min each. Finally, the wells were washed once with PBS, then 100 µl/well ABTS solution was added. The plate was shaken gently, then placed in the dark. After sufficient colour had developed (usually 20–40 min), the absorbance of each well at 410 nm was determined using an ELISA plate reader.

Comparison of VT₁B–Gb₃Cer oligosaccharide complexes

The structural data for the complex between VT₁B and Gb₃P^K were obtained from the Cambridge database [protein data bank (pdb) file for VT₁B is 1BOV and that for the VT₁B–Gb₃P^K complex is 1BOS], and the VT₁B and Gb₃Cer docking data were from P.-G. Nyholm (unpublished work). Peptide sequences surrounding Site 1 (residues 12–21, 28–35, 53–56 and 60–64) of the bound (in the case of X-ray data) or the docked (in the case of modelling data) glycoconjugates were constructed in Chem 3D[®]. The peptide backbones of these models were superimposed within 0.3 root mean square. Some residues were later removed for clarity.

Molecular mechanics calculations using MM3 [22] at a dielectric constant of 80 were carried out on galabiose and on 2'' acetamido galabiose in order to determine if the acetamido group affects the conformational preferences at the α1–4 linkage. A two-dimensional ϕ/Ψ driver calculation was carried out and an adiabatic ϕ/Ψ energy map was generated on the basis of the MM3 results. The ϕ and Ψ torsion angles were defined as H-1–C-1–O-1–C-4 and C-1–O-1–C-4–H-4.

RESULTS AND DISCUSSION

Soluble Gb₃Cer analogue for recognition of VT₁B

Evidence for the multivalent nature of VT–Gb₃Cer binding comes from structural [11], spectroscopic [12], analytical [14] and modelling [10] studies. These studies showed that each monomer may have up to three Gb₃Cer binding sites, giving a possible 15 receptor sites per VT₁B pentamer. Gb₃Cer binding on the cell membrane is the primary determinant of VT-induced cytotoxicity, and therefore understanding the molecular basis of this recognition is central to understanding and preventing pathogenesis. It was also shown that toxin recognition by Gb₃Cer is modulated by the lipid composition of the ceramide moiety [8]. Protein glycolipid binding dependency on the acyl chain composition of ceramide has been observed for other glycolipid receptor systems as well [23,24]. However, such dependency of glycolipid binding on the acyl chain composition of ceramide has not been apparent in all cases; for instance, lipid-free galabiose analogues inhibit the binding of *Streptococcus suis* [18] or PapG-containing *E. coli* [25] to Gb₃Cer.

Lipid modulation suggests that the optimal presentation [16] of the trisaccharide of Gb₃Cer for VT binding is altered, possibly due to changes in the lipid organization. Such organizational changes cause perturbation of the 'interface' region affecting solvation, relative head group organization and other supra-molecular features [16]. The 'interface' refers to the region of the

Figure 1 Structures of aglycones used in the synthesis of globotriaose conjugates (left), and three-dimensional model of a phantom, fully protonated 2'',4'',6'',6'-tetra-aminodeoxy AdaGb₃ (right)

membrane occupied by the serine-like moiety of the ceramide. The major obstacle to studying lipid modulation is the insolubility of natural glycolipids in water. Water-soluble glycoconjugates that mimic the structural and functional properties of natural glycolipids will facilitate the study of lipid modulation in glycolipids. In the case of Gb₃Cer, substituting the fatty acid chain of ceramide with an adamantyl group gave a conjugate (AdaGb₃) that was soluble in water and retained its receptor activity (Figure 1, aglycone 1). An adamantane derivative of sulphogalactosyl ceramide showed similar properties [16], suggesting that this may be a general approach for soluble glycolipids. AdaGb₃ inhibited VT binding to natural Gb₃Cer immobilized on an ELISA plate with an IC₅₀ of 10 μ M. Total chemical synthesis of AdaGb₃ has been reported [17,20], and this material was found to have the same inhibitory properties (Figure 2C) as the semi-synthetic AdaGb₃ reported previously [15].

To assess the effect of substituting a rigid adamantane group for a more flexible acyl chain, globotriaoses conjugated to an adamantaneacetamido (Figure 1, aglycone 2) or a *t*-butylacetamido (Figure 1, aglycone 3) aglycone were compared. These aglycones were conjugated directly to the trisaccharide and therefore did not have the serine-like interface present in ceramide. It was presumed that a single amide linkage present in these conjugates would be insufficient to form substantial interface organization, and therefore that these conjugates should reflect mainly the structural effects of the hydrocarbon units. The conjugate with aglycone 2 inhibited the binding of VT₁B to Gb₃Cer with an IC₅₀ of 500 μ M, whereas the conjugate with aglycone 3 showed no inhibition up to 10 mM (Figure 2B). These

findings suggest the influence of a compact aglycone. It is possible that, in the VT₁B–globotriaoside complex, rotational freedom of the aglycones of adjacent glycoconjugates could be restricted. This will result in a loss of entropy associated with the aglycone upon conjugate binding. The methyl groups of the *t*-butylacetamido conjugate are free to rotate, and therefore binding could result in a greater loss of entropy than for the adamantyl conjugate. Similar considerations could have relevance to membrane lipid microdomains [26], since microdomains having tighter packing could be in a lower-entropy state than the bulk of the membrane. Other conjugates with non-ceramide-like aglycones (Figure 1; univalent 4, bivalent 5, quadrivalent 6 and tervalent 7) failed to show any inhibitory activity up to a concentration of 10 mM. This emphasizes the importance of designing appropriate aglycones towards active glycoconjugates, particularly for instances where lipid modulation is observed. Galabiose conjugates derived from aglycones 4, 5, 6 and 7 were effective inhibitors of bacterial binding to Gb₃Cer [18].

Soluble deoxyamino AdaGb₃ conjugates

Substituting the hydroxy group involved in hydrogen bonding with an amino group could enhance the strength of the hydrogen bond. The basic amine group could act as a better proton acceptor, and the 2 NH bonds may enhance the chances of hydrogen bonding compared with a single OH bond in alcohol. However, under physiological conditions, the basic amine group will be protonated and the ammonium group will not have acceptor interactions, but will enhance hydrogen donor inter-

Figure 2 Binding of VT₁ and VT₂ to AdaGb₃, aminodeoxy AdaGb₃ and acetamidodeoxy AdaGb₃ derivatives adsorbed on a TLC plate

Glycoconjugates were separated on TLC plates and allowed to bind to the toxins. (A) Orcinol stain; (B) VT₁ binding; (C) VT₂ binding. Lanes: (1) Gb₃Cer; (2) AdaGb₃; (3) 6' aminodeoxy AdaGb₃; (4) 6' acetamidodeoxy AdaGb₃; (5) 2'' aminodeoxy AdaGb₃; (6) 2'' acetamidodeoxy AdaGb₃; (7) 4'' aminodeoxy AdaGb₃; (8) 6'' aminodeoxy AdaGb₃.

actions and charge interactions. Some of the important hydroxy groups of Gb₃Cer identified from binding studies with deoxy-Gb₃Cer and VT were substituted with amino groups. Relative VT₁ binding to 2'', 4'', 6'' and 6' deoxyGb₃Cer and natural Gb₃Cer was 41 %, 31 %, 3 %, 1 % and 100 % respectively [10]. Soluble deoxyaminoAdaGb₃ conjugates were derived by coupling deoxyamino globotriaoses with an adamantyl ceramide aglycone (aglycone 1, Figure 1) [17].

Aminodeoxysialosides have been shown to be effective inhibitors of influenza neuraminidase activity. In this case, the 4-deoxyamino group of the sialoside enhanced charge and hydrogen bonding interactions with an aspartic acid residue of neuraminidase [27]. Amino substitution of hydroxy groups of galabiose enhanced the binding of the PapG *E. coli* adhesin due to charge interactions [28]. Also, globotetraosyl ceramide (Gb₄Cer), which is not a receptor for VT₁, VT₂ and VT_{2c}, was transformed into a receptor for all VTs by deacetylation of the terminal *N*-acetylglactosamine to a galactosamine [10].

By TLC overlay assay, VT₁ and VT₂ bound to AdaGb₃ and to the 2'' and 6' aminodeoxy AdaGb₃ conjugates. The 4'' and 6'' aminodeoxy AdaGb₃ conjugates showed no detectable binding (Figures 2B and 2C). A comparison of binding intensities on the TLC plate suggests that VT₂ shows tighter binding to AdaGb₃ than to Gb₃Cer, but otherwise a similar binding pattern was seen for the two toxins. On TLC or a microtitre plate-based ELISA,

Figure 3 Inhibition of VT₁ binding to Gb₃Cer immobilized on an ELISA plate

(A) Multivalent galabiose conjugates with non-ceramide-like aglycones. Symbols: ■, monoclonal anti-VT₁; ○, conjugate of aglycone 4 (see Figure 1); □, conjugate of aglycone 5; ●, conjugate of aglycone 5; △, conjugate of aglycone 6. (B) Effect of rigid compact aglycone compared with flexible aglycone. Symbols: ◇, monoclonal anti-VT₁; ▲, trimethylsilylethyl galabiose; ■, *t*-butylacetamido ethyl globotriaoside (aglycone 2, Figure 1); □, adamantaneacetamido ethyl globotriaoside (aglycone 3); ◆, β -galactose conjugated to adamantyl ceramide aglycone, (aglycone 1). (C) Inhibition by aminodeoxy AdaGb₃ conjugates: ▲, 4'' aminodeoxy AdaGb₃; △, 6'' aminodeoxy AdaGb₃; ●, AdaGb₃; □, 6' aminodeoxy AdaGb₃; ■, 2'' aminodeoxy AdaGb₃.

Gb₃Cer showed greater binding to VT₁ than VT₂ [10]. AdaGb₃ binding as assessed by TLC showed a similar profile, i.e. it bound more to VT₁ than to VT₂. However, VT₂ bound more strongly to AdaGb₃ than to Gb₃Cer, which we speculate is due to the fact that a greater mole fraction of AdaGb₃ is presented favourably for recognition by VT₂. The binding of the deoxyamino conjugates as assessed by TLC assay corresponded with their potential to inhibit VT₁ binding to Gb₃Cer adsorbed on to an ELISA plate. By this latter assay, 2'' and 6' aminodeoxy AdaGb₃ inhibited VT₁ binding to Gb₃Cer with an IC₅₀ of 5–10 μ M (Figure 3), whereas 4'' and 6'' aminodeoxy AdaGb₃ showed no detectable inhibition up to a 10 mM concentration.

Figure 4 Depiction of expected perturbations of the interactions between globotriaose and VT₁B upon substituting each of the 2'', 4'', 6'' and 6' hydroxy groups (left) with an amine (right)

Under physiological and experimental conditions, amines will be protonated, and therefore are represented as ammonium groups. Only direct hydrogen bonds are depicted. All three sites were determined from the structure of the VT₁B–Gb₃P^K complex, and Site 1 was also determined by modelling the docking of Gb₃Cer. In Site 1, X and M refer to interactions predicted by the structure and by the model respectively. Double-headed arrows indicate charge attraction, and 'lenses' indicate repulsion.

Relevance of aminodeoxy AdaGb₃ binding data to proposed VTB binding sites

Figure 4 shows the hydrogen bonding interactions of the 2'', 4'', 6'' and 6' hydroxy groups of globotriaose with the three proposed sites of VT₁B, and the perturbations that may result from substituting the hydroxy group with an amino group. The amine groups under the analytical conditions employed in the present study will be protonated and therefore will represent ammonium groups.

Analysis of Site 3

In the structure of the complex between VTB and Gb₃P^K, the 4'' hydroxy group of the terminal galactose is hydrogen bonded to an Asp-18 residue. Substitution of this 4''-OH with an ammonium group should have retained the hydrogen bonding interaction and enforced additional charge interaction. However, the 4'' aminodeoxy AdaGb₃ conjugate did not inhibit the binding of VT₁ to Gb₃Cer. The 6'' hydroxy group is a hydrogen acceptor in the hydrogen bonds formed with the peptide bonds of Trp-34N

Figure 5 ϕ/ψ adiabatic energy maps for Gal α 1-4Gal β (A) and GalNAc α 1-4Gal β (B)

and Asn-35N. Lack of inhibition by the 6'' aminodeoxy conjugate could be attributed to the inadequacy of the ammonium group to act as a hydrogen acceptor. The 2'' and 6' hydroxy groups do not form any substantial hydrogen bonding interactions with the protein.

Other studies also suggest that Site 3 may not be the most important site in defining the interaction between VT₁ and Gb₃Cer. For instance, the Trp-34 → Ala mutant did not show any significant reduction in cytotoxicity, but showed a decreased Gb₃Cer binding capacity [13]. NMR studies with VT₁ and Gb₃P^K showed no significant occupancy of Site 3 [12]. These findings suggest that the interaction of Gb₃Cer with Site 3 is weak; probably, this site facilitates in the localization and/or orientation of the toxin for optimal binding by Sites 1 and 2.

Analysis of Site 2

The structure of the complex between VT₁ and Gb₃P^K shows extensive hydrogen bonding interactions between the globotriaose and Site 2 of VT₁ [11]. The 4'' hydroxy group is hydrogen bonded to the guanidine group of Arg-33 and to the acetamidooxygen, Asn-32OD1, of Asn-32. Substitution of this 4'' OH group with an ammonium group could result in a significant repulsive interaction with Arg-33, possibly sufficient to overwhelm other favourable hydrogen bonds. Consistent with this analysis, 4'' aminodeoxy AdaGb₃ did not inhibit the binding of VT₁ to Gb₃Cer. The 6'' hydroxy group is hydrogen bonded to the peptide-bond-nitrogen Asn-32N, the acetamido-oxygen Asn-32OD1 and the peptide-bond-oxygen Phe-63O. In the latter two interactions, the 6'' OH group acts as a hydrogen donor. Substitution of this 6'' OH group with an ammonium group should have retained the hydrogen donor interaction. However, the 6'' aminodeoxy AdaGb₃ conjugate did not inhibit the binding of VT₁ to Gb₃Cer.

In the structure of the VT₁B-Gb₃P^K complex, the 6'' hydroxide group occupies a pocket that is well shielded from solvent, partly due to the orientation of the phenyl ring of Phe-30. The 6'' OH is only involved in direct hydrogen bonds, and the pocket cannot accommodate water molecules for solvent-mediated hydrogen bonds. Therefore, for an ammonium group to occupy the same pocket, the group would have to undergo complete desolvation. Such desolvation will impose a significant kinetic barrier for binding. However, if the interactions with the protein were exposed to the bulk solvent, only partial desolvation would

be required, and thus pose less of a barrier. Therefore these aminodeoxy conjugates may distinguish between solvent-buried and solvent-exposed interactions.

The 2'' and 6' hydroxy groups interact with Asp-16 and Asn-55, involving many water-mediated hydrogen bonds. Substitution of the 2'' OH group with an ammonium group should have retained the hydrogen bonding interaction and enforced additional charge interaction with Asp-16. Substitution of the 6' OH group with an ammonium group should retain hydrogen donor interaction with Asn-55 and augment solvent-mediated interactions. Consistent with this analysis, the 2'' and 6' aminodeoxy AdaGb₃ conjugates inhibited the binding of VT₁ to Gb₃Cer.

Analysis of Site 1

Binding at Site 1 has been determined by modelling and from the structure of the VT₁-Gb₃P^K complex. In the structure, the 6' and 2'' hydroxy groups interact with Asp-17 and/or form many water-mediated hydrogen bonds with other residues. In the model these two hydroxy groups also interact with Asp-17. Substituting either of these two hydroxy groups with an ammonium group should retain the hydrogen binding interaction and enforce additional charge and/or solvent-mediated interactions. Consistent with this analysis, the 2'' and 6' aminodeoxy AdaGb₃ conjugates inhibited the binding of VT₁ to Gb₃Cer.

In the structure, the 4'' hydroxy group is hydrogen bonded to Lys-13 and Thr-21, and in the model it is bonded to Lys-13 and Glu-65. Substitution of this 4'' OH with an ammonium group could result in a significant repulsive interaction with Lys-13, possibly sufficient to overwhelm other favourable hydrogen bonds. Indeed, 4'' aminodeoxy AdaGb₃ conjugates did not inhibit the binding of VT₁ to Gb₃Cer. In the structure, the 6'' hydroxide group interacts with Glu-28 and has no solvent-mediated interactions [29], and in the model it interacts with Glu-28 and Thr-21. Therefore substitution of 6'' OH with an ammonium group should have retained the hydrogen donor interaction and enforced additional charge interactions with Glu-28. However, 6'' aminodeoxy AdaGb₃ did not inhibit the binding of VT₁ to Gb₃Cer. As the structure and the model reveal, Site 1 is located within a cleft formed by adjacent subunits, and has no water-mediated hydrogen bonds associated with the 6'' OH group. Therefore the 6'' ammonium group has to undergo complete

Figure 6 Relative orientation of the globotriaose in Site 1 determined by X-ray and modelling studies

Stick amino acids and stick bond sugar representations correspond to X-ray data for the VT₁B–Gb₃P^K complex, whereas line amino acids and ball-and-stick bond sugar representations correspond to modelling data. The lower panels depict the relationship between the modelled Gb₃-saccharide in Site 1 with an attached ceramide moiety, and the Gb₃-saccharide in the Site 1 co-crystal (left panel), where rotation of one by approx. 55° about the β -galactose will approach the orientation of the other (right panel).

desolvation before binding, which in turn would impose a kinetic barrier.

Soluble acetamido AdaGb₃

The effective inhibitors, 2'' and 6' aminodeoxy AdaGb₃ conjugates, were acetylated to the corresponding 2'' and 6' acetamido conjugates. This transformation was carried out to evaluate the effects of neutralizing the charge of the ammonium group, decreased solvation, increased steric bulk and the donor acceptor properties of the acetamido function. When compared with the binding of 2'' and 6' aminodeoxy AdaGb₃ conjugates (as assessed by TLC), the acetamido conjugates showed reduced binding to both VT₁ and VT₂. From the crystal structure (Sites 1 and 2) and by modelling (Site 1), these two groups are involved in charge interactions with Asp residues and/or involve water-mediated hydrogen bonds. Therefore perturbations due to the loss of charge, decreased solvation and increased steric hindrance will have offset any favourable hydrogen bonding interactions resulting from the substitution of the hydroxy groups with an acetamido group. Molecular mechanics calculations on GalNAc α 1-4Gal β showed that its ϕ/ψ adiabatic energy map was indistinguishable from that of Gal α 1-4Gal β (Figure 5). Conformational effects are therefore unlikely on substitution of an acetamido group for hydroxide.

Possible relationship between VTB binding to a globotriaose conjugated with a ceramide compared with a non-ceramide-like aglycone

Since Gb₃Cer is insoluble in water, the only available alternative to study its interaction with VTB is by modelling. Docking studies with Gb₃Cer identified Site 1 [10], which was similar to the Site 1 found in the structure of the VTB–Gb₃P^K complex. As depicted in Figure 4 (Site 1), only a few variations in the hydrogen bonding interactions were noted. However, the relative orientation of the trisaccharide as a whole was significantly different. Figure 6 shows a superimposition of Site 1 from the complex structure and that from modelling. Selected peptide sequences from the structure and from the model were superimposed to determine the relative orientation of the trisaccharide. The trisaccharide moieties from the structure and the model showed an area of overlap around the central β -galactose. One orientation, for example the orientation of the trisaccharide of Gb₃P^K in the structure, can transform into the other, i.e. into the orientation of the trisaccharide of Gb₃Cer predicted by the model. Rotating one trisaccharide by approx. 55° about a single axis virtually superimposes it on the orientation of the other trisaccharide. A notable difference between the superimposed trisaccharides is the torsion angle of the 5''–6'' bond of the α -galactose.

Although structural data are indisputable, the aglycone [8-(methoxycarbonyl)octyl moiety] of the Gb₃P^K conjugate is an oversimplification of the ceramide aglycone of Gb₃Cer. Both *in vivo* and *in vitro* evidence indicate the importance of ceramide in Gb₃Cer receptor function. A possible proposition for the relationship observed between the sugar moieties in Figure 6 is that the orientations observed for the trisaccharides in the structure and in the model are two limiting cases. If this were the case, then, depending on aglycone modulation, the trisaccharide could occupy an orientation within the limits predicted above.

Conclusions

Aminodeoxy AdaGb₃ conjugates are useful inhibitors of the binding of Gb₃Cer to VTs, and provide information regarding the effects of solvation on binding. This information will be useful in the design of other conjugates, such as selecting those hydroxy groups involved in solvent-mediated interactions with the protein. Results from the inhibition studies with these conjugates were consistent with interactions with Site 1 and Site 2, and to a lesser extent with Site 3. Since membrane Gb₃Cer is differentially organized in lipid microdomains [30], the ceramide influence on saccharide presentation may be important in modulating binding.

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