Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Definition of the carbohydrate binding capacities of the novel enterotoxin LT-IIc

Master thesis

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Abstrakt

Hnačka ako ochorenie je stále vedúcou príčinou malnutrície a jednou z hlavných príčin úmrtí u detí do 5 rokov v nízko príjmových krajinách. Rovnako je to najrozšírenejší zdravotný problém spájaný s cestovaním do rozvíjajúcich sa zemí. Vo všetkých týchto prípadoch je enterotoxigénna *E. coli* (ETEC) jednou z najčastejších príčin.

ETEC je definovaná ako patogénny kmeň *E. coli*, ktorý produkuje enterotoxíny. Dodnes boli identifikované dva typy enterotoxínov: teplo-stabilné (ST) a teplo-labilné (LT). LT enterotoxíny sa ďalej členia na základe podobnosti s cholerovým toxínom na dve kategórie, a to LT typu I (LT-I) a typu II (LT-II). U všetkých týchto toxínov bola potvrdená schopnosť viazať uhľovodíkové štruktúry prítomné na glykosfingolipidoch pomocou B podjednotiek, avšak ich jednotlivá väzbovosť sa medzi sebou líši. Zatiaľ čo LT-I, LT-IIa a LT-IIb boli už v minulosti študované z pohľadu uhľovodíkovej väzbovej špecificity, najnovší enterotoxín LT-IIc bol zatiaľ otestovaný len na pár komerčne dostupných gangliozidoch ganglio série.

V tejto práci bola znovu otestovaná väzbovosť tohto nového enterotoxínu pomocou série väzbových testov s využitím väčšieho množstva gangliozidov ganglio série, niekoľkých gangliozidov neolacto série a ďalších glykolipidov a glykoproteínov na zistenie rozpoznávanej štruktúry a charakterizáciu optimálnej sekvencia potrebnej na väzbu. Na záver boli tiež vykonané inhibičné štúdie s použitím čistých uhľovodíkov.

Ako už bolo popísané, gangliozidy ganglio série so sekvenciou Siaα3Galβ3GalNAc boli viazané B podjednotkami LT-IIc a najsilnejšiu väzbu vykazoval gangliozid Neu5AcGD1a. Podobne silná väzbovosť bola však zaznamenaná aj pre gangliozidy neolacto série Neu5Acα3nLc$_4$Cer a Neu5Gcα3nLc$_6$Cer s obdobnou sekvenciou Siaα3Galβ4GlcNAc. Žiadna väzba nebola zaznamenaná u gangliozidov s disialo motívom (Siaα8Siaα3-) alebo s α6-naviazanou kyselinou sialovou (Neu5Ac). Rovnako nebola zaznamenaná žiadna väzba u asialo glykolipidov a glykoproteínov, čo len potvrdzuje dôležitosť sialovej kyseliny v interakcií medzi B podjednotkami LT-IIc a uhľovodíkmi.

Abstract

Diarrhoea as a disease is still the leading cause of malnutrition and a major cause of deaths in children under 5 years of age in the low-income countries. Additionally, it is the most common health problem associated with travelling to the developing countries. In all the mentioned cases, enterotoxigenic *E. coli* (ETEC) is one of the most frequent causes.

ETEC is defined as a pathogenic strain of *E. coli* producing enterotoxins. So far, two types of enterotoxins have been identified: heat-stable (ST) and heat-labile (LT). LTs are further divided into two categories based on their relatedness with cholera toxin to type I (LT-I) and type II (LT-II). All of these enterotoxins have been found to bind to carbohydrate structures on glycosphingolipids by their respective B subunits, however, their binding patterns differ. While LT-I, LT-IIa and LT-IIb have been previously studied in terms of binding specificities, the newest LT-IIc was tested only on few commercially available ganglio-series gangliosides.

In this thesis, the binding capabilities of this novel enterotoxin were re-examined by series of binding assays using more ganglio-series and some neolacto-series gangliosides as well as other glycolipids and glycoproteins, to establish the basics of the recognition pattern and to characterize the optimal binding sequence. At the end, inhibition studies using pure carbohydrates were carried out.

As previously described, ganglio-series gangliosides with Siaα3Galβ3GalNAc carbohydrate chain sequence were bound by the B subunits of LT-IIc (LT-IIc-B) and the strongest binding was noted for Neu5AcGD1a. Similarly strong binding was noted for neolacto-core gangliosides Neu5Acα3nLc$_4$Cer and Neu5Gcα3nLc$_6$Cer with the similar terminal sequence Siaα3Galβ4GlcNAc. No binding to gangliosides carrying a disialo motif (Siaα8Siaα3-) or an α6-linked Neu5Ac occurred. Furthermore, no binding was noted for asialo glycolipids or glycoproteins, underlining the importance of the sialic acid in LT-IIc-B carbohydrate interactions.

TABLE OF CONTENTS

I. List of abbreviations 9
II. Aim 10
III. Introduction 11
1. Glycoproteins and proteoglycans 12
2. Glycolipids 12
2.1. Glycoglycerolipids 13
2.2. Glycophosphatidylinositols 13
2.3. Glycosphingolipids 14
2.3.1. Nomenclature of glycosphingolipids 14
2.3.2. Glycosphingolipid types 16
2.3.3. Biosynthesis and biodegradation 17
2.3.4. Physiological functions 19
2.3.5. Other notable roles in human physiology and pathology 20
3. AB_5 toxins 20
3.1. Structure of AB_5 toxins 20
3.2. Subfamilies of the AB_5 toxins 21
3.3. Mechanisms of action 21
3.4. Cholera toxin family 22
3.4.1. Type I and type II group 22
3.4.2. Biosynthesis of the toxins 23
4. Diarrhoea as a travellers' and low-income countries' disease 24
4.1. Etiology 24
4.1.1. Enterotoxigenic *Escherichia coli* 25
4.1.1.1. ETEC classification 25
4.1.1.2. Pathogenesis of ETEC caused diarrhoea 26
4.2. Symptoms and consequences of *E. coli* caused diarrhoea 26
4.3. Treatment of diarrhoeal infections 27
4.3.1. Specific treatment of diarrhoea 27
4.3.1.1. Antibiotics and antisecretory drugs 27
4.3.1.2. Vaccines 28
IV. Materials and Methods 29
1. Isotope labelling of LT-IIc-B 30
1.1. Preparations for the iodination 31
1.2. Radioiodination 31
1.3. Separation of the iodinated protein 31
2. Binding assays 31
2.1. HPTLC binding assay 32
2.1.1. Application of the sample and elution of the plate 32
2.1.2. Fixation of the plate and binding of LT-IIc-B 32
2.1.3. Visualization of the binding 33
2.2. Radioimmunoassay 33
2.2.1. Preparation of the assay 33
2.2.2. Binding of radiolabelled protein 33
2.2.3. Counting of γ radiation 34

3. **Glycoproteins binding assays** ... 34
3.1. **HPTLC binding assay of glycoproteins** ... 34
3.2. **Western blotting** ... 34
3.2.1. SDS-PAGE ... 34
3.2.1.1. Gel electrophoresis of the samples ... 35
3.2.1.2. Imperial staining ... 35
3.2.2. Principle of Western blotting ... 35
3.2.2.1. Blotting of the gel ... 36
3.2.2.2. Binding of LT-IIc-B ... 36
4. **Inhibition assays** ... 37
4.1. **Inhibition assays using HPTLC and RIA** ... 37
5. **Separation and purification of GSLs** ... 38
5.1. **Column chromatography** ... 38
5.1.1. Preparation of the sample ... 39
5.1.2. Preparation of the column for glycolipid separation ... 39
5.1.3. Separation of the glycolipids in the sample ... 39
5.2. **Thin-layer chromatography (TLC)** ... 39
5.2.1. Preparation of the fractions for TLC ... 40
5.2.2. Detection of the compounds ... 40
5.3. **Second column chromatography** ... 40
6. **Mass spectrometry** ... 41
6.1. **Mass spectrometry for glycosphingolipid analyses** ... 42
6.1.1. Analysis of intact glycosphingolipids ... 43
6.1.1.1. Preparation of the sample ... 43
6.1.2. Analysis of the carbohydrate part ... 43
6.1.2.1. Preparation of the sample ... 44
V. **Results** ... 45
1. **Binding of LT-IIc-B to reference glycolipids** ... 45
1.1. **Binding of LT-IIc-B to dilutions of selected glycolipids** ... 51
1.2. **CTx-B vs. LT-IIc-B binding specificity** ... 52
2. **Binding to glycoproteins** ... 53
2.1. **HPTLC binding** ... 53
2.2. **Gel electrophoresis and Western blotting** ... 53
3. **Inhibition studies** ... 54
3.1. **HPTLC and RIA inhibition assays** ... 54
4. **Isolation of GSLs from moose kidney** ... 55
VI. **Discussion** ... 60
VII. **Conclusion** ... 64
VIII. **References** ... 65

I. LIST OF ABBREVIATIONS

BSA	bovine serum albumin
Cer	ceramide
CFU	colony forming units
cpm	counts per minute
CTx	cholera toxin
CTx-B	B subunits of cholera toxin
ER	endoplasmic reticulum
ETEC	enterotoxigenic *Escherichia coli*
GA	Golgi apparatus
Gal/GalNAc	galactose/N-acetylgalactosamine
Glc/GlcNAc	glucose/N-acetylglucosamine
GPI	glycophosphatidylinositol
GSL	glycosphingolipid
Hex	hexose
HexNAc	N-acetylhexosamine
LT-IIc-B	B subunits of LT-IIc
MS	mass spectrometry
Neu5Ac/Neu5Gc	*N*-acetyl/*N*-glycolylneuraminic acid
PBS	phosphate-buffered saline solution
Sia	sialic acid (Neu5Ac or Neu5Gc)
SLTx	Shiga-like toxin
STx	Shiga toxin

II. AIM

The binding specificity of B subunits of LT-IIc enterotoxin (LT-IIc-B) had been previously examined using a few commercially available ganglio-series gangliosides.[1, 2] The aim of this thesis was to further define the carbohydrate binding specificity using the collection of purified glycosphingolipids available at the *Department of Medical Biochemistry and Cell Biology* at the *University of Gothenburg.* Determination of the carbohydrate sequence of glycosphingolipids with the highest affinity to the B subunits of LT-IIc enterotoxin could lead to the discovery of the anti-adhesives against LT-IIc, and thus help the treatment of severe ETEC-caused diarrhoeal infections.

III. INTRODUCTION

The study of carbohydrates has been an interest of many scientists for decades. However, because of inability to easily study and predict their complex structures, and the previous views of carbohydrates as energy and structural sources, major breakthroughs in the understanding of carbohydrates' roles came relatively late when compared to proteins or nucleic acids.[3] After the studies of Karl Landsteiner's ABO blood group antigens by Winifred M. Watkins and Walter T. J. Morgan in 1960s, who described their carbohydrate nature[4], it has become obvious that the roles of carbohydrates in human body exceed the previous incorrect beliefs. Thus, in the 1980s the new field of research, studying physiology, biology and biochemistry of all carbohydrate-related molecules was formed – the field of glycobiology.

Subsequent research has proven, that apart from being basic cellular energy sources (*e.g.* glycogen, glucose), structural foundations of extracellular matrix (*e.g.* hyaluronic acid), the carbohydrates in the human body also serve as receptors for physiologically active molecules, accessible on the cell surface. There they are linked to proteins and lipids, forming glycoproteins, glycolipids and proteoglycans and together with their non-carbohydrate parts, these glycoconjugates are responsible for several important functions of the cell.[3]

1. GLYCOPROTEINS AND PROTEOGLYCANS

Glycoproteins and proteoglycans are both defined as molecules with carbohydrate chains linked to proteins. However, while in glycoproteins, the carbohydrate represents only a small portion of the molecule, in proteoglycans the glycan is dominant. Therefore, properties of these glycoconjugates are very different.[5]

Glycoproteins are a very diverse group of glycoconjugates. They exist as *O*-glycosylated, where the glycosylation occurred on serine or threonine[6] and as *N*-glycosylated, with the carbohydrate chain linked to asparagine in a specific amino acid sequence.[7] In addition to the structure diversity produced by the carbohydrate chain, the glycan part of the glycoproteins may be further modified *e.g.* by sialylation or sulfation, further modifying their structure and as a consequence, their functions.[7, 8]

Proteoglycans consist of the protein core branched with the polycarbohydrate chains called glycosaminoglycans (GAG) because of high content of amino sugars. The properties of the proteoglycans are defined mainly by the GAG constitution and modifications, similarly to the glycoproteins.[9]

2. GLYCOLIPIDS

Glycolipids are molecules necessary for a healthy development and they play important biochemical and immunological roles.[10]

In **glycolipids**, carbohydrate chain is bound to a hydrophobic moiety such as acylglycerol, sphingoid, ceramide or a prenyl phosphate.[11] Apart from prenyl phosphate based glycolipids that seem to be just the biosynthetic intermediates for *N*-glycosylated glycoproteins[7], all the others have been identified as the final products of the metabolism, although not always human.

Based on the hydrophobic moiety, three types of naturally occurring glycolipids are known: **glycoglycerolipids**, **glycophosphatidylinositols** and **glycosphingolipids** (as seen in *Fig. 1*).[11]

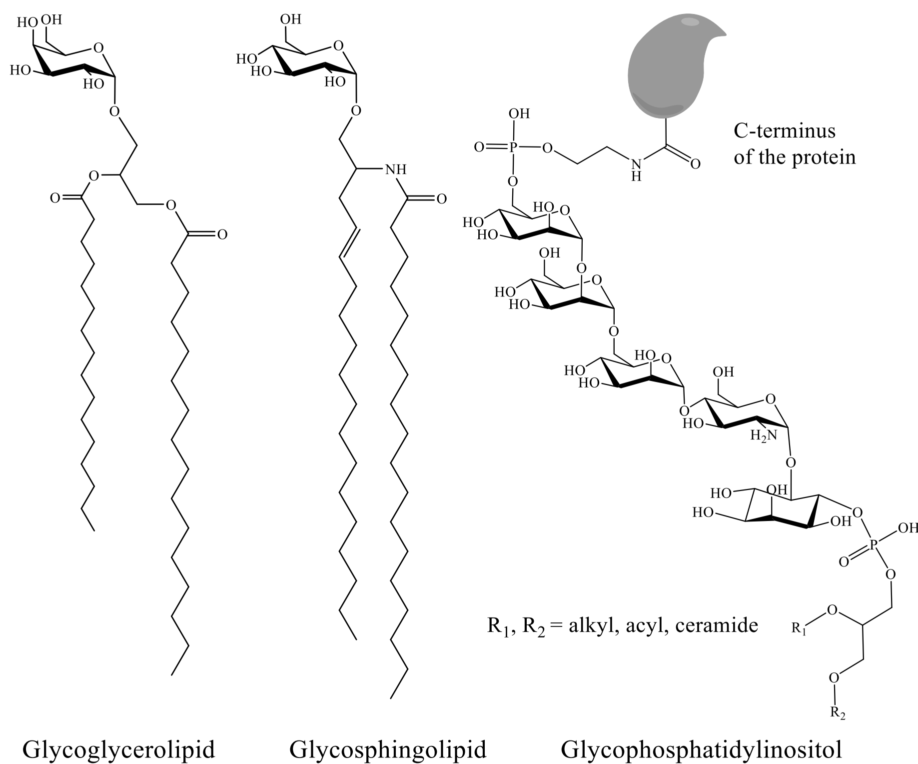

Fig. 1 Classification of glycolipids based on the lipid moiety.[11]

2.1. Glycoglycerolipids

Glycoglycerolipids are present mostly in plants, where they have an essential role in photosynthesis.[12] The most abundant and most important vegetal glycoglycerolipids are mono- (MGD, shown in *Fig. 1*) and digalactosyldiacylglycerol (DGD). In animals and humans, their presence is very rare.[13]

2.2. Glycophosphatidylinositols

Glycophosphatidylinositols (GPI) are a large and very diverse group. Their main role is to anchor proteins to the cellular plasma membrane, mainly from the extracellular part, thus they are sometimes called GPI-anchors. GPIs largely differ among organisms, but all share some common features represented in *Fig. 1*. GPIs are always based on a diacylglycerol lipid moiety. Multiple modifications of the basic structure have been identified. The most common are larger glycan part, esterification of inositol with palmitate and presence of another ethanolamine molecule on the glycan. These various changes have a great impact on the GPI functions, *e.g.* GPI esterified with palmitic acid is not susceptible to enzymatic cleavage of the protein-GPI bond with phospholipase C.[10]

2.3. Glycosphingolipids

Glycosphingolipids represent the majority of all glycolipids found in humans and they are an indispensable part of the cell membrane.[13] The carbohydrate chain is β-*O*-linked to the C1 hydroxyl of the sphingoid, which is the part of the ceramide.

Sphingoid, is a common term for all 18 carbon long amino alcohols, derivatives of sphinganine ((2*S*,3*R*)-2-aminooctadecane-1,3-diol, originally called dihydrosphingosine), differing among each other by the degree of saturation or by the presence of the hydroxy groups. Three natural sphingoids have been described: sphing-4-enine (sphingosine) common in mammals, already mentioned sphinganine (dihydrosphingosine), and 4*R*-hydroxysphinganine (phytosphingosine) present mostly in plants and fungi.[13]

The **ceramide** represents all *N*-acylated sphingoids with the fatty acid usually ranging from C16 to C24.[11]

2.3.1. Nomenclature of glycosphingolipids

Systematic nomenclature of glycolipids established by International Union of Pure and Applied Chemistry (IUPAC) differentiate GSLs based on their carbohydrate part, referred to as the glycan. Full systematic names like **β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-ceramide** are nowadays used very little, names of the carbohydrates are usually replaced by defined symbols (Gal – galactose, GlcNAc – *N*-acetylglucosamine etc.). Depending on what details are necessary to emphasize, multiple forms are used.

The extended form like **β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-Cer** (*p* stands for pyranose form) is clear and contains all information, although, it is unpractical for very complex, branched structures. **Condensed** and **short forms** are simpler, often used to name big and complex molecules but they omit the full stereo description of the saccharides and details of the glycosidic linkage (in short form). When using these names, all monosaccharides are presumed to be in cyclic D form, except rhamnose and fucose that are in cyclic L form. In the short form, the glycosidic linkage is simplified by omitting the number of the anomeric carbon, since it is invariable for each monosaccharide in naturally occurring glycosidic bonds in GSLs, usually C1, or C2 in sialic acid. Thus, the

same structure as before, using condensed form would be **Galβ(1-4)Glc(β1-1)Cer** or **Galβ4GlcCer** using the short form. The latter will be preferred in this thesis.

In **semi-trivial nomenclature**, each GSL is attributed to one of the categories described in *Fig. 2* based on the first sugars. The names are composed of the **root** followed by **root size** and suffix **osylceramide**, such as **GalNAcβ3Galα4Galβ4GlcCer** or simply **Gb$_4$Cer** refers to **globotetraosylceramide**. More detailed nomenclature principles may be found in reference [11].

Root	Symbol	Structure
ganglio	Gg	Galβ3GalNAcβ4Galβ4Glc-
isoganglio	iGg	Galβ3GalNAcβ3Galβ4Glc-
lacto	Lc	Galβ3GlcNAcβ3Galβ4Glc-
neolacto	nLc	Galβ4GlcNAcβ3Galβ4Glc-
globo	Gb	GalNAcβ3Galα4Galβ4Glc-
isoglobo	iGb	GalNAcβ3Galα3Galβ4Glc-
mollu	Mu	GlcNAcβ2Manα3Manβ4Glc-
arthro	At	GalNAcβ4GlcNAcβ3Manα4Glc-
muco	Mc	Galβ4Galβ4Glc-
schisto		GalNAcβ4Glc-
gala	Ga	Galα4Gal-
neogala		Galβ6Galβ6Gal-
spirimeto		Galβ4Glcβ3Gal-

Fig. 2 Classification of glycosphingolipids based on carbohydrate structure.[11, 14]

Even though semi-trivial names have been recommended, for gangliosides, the **trivial** names from Svennerholm system proposed for brain gangliosides in 1980 are used more frequently. All gangliosides begin with **G** followed by a letter indicating a number of sialic acids (**M** – mono-, **D** – di- etc.) and an Arabic numeral referring to migration order on a TLC plate during Svennerholm's research.[15] Consequently, **GM1** and **GM2** are gangliosides containing one sialic acid, with GM2 migrating on a TLC plate higher than GM1. These abbreviations may be somewhat confusing but they have been well accepted by the scientific community.[11]

2.3.2. Glycosphingolipid types

Two main categories of GSL have been described: **neutral glycosphingolipids** lacking a charged function group (ionic or acidic) and **acid glycosphingolipids** containing an acidic moiety in their molecule.[11]

Acid glycosphingolipids are sub-divided based on the acidic residue. **Gangliosides** are all GSLs containing one or multiple *O*- or *N*-acylated neuraminic acid (commonly named sialic acid), acetylated (Neu5Ac) in humans or glycolylated (Neu5Gc) in other mammals, as humans lack active Neu5Gc-hydroxylase converting Neu5Ac to Neu5Gc.[16] In humans, Neu5Gc presence may occur when taken up from food. The name ganglioside is not to be confused with ganglio-series, as not all gangliosides are necessarily ganglio-series glycosphingolipids. **Glycuronosphingolipids** contain residues of uronic acid, **sulfoglycosphingolipids** (previously called sulfatides) are molecules containing carbohydrate-sulfate ester groups, **phosphoglycosphingolipids** contain phosphate mono or diester groups and **phosphonoglycosphingolipids** contain (2-aminoethyl)hydroxyphosphoryl groups. The main differences of acid sphingolipids are represented in *Fig. 3*.[11]

Of all GSLs, gangliosides and sulfatides are the most common and abundant in humans.[3] Glycuronosphingolipids could also be found, possibly because of low specificity of glycoprotein glycosyltransferases[13], but alongside phosphoglycosphingolipids and phosphonoglycosphingolipids, these are the most common GSLs of the invertebrates.[17, 18]

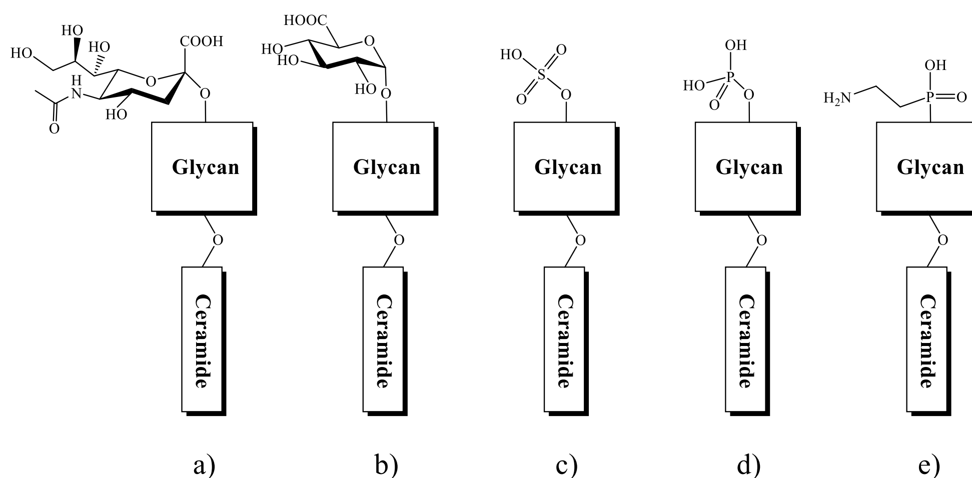

Fig. 3 Acid glycosphingolipids.[11]
a) gangliosides, b) glycuronosphingolipids, c) sulfoglycosphingolipids, d) phosphoglycosphingolipids, e) phosphonoglycosphingolipids

2.3.3. Biosynthesis and biodegradation

The biosynthesis of glycosphingolipids in mammals is a complicated, multiple-step process. Firstly, the ceramide part of the GSL is synthesized, then the carbohydrate chain is built up. Two main GSLs biosynthetic pathways have been described, forming derivatives differing with the carbohydrate linked to the Cer: **GlcCer derivatives** and **GalCer derivatives**.[13]

The ceramide biosynthesis takes place on the cytoplasmic face of endoplasmic reticulum by four essential pathways as shown in *Fig. 4*: *de novo* synthesis from serine and palmitoyl-CoA (***de novo* pathway**), salvaging complex sphingolipids (**salvage pathway**), **recycling exogenous ceramides** and pH-dependent enzymatic degradation of sphingomyelin (**sphingomyelin pathway**). When successfully synthesized, the ceramide either (1) flips to the luminal face of ER where Gal is added, creating GalCer, which is transferred to the Golgi apparatus (GA) to form GalCer derivatives (*e.g.* some sulfatides), or (2) the ceramide traffics to the GA where Glc is added on the outer side, then, the newly formed GlcCer is flipped to the luminal part of GA and forms GlcCer derivatives (the majority of GSLs). The last possibility for the ceramide is to be translocated to GA and eventually used for biosynthesis of sphingomyelin.[13]

Nevertheless, once the GlcCer is flipped to the GA and galactosylated, it forms a metabolic branch point for other classes of GSLs – lactosylceramide (LacCer, Galβ4GlcCer). The following synthesis depends on the composition of available nucleotide sugar donors (UDP-Glc, UDP-Gal etc.), and on the presence and levels of specific glycosyltransferases (α-2-3-sialyltransferase, α-1-4-galactosyltransferase etc.) in the GA compartments within the given cell. The carbohydrate chain may be further elongated, sialylated with CMP-Neu5Ac donor or sulfated with 3'-phosphoadenosine-5'-phosphosulfate (PAPS), thus creating more complex GSLs. At the end of the biosynthesis, the finished GSLs are transported and incorporated into the plasma membrane.[13, 19]

As for many other enzymes, the activity of the glycosyltransferases is variable in different tissues and changes during their development. Therefore, the composition of GSLs in the tissues strongly varies in time and place. This fact was best described in the human brain, where the simple GSL GM3 is gradually replaced by more complex ones,

like GT1c and others during ageing. Biosynthetic pathways of GSLs in the brain are presented in *Fig. 5*.[13, 19]

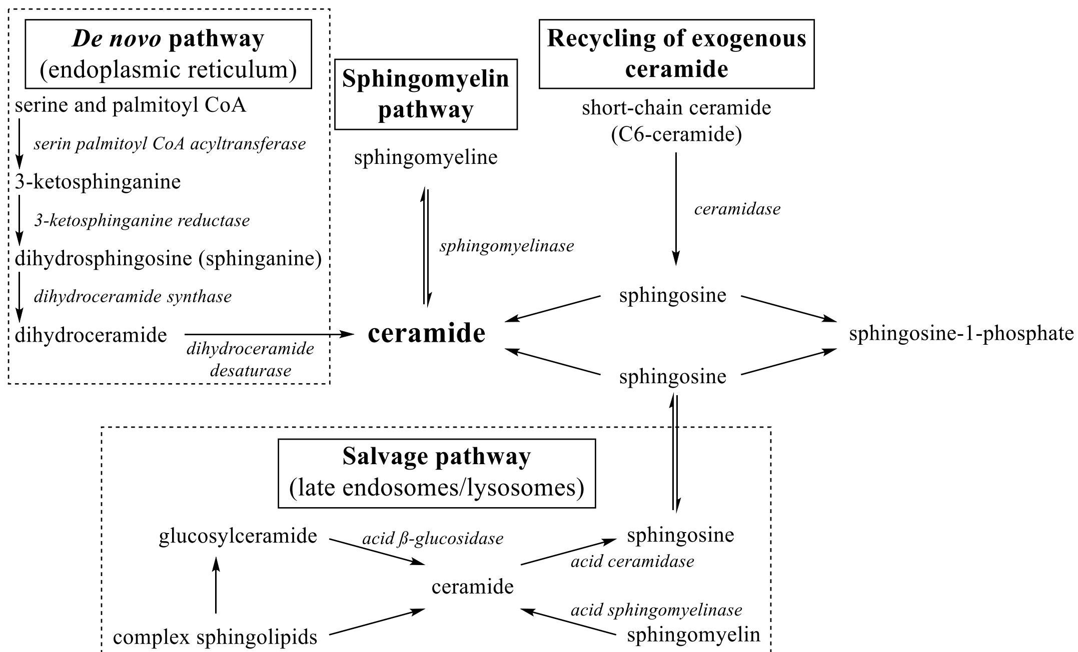

Fig. 4 Four essential ceramide biosynthesis pathways.
Adapted and modified after Kitatani, Idkowiak-Baldys and Hannun [20]

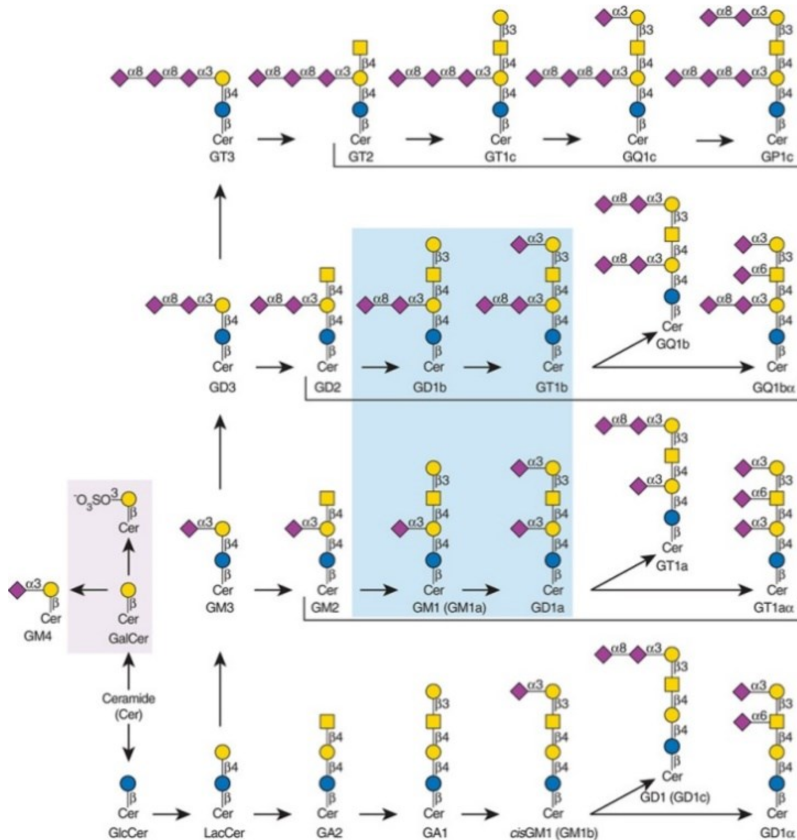

Fig. 5 The biosynthetic pathways of human brain GSLs.
Adapted from Schnaar and Kinoshita [13]

The degradation of the GSLs from the membrane begins by the formation of the vesicules, invaginated from the plasma membrane. These vesicules traffic through the endosomal compartments to finally fuse with lysosomes containing specific hydrolases that commence the stepwise cleavage of the carbohydrate chains. For the short-chained GSLs, the degradation process is assisted by specific membrane-active sphingolipid activator proteins (SAPs) disrupting the interactions between the membrane environment and GSLs, thus facilitating the access of the hydrolases to the glycan. When the GSLs are broken down to their individual components, these become available for reuse.[13, 21]

2.3.4. Physiological functions

GSLs may cover from 5% up to 20%[13] of the cell membrane lipids in higher animals and humans, and they are mostly distributed in the outer membrane layer with the carbohydrate part facing outwards.

The functions of GSLs can be divided into two categories: ***trans*** **recognition** responsible for cell-cell interactions and ***cis*** **regulation***,* which is responsible for modulation of protein activity within the same membrane.[13] Even though multiple experiments have proven that life without GSLs at the single cell scale is possible although limited, when similar experiments were done with multicellular organisms (mice), the life ended at the embryonic stage, underlining the essential role of GSLs in the development of the organism.[10, 22]

The *trans* recognition role is best described in the brain, where more than 50%[13] of all glycoconjugates are GalCer and its 3-*O*-sulfated derivatives, which are an essential part of the myelin structure. Their presence is necessary for proper myelin-axon interactions, although the exact mechanisms are yet to be understood.[13] However, inability to synthesize or properly utilize GalCer and its derivatives leads to myelin that is incorrectly attached to the axon in the Ranvier nodes, resulting in fault neurotransmission and leading to several neurological disorders, *e.g.* Krabbe disease.[23]

Cis regulatory functions of GSLs are described in interactions between gangliosides and the tyrosine kinase family receptors, *e.g.* the epidermal growth factor or insulin receptor. Best known is the influence of GM3 ganglioside on down-regulation of insulin receptor response[24] or the up-regulation of the high-affinity nerve growth factor receptor TrkA by GM1.[25]

2.3.5. Other notable roles in human physiology and pathology

Apart from membrane functions, GSLs are also required in other places. One of the most important is the role of GlcCer in the epidermal protection barrier formation. After being transported into dermal cells, the ceramide part of GlcCer is enzymatically separated from the carbohydrate and incorporated into stratum corneum (top skin layer). Once there, the ceramide forms an impenetrable barrier, minimizing water losses from the inside and protecting the organism from penetration of harmful compounds from the outside.[13]

Many pathological conditions are directly or indirectly linked to GSLs either by their deficiency (deficiency of GM3 leads to severe seizures and both motor and intellectual deficits in the infants), surplus (Gaucher disease with GlcCer accumulation in the liver or spleen).[13, 14] However, the majority of the mechanisms involved is still not fully understood.

Additionally, GSLs play an important role in pathophysiology of several diseases caused by toxins (cholera toxin and others), pathogenic bacteria (numerous strains of *E. coli, Helicobacter pylori, Mycoplasma pneumoniae*), fungi (*Candida albicans*) and viruses (human immunodeficiency virus 1 (HIV-1), influenza virus), specifically recognizing membrane GSLs, mostly gangliosides and with their help changing intracellular functions to provoke associated pathological changes.[26]

3. AB_5 TOXINS

AB_5 toxins are a well-known and clinically important toxin family. They are responsible for some of the most contagious diseases like cholera, shigellosis, whooping cough and others. Even though some AB_5 toxins prefer glycoproteins, the majority bind to glycans of GSLs. Thus, GSLs play a very important role in the pathophysiology of the AB_5 toxin-caused diseases.[27]

3.1. Structure of AB_5 toxins

Most of the toxins are composed of a homopentameric B-fragment (also referred to as B-pentamer), built of five identical non-covalently bound B subunits responsible for the binding and the delivery of the toxin to the target cell, and a heterodimeric A-fragment (often referred to as A subunit because of its structure), composed of A_1 and

A_2 polypeptide chains linked by disulphide bond. While A_1 polypeptide is the main catalytic part responsible for the enzymatic function, A_2 is a cross-linking agent between A_1 and the B-pentamer. The binding of the B fragment is possible without the catalytic A fragment, however, only a holotoxin can intoxicate the cell.[28] Only one subfamily, the pertussis toxins have a slightly different composition of the fragments. They have an enzymatic S_1 subunit and in place of B homopentamer, they have a heteropentameric fragment composed of $S_2S_3S_4S_4S_5$ subunits. However, since their folding motif, 3D structure and the mechanism of intoxication is closely related to other AB_5 toxins, these fragments are also referred to as A and B, respectively.[29, 30]

3.2. Subfamilies of the AB_5 toxins

Four subfamilies of the AB_5 toxins have been described so far: the **cholera toxin family (CT)**, the **shiga toxin family (ST)**, the **pertussis toxin family (PT)** and the **subtilase cytotoxin family (SubAB)**. These subfamilies were formed based on excessive A and B fragments' homology studies (some results may be seen in *Fig. 7*) and the mechanisms of action of the corresponding toxins. Although not all the subfamilies share high polypeptide sequence similarity, in case of PT toxins, not even the same structure, their folding motifs are very closely related as may be seen in *Fig. 6*. This motif was first observed and described by Murzin in 1993[31] who named it as OB-fold (oligonucleotide/oligosaccharide binding fold) and it can also be found in other toxins, *e.g.* staphylococcal nuclease or toxic-shock syndrome toxin 1 (TSST-1), which suggests its long-term phylogenetic effectiveness.[32]

3.3. Mechanisms of action

The B subunits recognize multiple carbohydrate patterns on plasma membrane coming mostly from the glycosphingolipids.[28] The recognition of the target structure by the B subunits triggers endocytosis of the toxin, transport to ER via GA and disassembly. Once the subunits are separated, the A_1 subunit is unfolded and transported to the cytosol, where it is refolded and then ready to exert its cytotoxic effect.[30, 33]

Each subfamily of the AB_5 toxins has a different mechanism of action. Even though CT and PT both have ADP-ribosylating effect on a G-protein subunit, each acts through a different subunit. While CT toxins ribosylate the $G_s\alpha$ subunit, PT is acting through $G_i\alpha$. In both cases, as a result of ribosylation, the G-protein's GTPase activity is

inhibited, which is responsible for subsequent overactivation of adenylyl cyclase and elevation of intracellular cAMP. The increased concentration of cAMP influences opening of the cystic fibrosis transmembrane conductance regulator (CFTR) leading to uncontrolled secretion of fluids and electrolytes (mainly chlorides) extracellularly. These molecular changes are evident primarily in the gut epithelial cells, as they clinically manifest as a severe diarrhoea.[30]

The ST family has RNA *N*-glycosidase activity, cleaving a specific adenine base from 28S rRNA resulting in inhibition of protein synthesis and subsequently in cellular death.[34]

SubAB, the newest and extremely cytotoxic AB_5 toxin subfamily consists of proteases, therefore it acts differently from both mechanisms mentioned above. Cellular target of SubAB toxins is BiP, a highly conservative chaperon protein located in ER, responsible for numerous functions in the cell. SubAB toxins cleave the BiP, thus disable its further activity, which has fatal consequence for the cell.[32]

3.4. Cholera toxin family

Cholera toxin family is the most common and the best described AB_5 subfamily. It comprises heat-labile enterotoxins produced by enterotoxigenic *E. coli* (ETEC), *V. cholerae* and other related enterotoxins from bacteria like *Campylobacter jejuni*.[35] These proteins were divided into two main groups based on their genetic, biochemical and immunological characteristics.

3.4.1. Type I and type II group

The older, type I group consists of cholera toxin (CTx) produced by *Vibrio cholerae,* heat-labile enterotoxin of type I (LT-I) produced by enterotoxigenic *E. coli* and other similar bacterial enterotoxins from *C. jejuni*. Because of high similarity and close relatedness of the B subunits, all of the type I group toxins show cross-linked reactivity, giving them similar binding properties, *e.g.* equally high affinity to the GM1 ganglioside.[36]

The type II group consists of three heat-labile enterotoxins: LT-IIa, LT-IIb and LT-IIc. As a result of feeble B subunits homology, the affinity of LT-II toxins towards GSLs is different from that of CTx/LT-I and no cross-reactivity is observed. In fact, each of the LT-II toxins has a unique binding pattern. While LT-IIa has the strongest affinity

to GD1b and LT-IIb to GD1a[36], LT-IIc binds strongly to GD1a and GM1 but not to GD1b. Homology differences between types I and II are summarized in *Fig. 7*.[37, 38]

Even though the B subunits are not much related, the type I and type II enterotoxins share the same mechanism of action, exerted by more or less homologous catalytic A_1 subunits.[30]

3.4.2. Biosynthesis of the toxins

Biosynthesis of the CT family toxins has been partially described. The two-gene operon *eltAB* coding A and B subunits of LT-I is plasmid encoded[39] while the *ctxAB* coding the CTx[40] and genes responsible for the LT-II toxins are encoded chromosomally.[41] The latter have been just recently discovered to be integrated into the genome by a lambdoid prophage.[41]

The subunits are synthetized in the cytosol separately as unfolded protein sequences. Once synthesized, the subunits of the CT and LT-I enterotoxins are translocated to the periplasmic space, where they are folded and assembled to form the holotoxin, which is then secreted through the outer membrane.[42, 43] A similar mechanism could be expected also for the LT-IIs, however, no research has been done in that matter so far.

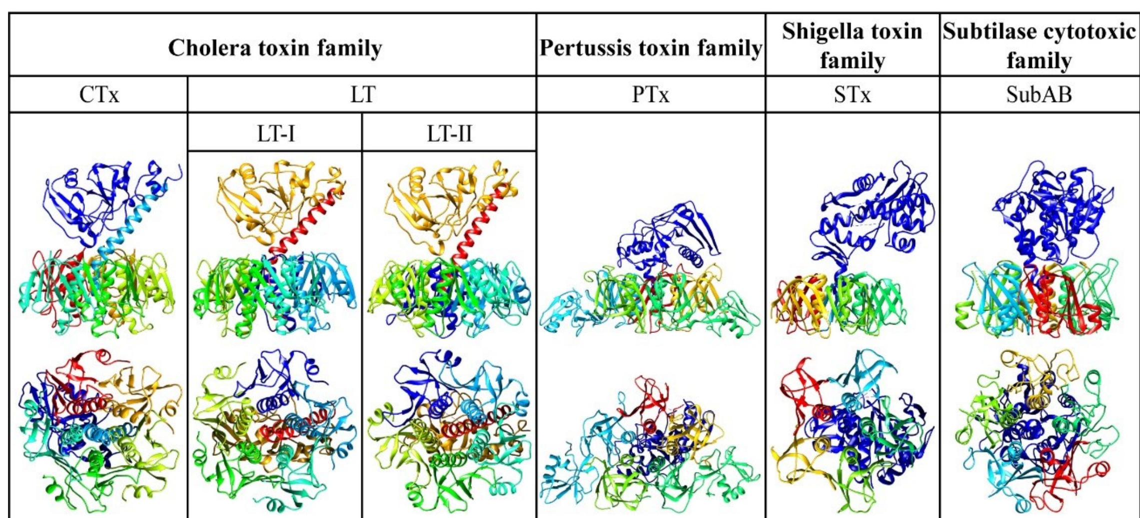

Fig. 6 The AB_5 toxin families with the 3D structures of their representative.
CTx – Vibrio cholerae toxin, LT-I/LT-II – ETEC enterotoxin LT-I/LT-II (LT-IIb is presented), PTx – Bordetella pertussis toxin, STx – Shigella dysenteriae toxin, SubAB – E. coli subtilase cytotoxin. (Structures were visualised and modified using UCSF Chimera software, available online at: http://www.rbvi.ucsf.edu/chimera/)

AB$_5$ Family	Toxins	Subunit	Sequence homology							
Cholera toxin family	***CTx***	*A*		*80%*	*57-59%*	*57-59%*	*47%*	*low*	*A to S_1: 15-20%*	*NA*
		B		*80%*	*15%*	*15%*	*7%*			
	LT-I	*A*			*57-59%*	*57-59%*	*49%*			
		B			*15%*	*11%*	*7%*			
	LT-IIa	*A*				*84%*	*79%*			
		B				*57%*	*53%*			
	LT-IIb	*A*					*72%*		*B to $S_{2\text{-}5}$: none*	
		B					*54%*			
	LT-IIc	*A*								
		B								
Shiga toxin family	***STx, SLTx***	*A, B*						*A: high B: ≈100%*	*A: NA B: none*	
Pertussis toxin family	***PTx***	*S_1, S_2, S_3, S_4, S_5*							*S_2 to S_3:70%*	
									S_2/S_3 to S_4, S_5: none	
Subtilase cytotoxic family	***SubAB***									
			CTx	*LT-I*	*LT-IIa*	*LT-IIb*	*LT-IIc*	*STx, SLTx*	*PTx*	*SubAB*
			Type I		***Type II***					

Fig. 7 Summary of AB$_5$ toxins' homology. *(NA – not available)*
(References: LT [28, 44]*, LT-*IIa [45]*, LT-IIb* [46], *LT-IIc* [38], *STx/SLTx* [28, 34], *PTx* [29, 32])

4. DIARRHOEA AS A TRAVELLERS' AND LOW-INCOME COUNTRIES' DISEASE

Diarrhoea is still the leading cause of malnutrition and the second leading cause of death in children under five years of age in low-income countries. For other countries, it represents the most frequent health problem associated with travelling to the developing countries. Its primary lethal effect used to be caused by fluid and salts losses, nowadays, fatal consequences are attributed to septic bacterial infections, dangerous mostly for children, elderly and patients with the impaired immune system.[47, 48]

4.1. Etiology

The World Health Organization (WHO) defines diarrhoea as a passage of three or more loose or liquid stools per day, with regard to the individual routine. Usually, it appears as a symptom of bacterial (*E. coli, Vibrio* spp., *Shigella* spp.) or viral (rotavirus) gut infection, although, other causes may also occur (parasites *e.g. Entamoeba histolytica*).[49] When it comes to bacterial diarrhoea, several pathogenic strains of *E. coli* are the most common causes.[47]

4.1.1. Enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) is responsible for the majority of all moderate to severe travellers' diarrhoeal infections as well as for endemic diarrhoeas in low-income countries in both humans and animals.[48] Its main distinction from the commensal and other *E. coli* strains is the production of enterotoxins that cause water and electrolytes secretion in the gut. The involvement of ETEC in diarrhoea was first described in the 1960s in animals[50], however, the same strains were later identified also in humans, mostly in children.[51]

4.1.1.1. ETEC classification

Based on O (lipopolysaccharide) and H (flagellar) antigen, multiple serotypes of ETEC have been described. However, their prevalence, as well as the type of produced virulence factors, are strongly dependent on the locations, where they are isolated. The generally recognised virulence factors of the ETEC are enterotoxins and colonization factors (CF). Some other virulence factors have been described recently, although their exact roles are still unclear.[52] As many CFs have been described so far and many more are still expected to be uncovered, the most basic and commonly used differentiation of ETEC is based on the production of enterotoxins.

Two types of enterotoxins in ETEC have been described so far – **heat-stable (STs)** and **heat-labile (LTs)**. The majority of all ETEC strains produce ST (more than 75%, with 45% ST-only and 33% ST/LT producing) and these are found in severe diseases more often than LT-only producing strains.[52]

ST enterotoxins are small peptide molecules composed of 18-19 amino acids divided into ST-I and ST-II. In humans, only ST-I (or STa) has been discovered, although in two variants: STp and STh. The ST-II (STb) type infects only animals. The toxic effects of ST-I toxins is due to the binding to the guanylyl cyclase C receptor (GC-C) causing accumulation of cGMP, which indirectly promotes the opening of CFTR, secretion of chloride anions and indirect inhibition of the sodium-hydrogen exchanger. All of these changes are responsible for the creation of the watery diarrhoea.[52] Mechanism of action of the ST-II is unknown, however, research of Fujii et. al suggest the involvement of the Ca2+-calmodulin-dependent protein kinase II.[53]

LT enterotoxins and their mechanism of intoxication were discussed above.

4.1.1.2. Pathogenesis of ETEC caused diarrhoea

The pathogenesis of ETEC caused diarrhoea starts with orofecal transmission mostly from food or water, and is similar to cholera in many aspects, as may be seen in *Fig. 8*. This relatedness is important from both scientific and therapeutic views. Even though *E. coli* has shown resistance to the acidic pH[54], the commonly presumed infectious dose of ETEC for adults is relatively high, 10^8 CFU.[55] When ETEC reaches the small intestine, it adheres to the epithelial cells with the help of CFs, specifically recognize glycans of glycoproteins or GSLs.[52, 56] Subsequently, ETEC releases enterotoxins that bind to gangliosides or specific receptors, which causes the intoxication of the cells and secretion of water and electrolytes into the intestinal lumen by mechanisms described above. Thus a watery diarrhoea is produced.[48]

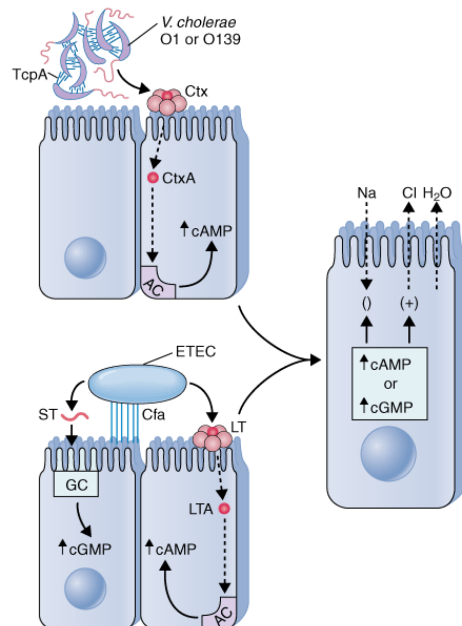

Fig. 8 Pathogenesis of ETEC diarrhoea compared to the cholera infection.
A great degree of similarity may be observed in the pathogenesis of these infections.
(AC – adenylyl cyclase, CTx – cholera toxin, TcpA – toxin co-regulated pilus A)
Downloaded from https://5minuteconsult.com/data/GbosContainer/49/m_0860.fig16.02.gif

4.2. Symptoms and consequences of *E. coli* caused diarrhoea

The symptoms of diarrhoea strongly vary depending on the etiology. However, the ETEC caused diarrhoea has usually milder symptoms. The incubation period of the infection is 1-3 days, and the disease lasts usually 3-4 days, rarely more than a week.[57] Typically, a watery diarrhoea and abdominal cramping are observed. More severe symptoms like nausea, vomiting, fever or muscle aches are less common. Escalation to dehydration and life-threatening condition due to electrolyte imbalance is rare in developed countries.[52]

Other *E. coli* pathogens responsible for travellers' or low-income countries citizens' diarrhoea vary very little in incubation periods and symptoms from ETEC. In rare cases, blood in the stools is observed in EAEC (enteroaggregative *E. coli*) and EPEC (enteropathogenic *E. coli*) infections. Only STEC (Shiga toxin-producing *E. coli*) caused infections tend to be more severe, and in some cases, they may escalate to haemorrhagic colitis or life-threatening haemolytic uremic syndrome (HUS).[52]

4.3. Treatment of diarrhoeal infections

Prevention should always be in the first place. The main suggestions on how to prevent contamination of food or water and how to stop human-to-human transmission were summarized by WHO in the publication "Five keys to safer food manual" from 2006. Personal hygiene and sufficient water and food boiling were emphasized.[58]

In general, when the prevention fails, no specific treatment is necessary. Oral rehydration solutions (ORS) for correction of fluid and electrolyte disbalance and appropriate energy diet are sufficient. For more severe cases, specific antibiotic or other treatment may be useful. However, possible side-effects as selection of resistant bacteria, increased predisposition to develop other intestinal infections and all other factors should be carefully considered.[52]

4.3.1. Specific treatment of diarrhoea

The use of antibiotics had been thoroughly studied and it had demonstrated to significantly decrease the number of water losses and to shorten the duration of the illness.[59] However, as ORS therapy is usually sufficient, administration of antibiotics is reserved for the life-threatening conditions.[57] Furthermore, choice of the drug should always follow local resistance patterns.

4.3.1.1. Antibiotics and antisecretory drugs

For the treatment of ETEC-caused diarrhoea, a growing antimicrobial resistance has become problematic. In a recent study, almost 60% of the isolates were resistant to co-trimoxazole and tetracyclines, and 50% to ampicillin with a growing number of ciprofloxacin-resistant isolates.[60] Despite these findings, ciprofloxacin followed by rifaximin and azithromycin are most commonly used.[52, 59]

In acute infectious travellers' diarrhoea, antibiotics may safely be combined with antimotility drugs like loperamide or with antisecretory drugs like bismuth-subsalicylate, or the newer zaldaride maleate and racecadotril. The latter may also be used in monotherapy for less severe cases.[59, 61]

4.3.1.2. Vaccines

The development of vaccines against ETEC had proven to be challenging due to the great variability of the ETEC strains. So far, only Dukoral® *(Valneva Sweden AB, Sweden)* is effective and marketed in the EU. This oral whole-cell cholera/recombinant CTx-B vaccine, originally developed for cholera can also protect against some ETEC strains as a result of the cross-reactivity of the secreted anti-CTx-B antibodies with CTx and LT-I.[62] However, the effectiveness of the vaccine against ETEC is low, only up to 67% (compared to 85% for cholera), and because of the mechanism involved, it can protect only against LT-I-producing strains. The duration of the protective effect is also low when compared to protection against cholera.[63] Other vaccines against ETECs are still being developed, targeting other enterotoxins as well as other virulence factors like CSs/CFs.[52, 64]

IV. MATERIALS AND METHODS

Several reagents and solutions were used for studies of LT-IIc-B binding specificity. Hereby, all the reagents and their compositions are described, divided based on the assay, where they were used:

Binding and inhibition assays:

Anisaldehyde reagent

- 1 ml 4-methoxybenzaldehyde (anisaldehyde) (*Sigma-Aldrich, USA)*
- 2 ml sulphuric acid 95-97%
- 98 ml glacial acetic acid

Resorcinol reagent

- 10 ml resorcinol solution (1 g in 50 ml of water) (*Fluka AG, Switzerland)*
- 80 ml concentrated hydrochloric acid
- 0.25 ml 0.1M $CuSO_4$

Plastic solution

- 0.5% mixture of poly(isobutyl)methacrylate in *n*-hexane-diethylether 1:5 (*V/V*) (*Sigma-Aldrich, USA*)

PBS-BSA solution

- a solution of phosphate buffer with bovine serum albumin (2% *w/V*), buffered at pH=7.2

Scintillation liquid

- ULTIMA GOLD™ solution used for better counting efficiency (*PerkinElmer Inc., USA)*

Gel electrophoresis and Western-blotting:

Sample buffer

- 500 µl NuPAGE® LDS Sample Buffer (4X) (*Life Technologies Corporation, Canada*)
- 200 µl dithiothreitol 1 mol/l
- 300 µl ultra-pure water

Transfer buffer

- 40 ml 25X Novex® Tris-Glycine Transfer Buffer
- 100 ml methanol
- 860 ml ultra-pure water

Running buffer

- 50 ml NuPAGE® MOPS SDS Running Buffer (20X) (*Life Technologies Corporation, Canada*)
- 950 ml ultra-pure water

Staining reagent

- Imperial™ Protein Stain (*Thermo Scientific, USA*)

Mass spectrometry:

Buffer A

- 10 mM NH_4HCO_3

Buffer B

- 100% acetonitrile

1. ISOTOPE LABELLING OF LT-IIc-B

Over the past years, radioiodination has become a commonly used procedure in the research of proteins and several methods have been described.[65, 66] Although working with radioactive materials has its dangers, the methods involving catalysed iodination of the proteins using the Iodogen® molecule, first described in 1978, is considered an easy, effective and relatively safe method.[67, 68]

The procedure of iodination is illustrated in *Fig. 9*. The Iodogen® reagent was obtained from *Thermo Scientific, USA*.

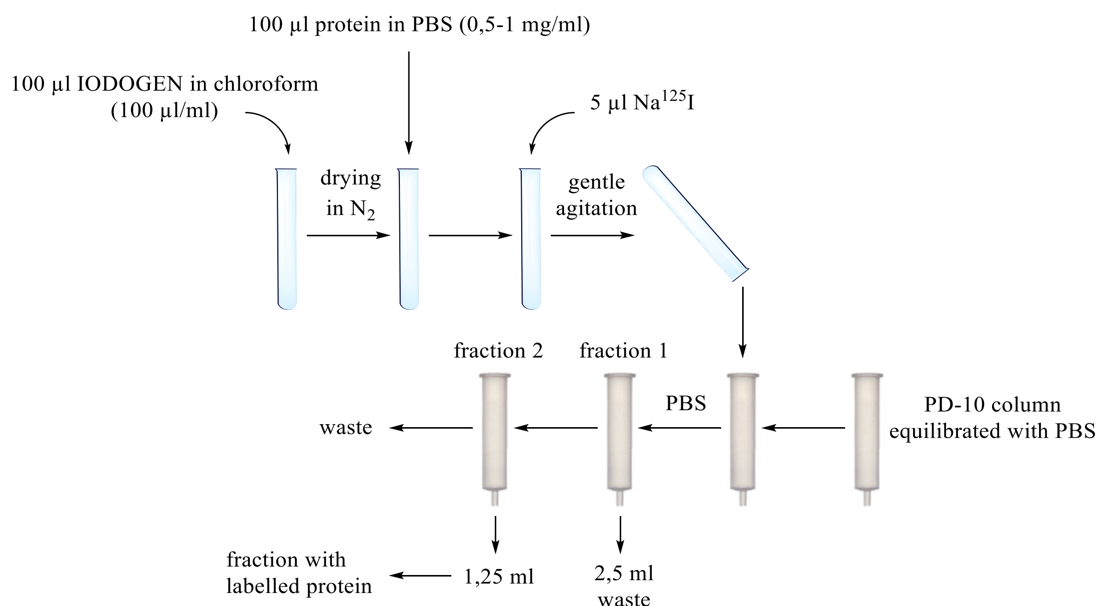

Fig. 9 Step by step illustration of iodination of the proteins using Iodogen® reagent.

1.1. Preparations for the iodination

100 µl of Iodogen® diluted in chloroform to 7.5 mg/100 ml was dried in the test tube using a flow of N_2 and thus, the catalyser was adsorbed and fixed on the walls of the test tube not to be washed out later.

1.2. Radioiodination

The main process of radioiodination was done at the *Sahlgrenska University Hospital, Gothenburg, Sweden*. When the catalyser was fixed in the test tube, 100 µl of the protein, diluted to 0.5 mg/ml in PBS was introduced, followed by 5 µl of $Na^{125}I$. The mixture was slightly agitated to ensure sufficient reaction of the components and incubated for 10 min. After this step, a PD-10 SPE-column, filled with Sephadec™ G-25M (*GE Healthcare, UK)* was saturated with PBS, and the incubated solution was gently poured on top.

1.3. Separation of the iodinated protein

By repeatedly adding PBS on the column, three main fractions were obtained. The first void fraction, 2.5 ml from the column, was discarded. The second fraction of 1.25 ml contained the labelled protein. The last fraction (free $Na^{125}I$) was discarded as radioactive waste. By this procedure, B subunits of LT-IIc (referred to as LT-IIc-B), obtained from prof. Terry Connell (*University of Buffalo, USA*), and B subunits of CTx (*List Labs, USA*) (referred to as CTx-B) were labelled and used in the binding experiments, as described below.

2. BINDING ASSAYS

The group of prof. Terry Connell has demonstrated that the LT-IIc toxin produced by ETEC bind to glycolipids GM3, GD1a, GM1, GT1b.[2] In collaboration with this group, we decided to test the binding of LT-IIc B subunits to reference GSLs previously purified and identified at the Department, and to our purified and identified GSLs from the column chromatography as described further. The binding of B subunits of LT-IIc to glycoproteins was also investigated.[2, 38]

Two types of assays were used when examining the binding of LT-IIc-B to the GSLs. The simpler and faster **HPTLC binding assay** provided only qualitative results that did not give much information about the strength of the binding, thus it was used to

define the binding of the new glycolipids. Aluminium-backed HPTLC plates *HPTLC Silica gel 60* (*Merck, Germany)* were used to get better resolution and separation on the plate due to finer layer and smaller particle sized silica gel. The more complicated **radioimmunoassay (RIA)** on the other hand, produced quantitative data, comparable among the glycolipids, hence it was used to describe the extent of the binding of the known GSLs quantitatively. Each GSL was tested multiple times, and compared to the reference in order to ensure the correctness of the results.

2.1. HPTLC binding assay

The principle of this method, described by Magnani in 1980[69], combines separation and migration of the glycolipid on the HPTLC plate and then binding of the radiolabelled B subunits of LT-IIc to the separated glycolipids. Thanks to the high specificity of ligand-receptor bonds, a very small amount of the sample is sufficient for clear results. As both glycolipids and radiolabelled LT-IIc-B are colourless, results of this assay needed to be visualised on a photographic film. Here *BIOMAX MR Film (Carestream, USA)* was used.

2.1.1. Application of the sample and elution of the plate

Firstly, the HPTLC plates (*Merck, Germany*) were pre-eluted with methanol, to ensure the purity of the plate and comparability of the results between the plate batches, and dried. Afterwards, 0.4 to 10 µg of the sample (pure glycosphingolipid or fraction from the column) was applied on the baseline of the plate with microsyringe to form a thin line about 1-1.5 cm from the bottom. The plate was eluted with chloroform-methanol-water 60:35:8 (*V/V/V)* as mobile phase and dried. The elution took about 30-35 minutes.

2.1.2. Fixation of the plate and binding of LT-IIc-B

When the mobile phase had evaporated, the plate was submerged into the plastic solution for exactly 1 minute. The plastification of the TLC plate ensured that the next steps would not destroy the silica gel on the plate.[70] The time of submersion had been optimized at the Department. Afterwards, the plate was covered with a solution of PBS-BSA for 2-2.5 hours to prevent non-specific binding of the labelled protein to the plate during the next step, thus to diminish the background.

After the fixation, the plate was covered with the solution of 50 µl of labelled LT-IIc-B diluted with 5 ml of PBS-BSA (approximately 2×10^6 cpm/ml), and incubated

for 1-1.5 hours at room temperature. After the incubation, the plate was washed six times with PBS and let to dry overnight.

2.1.3. Visualization of the binding

When the plate was dry, it was superimposed with a photographic film. The γ radiation from ^{125}I bound to B subunits of LT-IIc would change the local composition of the photographic layer and thus allow to see whether the binding took place or not. After a sufficient time, which we found to be around 4 hours, the photographic film was developed using a *Kodak® X-Omat 1000 Processor* developing machine.

2.2. Radioimmunoassay

Radioimmunoassay (RIA) is a very sensitive method, invented by Rosalyn Yalow and Solomon Berson during their study of insulin in the 1960s, commonly used when investigating biological molecules like hormones or antibodies and their abilities to selectively bind to a receptor or an antigen.[71]

2.2.1. Preparation of the assay

Because the original solvent of the samples contained chloroform (2:1 chloroform-methanol *V/V*) that would interfere with the plastic plates used during the assay, all the samples were dried using flow of N_2 and dissolved in pure methanol. Then, 50 μl of serial dilutions of GSLs (each dilution in triplicate) were pipetted into the wells of the microtiter plates and left to dry overnight. The first three wells were always used as a background (methanol only).

2.2.2. Binding of radiolabelled protein

When all the wells were dry, 200 μl of PBS-BSA was pipetted to each and left to incubate for 2 hours at room temperature in order to diminish the background. Afterwards, the solution of PBS-BSA was discarded and replaced with 50 μl of the new solution containing 1 μl of radiolabelled LT-IIc-B or CTx-B in 50 μl of PBS-BSA (2×10^6 cpm/ml for both proteins) for each well. After incubation for 4 hours, the microtiter plate was washed 6 times with PBS to ensure the elimination of all unbound B subunits and left to dry overnight.

2.2.3. Counting of γ radiation

When the plate was ready, each well was transferred to a specifically designed vial filled with 3 ml of scintillation liquid. The counting was done using a *Liquid Scintillation Analyzer Tri-Carb® 2810 TR, PerkinElmer, USA* (γ-counter).

3. GLYCOPROTEINS BINDING ASSAYS

For further investigation of LT-IIc B subunits binding specificities, the sialylated and non-sialylated (asialo) form of the glycoprotein fetuin was used. Fetuin is a major glycoprotein present in cattle and sheep fetal serum and it is a homologue of human α2-HS-glycoprotein that possesses many physiological functions *e.g.* a tyrosine-kinase insulin receptor inhibitor.[72] As a result of multiple possible carbohydrate chains described in 1979[73] its structure is variable and multiple bands may be observed after gel electrophoresis.

3.1. HPTLC binding assay of glycoproteins

At first, the potential binding of LT-IIc-B to fetuin and asialofetuin was tested on the HPTLC plates. The binding assay with glycoproteins was done following the same steps as glycolipids, but as proteins do not migrate on an HPTLC plate, the whole TLC chromatography step was omitted. The results were considered as preliminary, and the experiment was repeated later using gel electrophoresis and Western blotting technique.

Three sets of concentrations for sialo and asialofetuin (*Sigma-Aldrich, USA)* were prepared: 10, 1 and 0.1 mg/ml diluted with PBS. 2 μl of each solution was dotted on HPTLC plate and left to dry overnight. When the plate was dry, the process continued as described above, starting from *Fixation of the plate*.

3.2. Western blotting

Western blotting is a technique used in the analysis of protein binding capabilities described by Towbin in 1979[74] and it is usually coupled with the gel electrophoresis.[75]

3.2.1. SDS-PAGE

The process of SDS-PAGE (dodecyl sulfate polyacrylamide gel electrophoresis) is the most used electrophoretic technique in protein experiments, nowadays. The

principle is based on migration and separation of the charged proteins in the pores of polyacrylamide gel in the strong electric field, based solely on their molecular weight.[76] This is due to anionic detergent SDS (sodium dodecyl sulfate), which denatures the proteins and forms big micellar structures with them, giving them a negative charge proportional to their molecular weight. A reducing agent like dithiothreitol (DTT) is often added during the preparation of the sample to cleave disulphide bonds to further separate potential subunits of the complex proteins.[77]

The gel electrophoresis is always buffered, ensuring the optimal conditions for the separation. Mainly pH and ionic strength of the solutions are important as they influence the solubility of the proteins and thus efficacy of the separation.[78]

3.2.1.1. Gel electrophoresis of the samples

Two different sets of dilutions of the proteins were used. For the first assay, we used the same concentration as for HPTLC binding: 10, 1 and 0.1 mg/ml. However, because none of these concentrations produced satisfactory results, a new set of concentrations: 1, 2.5 and 5 mg/ml was prepared and used for the binding assay. 12 µl of the protein sample was mixed with 12 µl of the sample buffer. The mixture was heated at 95°C for 5 minutes, centrifugated for a few seconds and loaded on *nuPAGE™ 4-12% Bis-Tris gels (Life Technologies Corporation, Canada)*. Into the first well of the gel plate was pipetted 5 µl of the ladder *Precision Plus Protein™ Dual Color (Bio-Rad Laboratories, Inc., Germany).*

After loading the samples, the gel plate was inserted into the electrophoretic cell filled with the running buffer and ran using 150 V for 90 minutes.

3.2.1.2. Imperial staining

Staining was done according to the original protocol from the manufacturer as follows. After the separation, the gel was washed three times with ultra-pure water, covered with sufficient amount of the Imperial staining reagent, and left for one hour while slightly agitated. After one hour, Imperial staining reagent was discarded and the gel was being washed with ultra-pure water, until the staining was acceptably strong, usually for 1 hour.

3.2.2. Principle of Western blotting

Western blotting is based on the electroblotting principle. It uses either natural charge or the charge made by SDS during SDS-PAGE to transfer proteins from the gel to

the suitable membrane in the electric field. Multiple methods have been described. The **wet** method that was used during our research, uses a soaked "sandwich" containing gel and the membrane, which is put in a tank filled with transfer buffer. As a result of the design used by Towbin, the wet technique is done vertically and it uses stronger voltages than the other two. The **semi-dry** method, usually done horizontally, uses different electrodes and no buffer-filled tank is required. For the blotting, only the "sandwich" is soaked in the transfer buffer. [75, 79] The last, **dry** method using *iBlot® Dry Blotting System* from *Life technologies, USA* is the newest and fastest blotting method that does not require any transfer buffer and is done using specific gel blotting system.[80]

3.2.2.1. Blotting of the gel

The gel obtained from SDS-PAGE was placed on top of the membrane in so-called "transfer sandwich", a multilayer blotting structure consisting of 3 types of layers: (1) **porous polyethylene sheet (sponge)**, (2) **filtration paper** and (3) **0.2 µm nitrocellulose membrane** *(Bio-Rad Laboratories, Inc., Germany)* with the superimposed gel (*Fig. 10*). Then the layers (1) and (2) were repeated vice-versa so that the polyethylene sheet was on top. We used 3 polyethylene sponges on the bottom and two on top with 1 filtration paper on each side. Every component of the "sandwich" was prewetted with transfer buffer to avoid the formation of the air bubbles and destruction of the results. Subsequently, the sandwich was placed between the electrodes of the blot module with the membrane facing the anode (+). The fixed blot module was inserted into the blotting cell and covered with transfer buffer. The cell was maintained under a constant voltage of 30 V for 1.5 hours. At the end, the membrane was taken out and left to dry.

3.2.2.2. Binding of LT-IIc-B

The dried membrane from Western blotting was covered with a solution of radiolabelled LT-IIc-B in 2% PBS-BSA 0.1% Tween (approx. 2×10^6 cpm/ml) for 3 hours or overnight. When the time had elapsed, the membrane was washed 6 times with PBS, dried out, fixed in the photographic box and overlaid with photographic film for 6 hours, similarly to HPTLC binding assay.

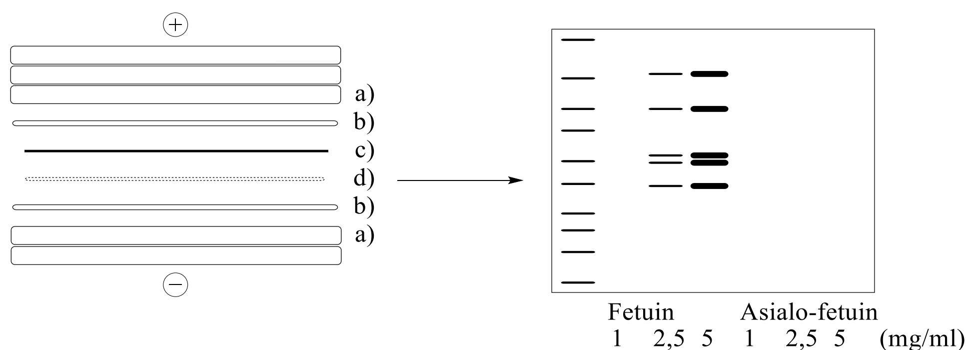

Fig. 10 Blotting sandwich layers used for Western blotting and illustration of the obtained membrane with bound LT-IIc-B.*(a - polyethylene sponges, b - filtration paper, c - nitrocellulose membrane, d - gel)*

4. INHIBITION ASSAYS

We attempted to investigate the possibility of interactions between LT-IIc-B and pure sugar fragments, not attached to the ceramide. The main purpose was to inhibit the binding of B subunits to the glycosphingolipids. As for the binding assay, HPTLC was very useful for its simplicity, although, RIA was later used as a quantitative method.

4.1. Inhibition assays using HPTLC and RIA

Before the assays, three inhibitory solutions were prepared and incubated at room temperature for 2 hours:

- **solution A** containing 50 µl of radiolabelled LT-IIc-B and 50 µl of sialic acid 100mM (Neu5Ac)
- **solution B** containing 50 µl of radiolabelled LT-IIc-B and 50 µl of Neu5Acα3-lactosamine (Neu5Acα3Galβ4GlcNAc) 100mM
- **solution C** containing 50 µl of radiolabelled LT-IIc-B and 50 µl of PBS (blank)

TLC plates and microtiter wells were both prepared and blocked as described above under respective binding assays. Solutions A, B and C were diluted to 5 ml and used for binding on TLC plates and microtiter wells.

5. SEPARATION AND PURIFICATION OF GSLs

Even though the emergence of new and automated separation techniques, like preparative high-performance liquid chromatography (HPLC), has allowed fast and efficient separation and purification of the samples obtained from biological sources, many of their positives are outweighed by the necessity of specialised equipment or sometimes continuous loss of small amounts of the analysed sample. This becomes problematic during the analysis of the biomolecules, like glycosphingolipids that are often present in the sample in very small amounts.[81, 82]

Thus, in separation and purification of glycosphingolipids, old-fashioned column chromatography has still its rightful place and thanks to its simplicity and ability to produce very pure fractions with minimal loses during the process it is still used.

5.1. Column chromatography

The principle of the column chromatography is very simple. The components in the mixture are separated from each other on the column filled with suitable stationary phase while suitable mobile phase is added. Therefore, the amounts of added mobile phase define the volumes of the obtained fractions and as a consequence, their purity.

Four basic separation mechanisms apply. The first and the most common is **adsorption**, where the separation occurs due to nonspecific surface adsorption to the solid stationary phase while the components are carried in liquid or gas mobile phase. Other mechanisms involve **partition** that separates the components based on their solubility in liquid or gaseous mobile phase and immobilised immiscible liquid stationary phase, **ion-exchange**, separating the components based on the ionic interactions between the stationary and mobile phase and lastly, **size-exclusion** that separates the components based on their size.[83]

Column chromatography may be further defined based on the polarity of the phases. In **normal phase** chromatography, a non-polar mobile phase and a polar stationary phase are used, in **reverse phase,** it is vice-versa. Depending on the state of the mobile phase, column chromatography may be **liquid** (LC) or **gaseous** (GC).[83]

The balance between retention exerted by the stationary phase and affinity of the components to the mobile phase determines the separation efficacy and subsequently the purity of the fractions.[83, 84]

In general, hydrophilic substances are more soluble in polar phases (*e.g.* water, salt solutions) and lipophilic in non-polar (*e.g.* chloroform). As glycolipids are amphiphilic, contain both polar (sugar chain) and non-polar part (ceramide), choosing the right polarity of the mobile and stationary phase is crucial for optimal conditions in the chromatographic system.

5.1.1. Preparation of the sample

The starting material was approximately 50 mg of a complex ganglioside sub-fraction extracted from moose kidney. A preliminary LC-ESI/MS analysis had indicated the presence of disialoglobopentaosylceramide (Neu5Acα3Galβ3(Neu5Acα6) GalNAcβ3Galα4Galβ4GlcCer) in this material. The intention was to isolate this ganglioside from the sample, purify it and use for further testing of LT-IIc-B binding.

5.1.2. Preparation of the column for glycolipid separation

As a stationary phase, 10 g of non-modified silica gel Iatrobeads® (*Iatron Laboratories Inc., Japan*) was used. This unique type of unmodified silica gel has been extensively used in the chromatographic analysis of glycolipids as it possesses high separation capabilities due to small, homogenous, round and highly porous particles (average size 60 μm).[85]

As a mobile phase, a mixture of chloroform-methanol-water 60:35:8 (*V/V/V*) was used. Before the introduction of the sample, the column filled with Iatrobeads® was saturated with the mobile phase.

5.1.3. Separation of the glycolipids in the sample

When the column was prepared as described above, the sample containing the mixture of glycosphingolipids was gently poured on top, and by repeatedly adding 1 ml of the mobile phase and taking eluted fractions, we obtained 50 fractions of 1 ml. At the end, the column was washed out with 50 ml of mobile phase to ensure all the glycolipids from the sample were eluted.

5.2. Thin-layer chromatography (TLC)

Even though TLC is considered an easy and unreliable method for identification of unknown compounds, it becomes a very fast and satisfactory method for estimation of fractions content when used with specific staining procedures alongside a references.[86]

To get better resolution and separation on the plate, we have been using glass-backed plates *HPTLC Silica gel 60* (*Merck, Germany)*, which compared to usual TLC plates have finer layer and smaller particle sized silica gel, resulting in better separation.

5.2.1. Preparation of the fractions for TLC

The fractions collected in the test tubes were dried using N_2 on a heat-block at 50°C. When fully dried, 200 µl of chloroform-methanol 2:1 (*V/V*) was pipetted into the test tube. 4 µl of each dissolved fraction was then applied on HPTLC plate with a microsyringe to form small lines about 1-1.5 cm from the bottom. The plate was put inside the chromatography tank saturated with the same mobile phase as used on the column. Elution of the plate took about 20-25 minutes.

5.2.2. Detection of the compounds

Detection of the migrated compounds was done on a dried plate by staining it with the anisaldehyde or resorcinol reagent and shortly heating in the oven at 200°C. These reagents, or ones with analogical compositions, are often used for detection of glycolipids. The anisaldehyde reagent reacts with all types of glycolipids giving green colour for carbohydrate-containing compounds and blue to bluish colour for others, *e.g.* phospholipids or fatty acids. The resorcinol reagent is specific to gangliosides as it also requires the presence of sialic acid for its reaction.[86]

When all fractions were analysed as described above, fractions were pooled together according to the TLC migration, weighed and diluted with chloroform-methanol 2:1 (*V/V*) to 2 mg/ml and underwent the same TLC detection of purity. In this way, we obtained 7 samples. The fractions were analysed by mass spectrometry as will be discussed further.

5.3. Second column chromatography

By MS analysis of the fractions obtained above, we found that two out of the seven fractions (fraction 6 and 7) contained the target glycosphingolipid, but also other glycosphingolipids. The two fractions were pooled and submitted to a second chromatographic separation. We used the same method as described above. This time, however, the amount of added mobile phase was lowered to obtain purer fractions. As a result, we obtained 135 fractions of 0.5 ml.

After pooling together those fractions that migrated at the same level on the TLC, we obtained 10 new samples. These were weighed and diluted with 2:1 chloroform-methanol (*V/V*) to 1 mg/ml and analysed by LC-ESI/MS/MS.

6. MASS SPECTROMETRY

Mass spectrometry (MS) is one of the most important tools in biomolecular research. The principle is to differentiate the molecules based on their mass/charge ratio (*m/z*), or *m/z* of their fragments, depending on the composition of the sample. This is achieved by volatilization and ionization of the molecules/fragments, their separation in mass analyzer and detection of the ions in strict vacuum afterwards. The ionization creates smaller ions/fragments or so-called molecular ions with the same molecular weight (M) as the original compound, created by losing electrons. In case of smaller molecules, M of the molecular ion (noted M^+) is roughly the same as M of original compound, for big macromolecules, several electrons may be lost, which creates proportionally smaller *m/z* molecular ions (noted M^{n+}).[87]

In general, ionization methods are categorized as soft or hard, depending on their strength to fragmentize the molecule. **Hard ionization techniques**, such as electron ionization (EI) or chemical ionization (CI) are known to produce a high degree of fragmentation, however, they rarely produce enough molecular ions. **Soft ionization techniques** such as fast atom bombardment (FAB) or electrospray ionization (ESI) carry less energy, therefore their fragmentation is milder and molecular ions are usually observed. These methods combined give ideal structural data.[87]

After the ionization of the molecule, the ions are separated and analysed based on their *m/z* ratio, according to the physical property monitored by the analyzer *e.g.* kinetic energy, resonance frequency etc. All analyzers use an electric and/or magnetic field to achieve the separation of the ions. Nowadays, the most used analyzers are quadrupole, quadrupole ion-trap (QIT), time-of-flight (TOF), Orbitrap and others.[88] When the ions are separated based on their *m/z* ratio, each of them is detected on the detector in form of electric charge proportional to its abundance.[88]

Additionally, multiple-step analysis, so-called **tandem MS, MS/MS** or **MS^n** are commonly used to automatically analyse the most abundant ions based on specified criteria and thereby obtain more detailed structural data using one sample. The ions are separated in the mass analyser and undergo further fragmenting by collision-induced

dissociation (CID) or other techniques. Newly formed ions are analysed and detected as before based on their *m/z*.[87]

For analysis of the mixtures, mass spectrometry may be coupled with chromatography, giving GC-MS or LC-MS. While the LC/GC separates the components from the mixture**,** the MS analyses every component with a high degree of precision and sensitivity, giving unparalleled results.[87, 88]

6.1. Mass spectrometry for glycosphingolipid analyses

The development of MS in glycosphingolipidomics is tightly linked to the development of the MS in other fields. The first GSL mass spectra were obtained using rather high-energy EI mass spectrometry of derivatized, mainly permethylated and permethylated and reduced GSLs. This was necessary to ensure heat-stability of the analysed GSL.[89, 90] Even though these methods were accurate at that time, elucidation of the GSL structure was complicated, involving multiple-step derivatization and purification processes.

A major breakthrough in GSL analysis came after more than 10 years after these first analyses with the arrival of the softer FAB ionization methods in the 1980s, which finally allowed MS of an underivatized glycosphingolipid.[91] Further development of MS in the late 90s, notably coupling ESI-MS with LC has presented an easier and more sophisticated method for GSL structural analyses, and they have taken the elucidation of GSL presence and determination of their structure in mixtures to the new levels.[92]

Two basic types of GSL analysis are possible. The first analyses the GSL as a whole molecule. This method is preferred for its simplicity and because it gives the data for carbohydrate part as well as for the ceramide part. The second method uses only the glycan part of the GSL and requires complicated sample preparation, therefore it is used mostly for analysis of new, unknown GSLs, or when the first method fails.

The ion fragments observed in the mass spectra of glycosphingolipids are named according to the systematic nomenclature proposed for glycoconjugates by Domon and Costello in 1988 as seen in *Fig. 11*.[93]

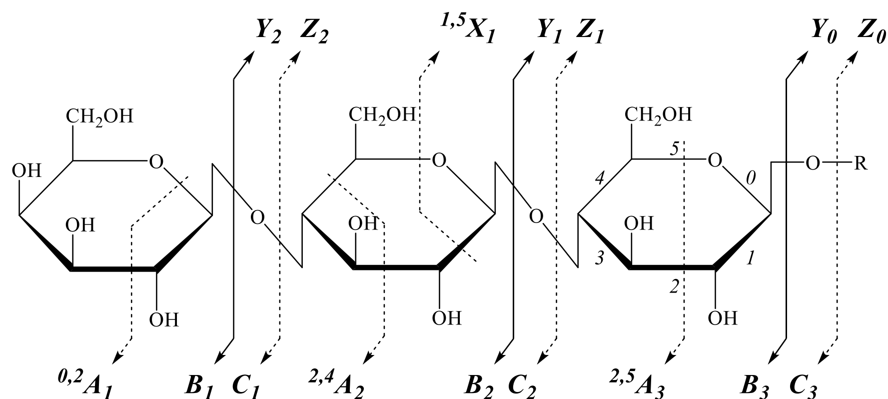

Fig. 11 Nomenclature of the carbohydrate fragmentations in mass spectrometry.
Adapted and modified after Domon and Costello [93]

6.1.1. Analysis of intact glycosphingolipids

For the analysis of intact glycosphingolipid, we used HILIC-based LC-MS/MS using *Agilent 1100 Series* HPLC (*Agilent, USA*) and column made of *fused silica capillary* (*Scandinavian Genetec AB, Sweden*) with inner diameter 250 µm × 10 cm, filled with *Polyamine II* (*ScantecLab AB, Sweden*), a modified silica gel with particle size of 5µm. HILIC, or hydrophilic interaction chromatography, is a variant of normal phase chromatography described by Andrew Alpert in 1990, which found use in chromatographic separation of polar biomolecules like nucleic acid, proteins or carbohydrates.[94] Mass spectrometry was done on a *Finnigan LTQ mass spectrometer (Thermo Electron Corporation, USA)* with an ion-trap analyser, ESI ionization and CID fragmentation for MS/MS.

6.1.1.1. Preparation of the sample

Firstly, an amount of the GSL solution containing 30 µg of the sample was pipetted into a small vial and fully dried in the heat block at 50°C. Then, the dried sample was dissolved in a mixture of acetonitrile-methanol 3:1 (*V/V*) and inserted into the auto-sampler of the LC-MS/MS. The whole LC-MS/MS system was equilibrated for 10 minutes using buffer A (100-120 bar), followed by equilibration with buffer B (50-60 bar) for 20 minutes. Measurements of *m/z* values were set to 600-2000.

6.1.2. Analysis of the carbohydrate part

Analysis of the carbohydrate part is the next method in GSL analysis. After the enzymatic hydrolysis of the ceramide with recombinant endoglycoceramidase II (rEGCase II), the carbohydrate is more easily characterized, than with the method using

whole GSLs. However, enzymatic hydrolysis requires specific conditions and takes longer time. The same LC-MS/MS system was used, although, the capillary making the column (same size) was filled with *Hypercarb (Shandon HPLC, UK)* with 5µm particle size. Hypercarb or porous graphitic carbon columns are commonly used in glycomics MS analysis for their high sensitivity and robustness.[95] Furthermore, graphitic carbon columns give a resolution of isomeric oligosaccharides, and the carbohydrate sequence can be deduced from series of C-type fragment ions obtained by MS^2.[96] Additionally, differentiation of linkage positions is possible since MS^2 spectra of oligosaccharides with Hex or HexNAc substituted at C4 have diagnostic cross-ring ^{0,2}A-type fragment ions.[96]

6.1.2.1. Preparation of the sample

Firstly, the glycolipid solution was transferred to a glass tube and dried under N_2. Then, 70 µl of water, 10 µl of sodium-acetate buffer (50 mM, pH=5) and 10 µl of sodium-cholate (12 mg/ml) was added, and the solution was briefly sonicated. After the that, 10 µl of *rEGCase II (Takara Bio Inc., Japan)* was pipetted into the solution, and the solution was incubated at 37°C for two days. After the incubation, 0.9 ml of water and 4 ml of chloroform-methanol 2:1 (*V/V*) was added to the mixture and left overnight (Folch partition for lipid separation[97]). The upper phase was taken out and dried using N_2. Afterwards, the sample was desalted. 30 µl of water was added to the dried sample a briefly sonicated. In the meantime, *Sep-Pak Accell Plus QMA Plus Short Cartridge* (*Waters, USA*) was washed twice with 0.8 ml of methanol, followed by washing twice with 0.8 ml of water. The sample was then transferred to the cartridge, pushed with approx. 0.15 ml of air so 1-2 drops went to the fresh sample tube. Then, 50 µl of water was pipetted into the sample tube, vortexed briefly and transferred back to the cartridge. Then, some of the air from the cartridge was taken out, and 0.8 ml of water was injected into the cartridge to elute the sample. Finally, the eluate was dried using N_2, dissolved in 50 µl of water and analysed.

V. RESULTS

1. BINDING OF LT-IIc-B TO REFERENCE GLYCOLIPIDS

Initially, a number of reference GSLs were tested for binding of B subunits of LT-IIc in the chromatogram binding assay. In the first assays, relatively high amounts of GSLs (2-4 μg) were applied on the chromatograms and then the B subunits bound to almost all GSLs in an unspecific manner. We then lowered the amounts applied to less than 1 μg, to be able to discern if there was a preferential binding to some GSLs. Using these assay conditions we found, that LT-IIc-B bound only to gangliosides (*Fig. 12* to *Fig. 14*), whereas no binding to sulfated or neutral GSLs was obtained (*Fig. 15*). Thereafter, the appearance of a band at a concentration lower or equal to 1 μg was considered as a positive binding. Binding-active GSLs according to the TLC chromatogram binding assays are summarized in *Tab. 1,* and non-binding glycolipids in *Tab. 2*. The relative binding strength at 300 ng obtained by RIA are presented in *Tab. 3*.

Thus, as reported previously[2] the LT-IIc-B bound to the ganglio-series gangliosides *e.g.* Neu5AcGD1a and Neu5AcGT1b (see *Tab. 4* for ganglioside structures). In addition, we obtained binding to a number of neolacto-series gangliosides (*e.g.* Neu5Acα3nLc$_4$, Neu5Gcα3nLc$_4$, Neu5Gcα3nLc$_6$, Neu5Ac-G-10), demonstrating that the B subunits of LT-IIc binds to both terminal Neu5Ac(Gc)α3Galβ3GalNAc and Neu5Ac(Gc)α3Galβ4GlcNAc sequences, with slightly more preference to Neu5Ac as seen in *Fig. 18*. In addition, the sequence Neu5Ac(Gc)α3Gal seems to be recognized quite well (Neu5AcGM3, Neu5GcGM3 in *Fig. 13* and later in *Fig. 18*).

No binding of the B subunits to the derivatives of Neu5Gc-GM3, where the carboxylic acid of Neu5Gc has been converted to a methylamide (CH_3NH-Neu5GcGM3) and ethylamide (C_2H_5NH-Neu5GcGM3) occurred (*Fig. 13*), demonstrating that the carboxylic acid group of the sialic acid is involved in the protein-carbohydrate interactions.

No binding to gangliosides with a disialo motif (Neu5Acα8Neu5Ac or Neu5Gcα8Neu5Gc), as the Neu5AcGD3 or Neu5AcGD1b gangliosides, was obtained, and there was no binding to the ganglioside with terminal Neu5Acα6Galβ4GlcNAc as on Neu5Acα6nLc$_6$Cer (see *Tab. 2*). Furthermore, gangliosides with an internal sialic acid (Neu5AcGM1 and Neu5AcGD1b) were not recognized.

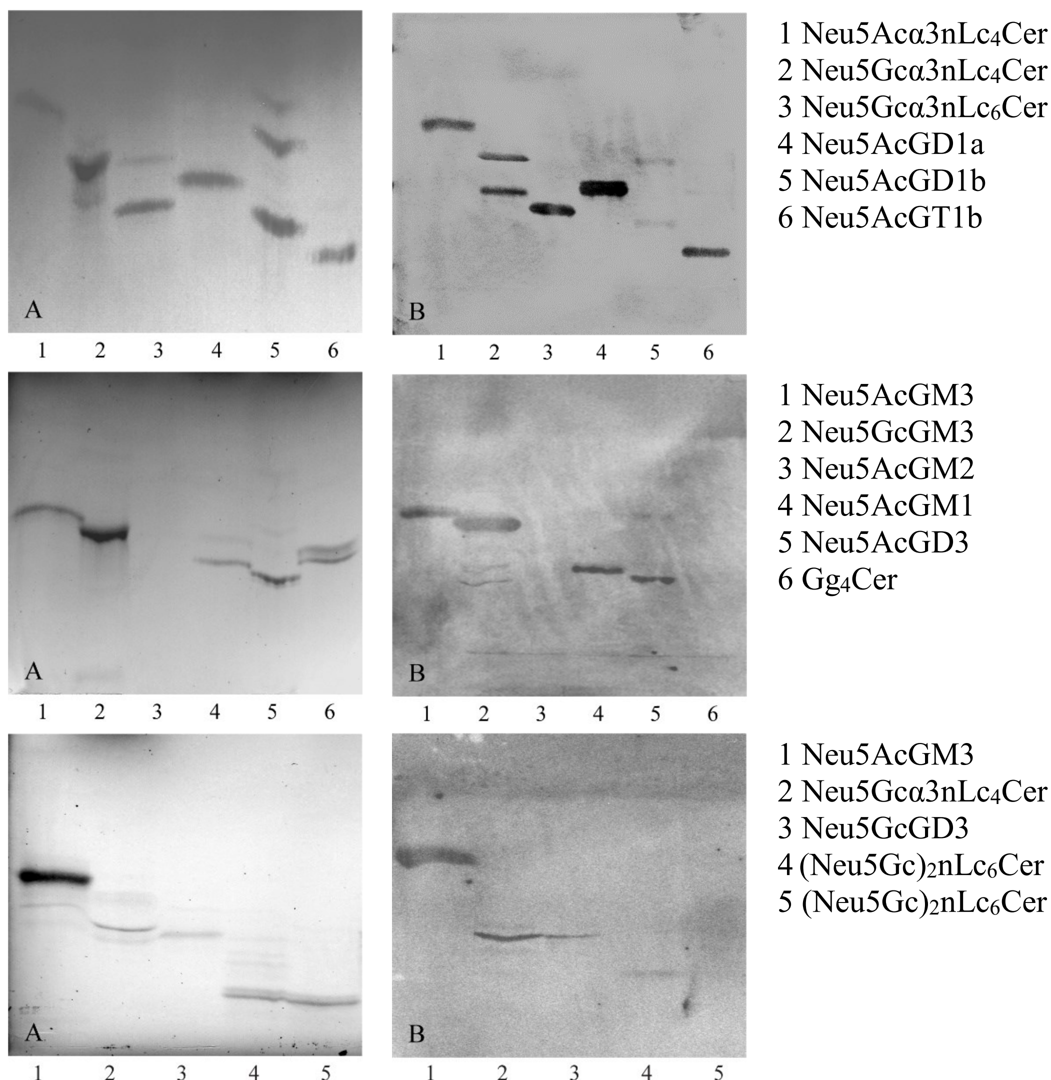

Fig. 12 LT-IIc-B binding to gangliosides. *On the chromatograms (A), higher concentrations (1.6 µg to 2 µg) were used to allow visualization by anisaldehyde staining. The binding experiments in (B) were done using 0.4 µg to 2 µg of GSL.*

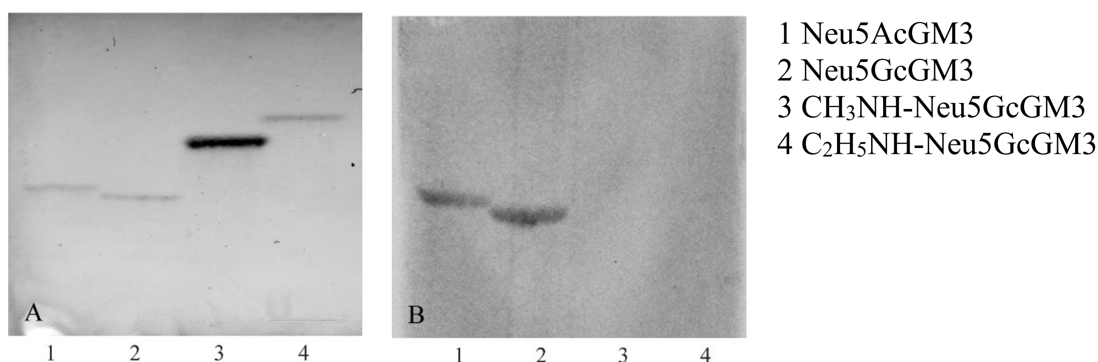

Fig. 13 No binding of LT-IIc-B to derivatives of ganglioside Neu5GcGM3.
0.8 µg of Neu5AcGM3 and Neu5GcGM3 was used for anisaldehyde staining (A) as well as for the binding assay (B). The derivatives were applied in amounts of 4 µg.

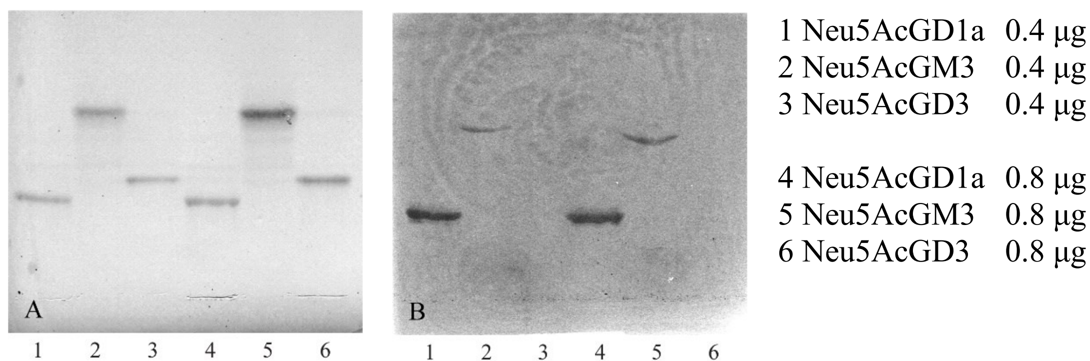

Fig. 14 Comparison of binding of LT-IIc-B to the gangliosides Neu5AcGD1a, Neu5AcGM3 and Neu5AcGD3 at different concentrations.
TLC plate stained with anisaldehyde (A) and a plate incubated with LT-IIc-B (B). The amounts of GSLs were 0.4 µg for lanes 1-3 and 0.8 µg for lanes 4-6 on both chromatograms.

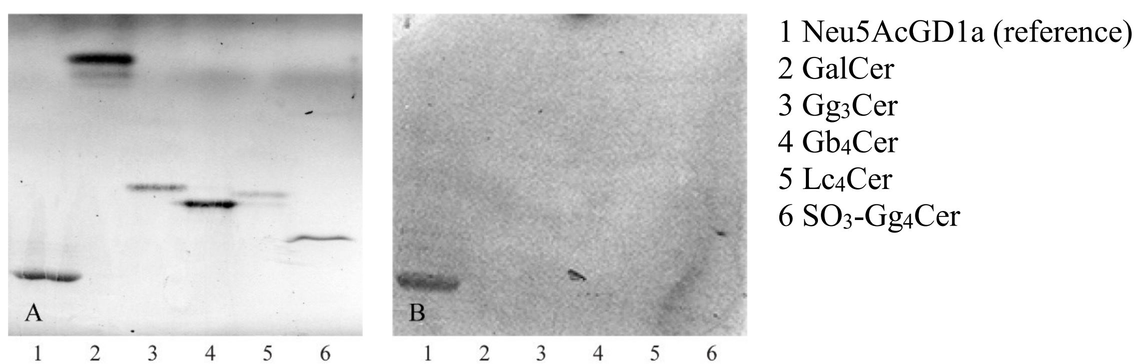

Fig. 15 The absence of binding of LT-IIc-B to non-acid glycosphingolipids.
TLC plates stained with anisaldehyde reagent (A) and incubated with LT-IIc-B (B), containing 0.8 µg of each glycolipid.

The relative binding strength cannot be told from the TLC binding experiments. Therefore, binding strength was further investigated by binding to GSLs in microtiter wells in a RIA. Results of these experiments are presented in *Fig. 16*.

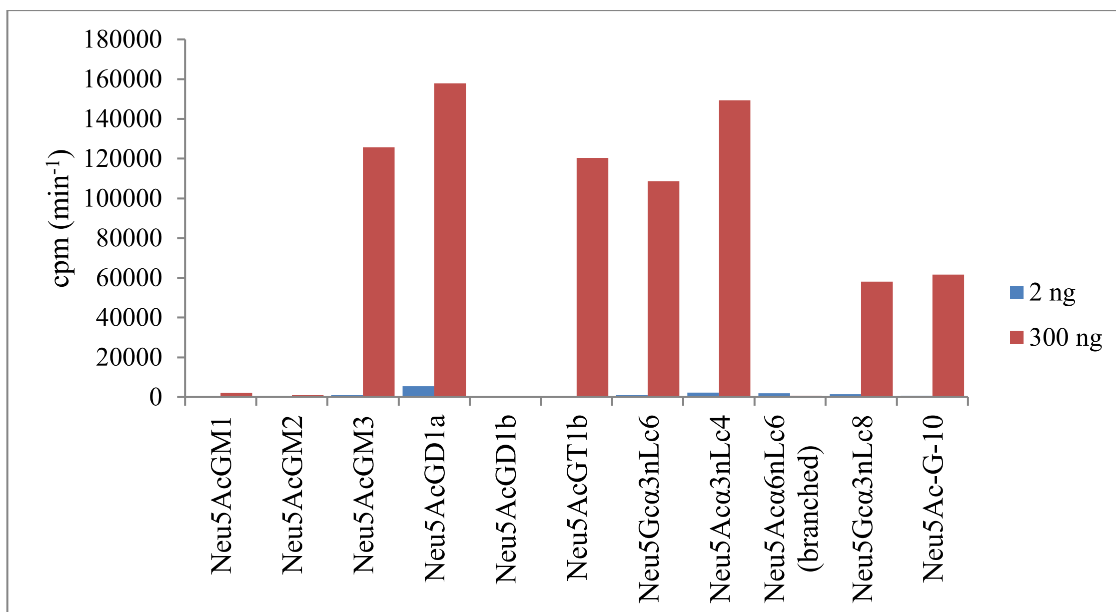

Fig. 16 Summary of the binding strength. *Graphic representation of relative binding strengths of LT-IIc-B to several GSLs at 2 ng and 300 ng.*

Of all gangliosides tested at 300 ng, Neu5AcGD1a showed the strongest binding, followed by Neu5Acα3nLc$_4$Cer, Neu5AcGM3, Neu5AcGT1b and Neu5Gcα3nLc$_8$Cer.

The following set of tables contains results from all binding assays with structures of the glycolipids and a summary of the relative binding strength obtained for several glycolipids at 300 ng. The exact concentration of the B subunits was not determined, however, the radioactivity of the solution used for the assay was 2×10^6 cpm/ml.

	STRUCTURE
Neu5AcGM3	Neu5Acα3Galβ4GlcCer
Neu5GcGM3	Neu5Gcα3Galβ4GlcCer
Neu5Acα3nLc$_4$Cer	Neu5Acα3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5Gcα3nLc$_4$Cer	Neu5Gcα3Galβ4GlcNAcβ4Galβ4GlcCer
Neu5AcGD1a	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer
Neu5Gcα3nLc$_6$Cer	Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5AcGT1b	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4GlcCer
Neu5Gcα3nLc$_8$Cer	Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5Ac-G-10	Neu5Acα3Galβ4GlcNAcβ3(Neu5Acα3Galβ4GlcNAcβ6)Galβ4GlcNAcβ3Galβ4 GlcCer

Tab. 1 LT-IIc-B binding GSLs. *Underlined section of the molecule demonstrates probable binding sequence.*

	STRUCTURE
GalCer	Galβ4Cer
CH_3NH-Neu5GcGM3	CH_3NHCO-Neu5Gcα3Galβ4GlcCer
C_2H_5NH-Neu5GcGM3	C_2H_5NHCO-Neu5Gcα3Galβ4GlcCer
Gg_3Cer	GalNAcβ4Galβ4GlcCer
Neu5AcGD3	Neu5Acα8Neu5Acα3Galβ4GlcCer
Neu5AcGM2	GalNAcβ4(Neu5Acα3)Galβ4GlcCer
Gg_4Cer	Galβ3GalNAcβ4Galβ4GlcCer
Neu5GcGD3	Neu5Gcα8Neu5Gcα3Galβ4GlcCer
Gb_4Cer	GalNAcβ3Galα4GalβGlcCer
Lc_4Cer	Galβ3GlcNAcβ3Galβ4GlcCer
SO3-Gg_4Cer	SO_3-Galβ3GalNAcβ4Galβ4GlcCer
Neu5AcGM1	Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer
Neu5AcGD1b	Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4GlcCer
$(Neu5Gc)_2$nLc_4Cer	Neu5Gcα8Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5Acα6nLc_6Cer (branched)	Galβ4GlcNAcβ6(Neu5Acα6Galβ4GlcNAcβ3)Galβ4GlcCer
$(Neu5Gc)_2$nLc_6Cer	Neu5Gcα8Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcCer

Tab. 2 LT-IIc-B non-binding glycolipids.

	BINDING STRENGTH
Neu5AcGD1a	100%
Neu5Acα3nLc_4Cer	95%
Neu5AcGM3	80%
Neu5AcGT1b	76%
Neu5Gcα3nLc_6Cer	69%
Neu5Ac-G-10	39%
Neu5Gcα3nLc_8Cer	37%
Neu5AcGM1	1%
Neu5AcGM2	1%
Neu5Acα6nLc_6Cer (branched)	0%
Neu5AcGD1b	0%

Tab. 3 Estimated relative binding strength compared to Neu5AcGD1a. *All GSLs were tested at 300 ng.*

	STRUCTURE
GalCer	Galβ4Cer
Neu5AcGM3	Neu5Acα3Galβ4GlcCer
Neu5GcGM3	Neu5Gcα3Galβ4GlcCer
CH_3NH-Neu5GcGM3	CH_3NHCO-Neu5Gcα3Galβ4GlcCer
C_2H_5NH-Neu5GcGM3	C_2H_5NHCO-Neu5Gcα3Galβ4GlcCer
Gg_3Cer	GalNAcβ4Galβ4GlcCer
Neu5AcGD3	Neu5Acα8Neu5Acα3Galβ4GlcCer
Neu5AcGM2	GalNAcβ4(Neu5Acα3)Galβ4GlcCer
Gg_4Cer	Galβ3GalNAcβ4Galβ4GlcCer
Neu5GcGD3	Neu5Gcα8Neu5Gcα3Galβ4GlcCer
Gb_4Cer	GalNAcβ3Galα4Galβ4GlcCer
Lc_4Cer	Galβ3GlcNAcβ3Galβ4GlcCer
SO_3-Gg_4Cer	SO_3-Galβ3GalNAcβ4Galβ4GlcCer
Neu5Acα3nLc_4Cer	Neu5Acα3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5AcGM1	Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer
Neu5Gcα3nLc_4Cer	Neu5Gcα3Galβ4GlcNAcβ4Galβ4GlcCer
Neu5AcGD1a	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer
Neu5AcGD1b	Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4GlcCer
$(Neu5Gc)_2$nLc_4Cer	Neu5Gcα8Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5Gcα3nLc_6Cer	Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5AcGT1b	Neu5Acα3Galβ3GalNAcβ4(Neu5Acα8Neu5Acα3)Galβ4GlcCer
Neu5Acα6nLc_6 (branched)	Galβ4GlcNAcβ6(Neu5Acα6Galβ4GlcNAcβ3)Galβ4GlcCer
$(Neu5Gc)_2$nLc_6Cer	Neu5Gcα8Neu5Gcα3 Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5Gcα3nLc_8Cer	Neu5Gcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcCer
Neu5Ac-G-10	Neu5Acα3Galβ4GlcNAcβ3(Neu5Acα3Galβ4GlcNAcβ6)Galβ4GlcNAcβ3 Galβ4GlcCer

Tab. 4 Overview of glycolipid structures. *Summary of all glycolipids tested for LT-IIc-B binding.*

1.1. Binding of LT-IIc-B to dilutions of selected glycolipids

After obtaining first results from RIA and HPTLC, a few of the strongest binding GSLs were selected for the testing of concentration-binding relationship. As expected, Neu5AcGD1a presented the strongest binding of all tested GSLs and Neu5AcGD1b did not bind at all. The relative binding strength of the two selected neolacto-series GSLs was closely related, however, weaker than Neu5AcGD1a. The following concentration-binding curves were drawn using Microsoft Excel 2016 (*Microsoft Corporation, USA)*.

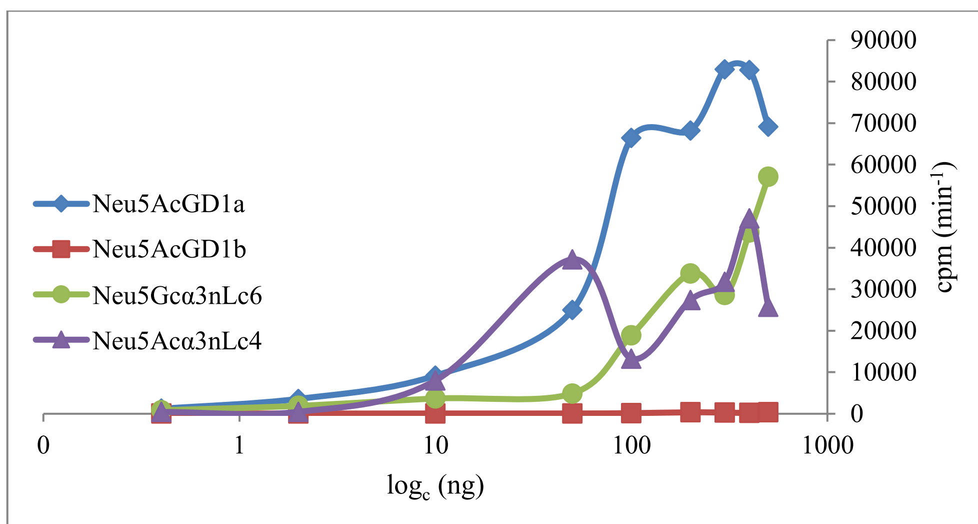

Fig. 17 Concentration-binding curves of four selected gangliosides. *Comparison of selectivity of Neu5AcGD1a, Neu5AcGD1b and two neolacto-series gangliosides Neu5Gcα3nLc$_6$ and Neu5Acα3nLc$_4$.*

By binding of LT-IIc-B to dilutions of Neu5AcGM3 and Neu5GcGM3, a slight preference for Neu5Ac was noted. The disialo ganglioside Neu5AcGD3, having a terminal Neu5Acα8Neu5Ac sequence was, on the other hand non-binding.

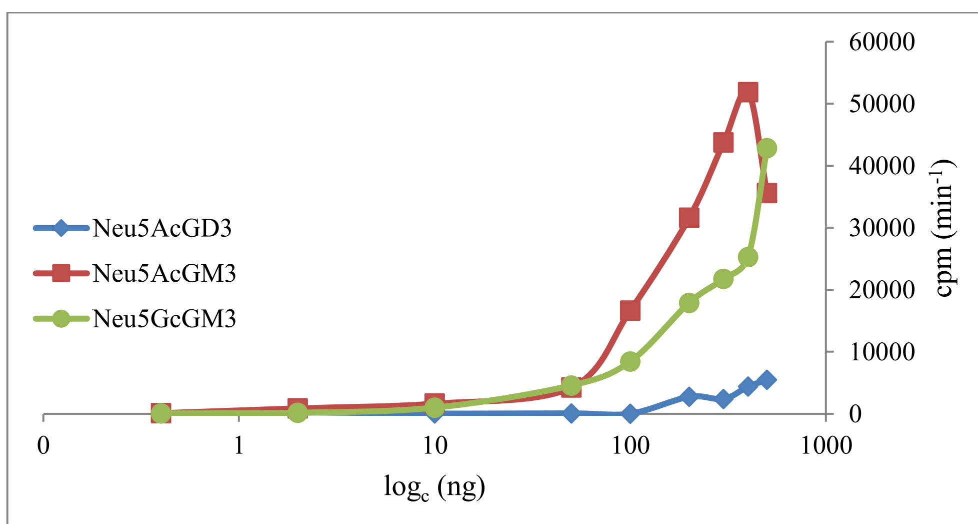

Fig. 18 Neu5Ac vs Neu5Gc binding strength comparison.

1.2. CTx-B vs. LT-IIc-B binding specificity

The binding of cholera toxin B subunits to the Neu5AcGM1 ganglioside is a high-affinity protein-carbohydrate interaction ($K_d = 0.43$ nM).[98] In the next set of RIAs, we compared the binding of LT-IIc-B to Neu5AcGD1a and the binding of CTx-B to Neu5AcGM1. Here, LT-IIc-B bound mainly to Neu5AcGD1a with a half-maximal binding (C_{50}) at ≈ 150 ng, while CTx-B bound mainly to Neu5AcGM1 with a half-maximal binding at ≈ 75 ng.

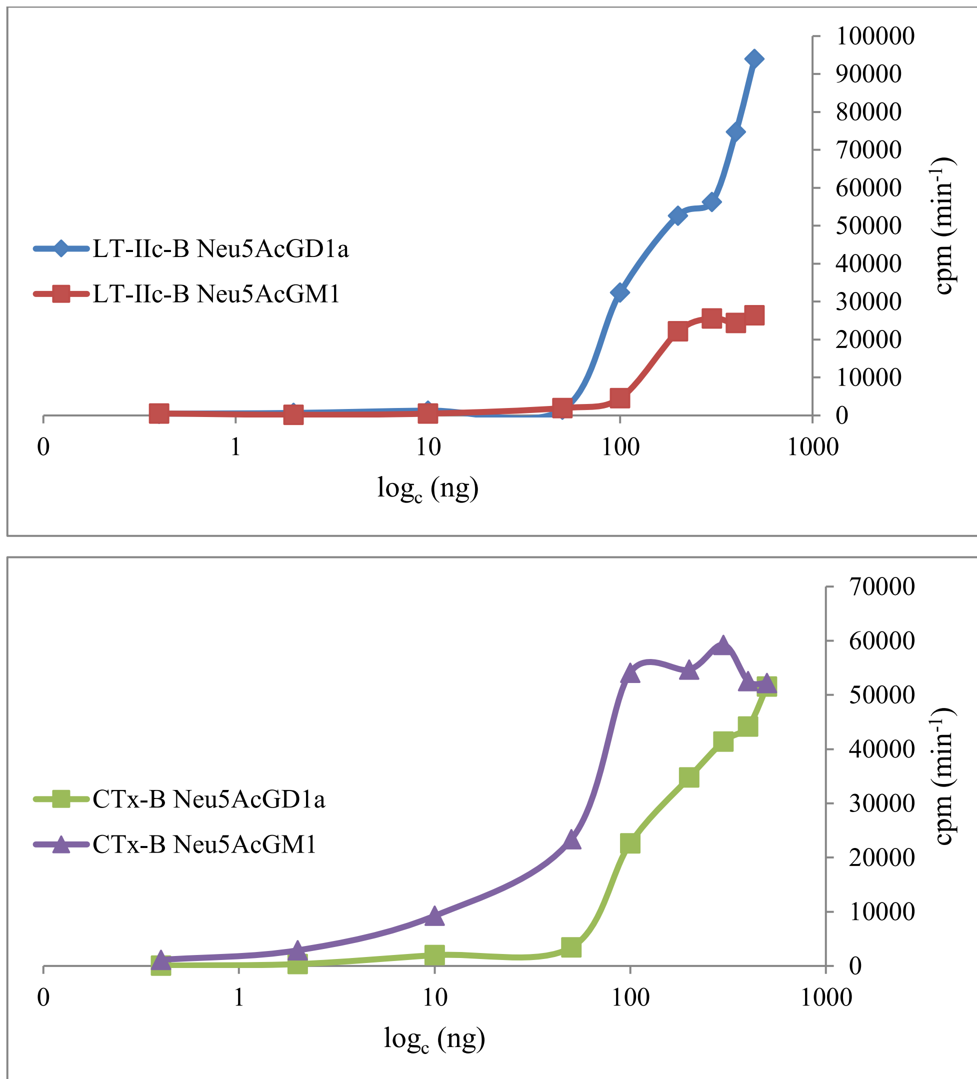

Fig. 19 Comparison of cholera toxin B subunits (CTx-B) and LT-IIc B subunits (LT-IIc-B) binding selectivity for Neu5AcGD1a and Neu5AcGM1.

2. BINDING TO GLYCOPROTEINS

2.1. HPTLC binding

The glycosphingolipid binding assay demonstrated that the minimal binding epitope required for LT-IIc B subunits binding was a terminal Neu5Acα3Gal or Neu5Gcα3Gal sequence. Since these sequences are also found on glycoproteins we next tested if the LT-IIc B subunits could also bind to glycoproteins, using fetuin and asialofetuin. The initial test with the different concentrations of the proteins dotted on a TLC plate showed binding to fetuin at 20 µg (indicated by an arrow), whereas asialofetuin was non-binding. (*Fig. 20*).

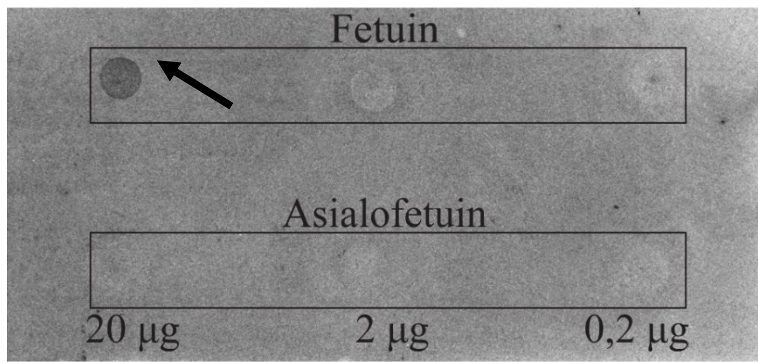

Fig. 20 Binding of LT-IIc-B to fetuin and asialofetuin. *Amounts of fetuin and asialofetuin are indicated in the figure.*

2.2. Gel electrophoresis and Western blotting

The initial glycoprotein binding screening was followed by gel electrophoresis and Western blot. Once again, the LT-IIc B subunits bound to fetuin but not to asialofetuin (*Fig. 21*).

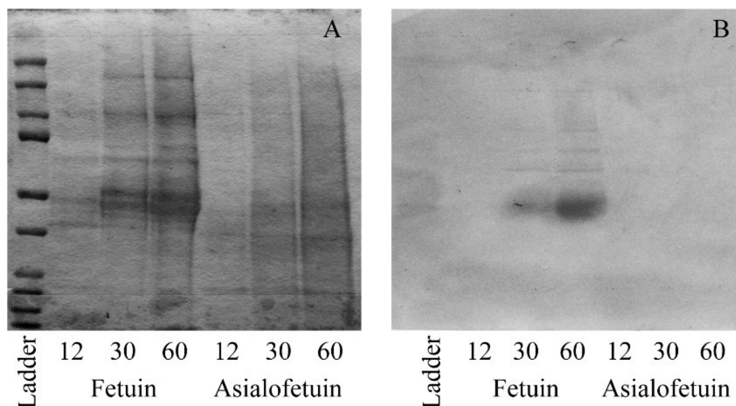

Fig. 21 SDS-PAGE (A) and binding of LT-IIc-B to fetuin and asialofetuin (B). *Three different amounts of fetuin and asialofetuin in µg are presented.*

3. INHIBITION STUDIES

3.1. HPTLC and RIA inhibition assays

Finally, we examined if it was possible to influence the protein-carbohydrate interaction by pre-incubating the B subunits of LT-IIc with soluble saccharides, using Neu5Ac and Neu5Acα3Galβ4GlcNAc. Some effects were observed, however, the two saccharides had different effects in the two different assays.

Thus, in the chromatogram binding assay, preincubation with free Neu5Ac gave a general weakening of the LT-IIc-B binding (*Fig. 22*). In the RIA, on the other hand, the Neu5Acα3Galβ4GlcNAc saccharide had a more pronounced inhibitory effect (*Fig. 23*).

The following figures summarise results from inhibition studies. The first RIA assays were done using higher concentrations of GSLs (300 ng). After obtaining results from concentration-binding curves, the concentrations were lowered accordingly to 10 ng. For HPTLC inhibition, 0.4 µg of GSLs were used. The assays were repeated twice.

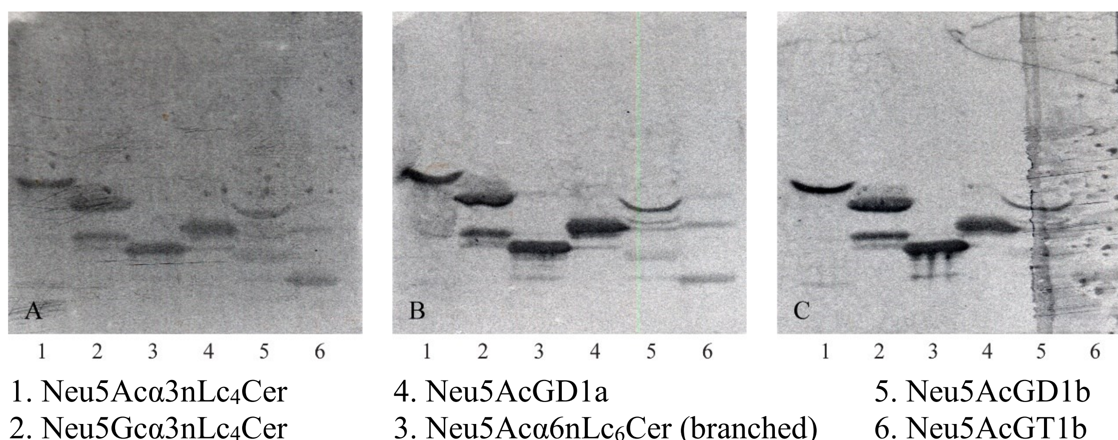

Fig. 22. Inhibition assay using 0.4 µg of glycolipids*.*
On the plate A, LT-IIc-B was incubated with 5 nmol of Neu5Ac, on the plate B, the same amount of Neu5Acα3Galβ4GlcNAc was used. The third plate represents a blank, LT-IIc-B incubated with PBS.

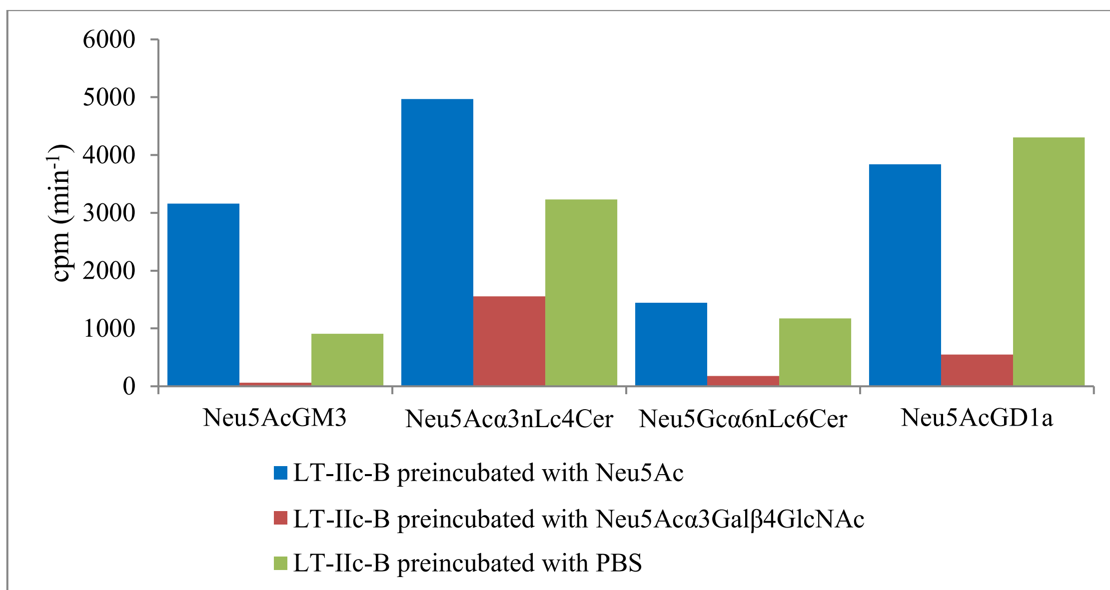

Fig. 23 Comparison of the inhibitory effects of Neu5Ac and Neu5Acα3Galβ4GlcNAc obtained from RIA inhibition studies. *For each tested glycolipid, the first two columns represent binding of the LT-IIc-B incubated with Neu5Ac and Neu5Acα3Galβ4GlcNAc, respectively. The third column is a blank. 10 ng of GSLs was used for the assay.*

4. ISOLATION OF GSLs FROM MOOSE KIDNEY

A previous LC-ESI/MS analysis of a complex ganglioside fraction from moose kidney had indicated the presence of the ganglioside disialoglobopentaosylceramide (NeuAcα3Galβ3(NeuAcα6)GalNAcβ3Galα4Galβ4Glcβ1Cer) in this sample. Since the structure of this ganglioside was different from the available reference gangliosides we attempted to isolate this compound to test it for LT-IIc-B binding.

The ganglioside fraction from moose kidney (50 mg) was separated by repeated chromatographies on Iatrobeads® columns, and the preparative procedure was monitored by binding of radiolabelled LT-IIc B subunits on thin-layer chromatograms. The sub-fractions obtained were pooled according to their mobility on thin-layer chromatograms and their LT-IIc-B binding activity, and the fractions thereby obtained were analysed by LC-ESI/MS/MS.

The second separation gave nine ganglioside-containing sub-fractions shown in *Fig. 24*. The results from LC-ESI/MS/MS are summarized in *Tab. 5*. Thus, the fractions 2 to 5 contained the Neu5AcGD3 ganglioside and certain amount of Neu5AcGM3 was observed in fraction 5, probably an impurity from the column chromatography separation.

The base peak chromatogram from LC-ESI/MS of fraction 6 (*Fig. 25A*) had four doubly charged molecular ions. These ions were subjected to MS^2 and thereby the gangliosides Neu5AcGD3 (*m/z* 729), Neu5AcGD1a (*m/z* 976) and Neu5AcGD1b (*m/z* 926) were identified. The doubly charged molecular ion at *m/z* 1040 corresponds to a singly charged molecular ion at *m/z* 2080, indicating a ganglioside with one Neu5Ac, one HexNAc, four Hex and d18:1-h24:0 ceramide. MS^2 of this ion (*Fig. 25B*) gave $Y_{5\alpha}$ ion at *m/z* 1790, and MS^3 of *m/z* 1790 (*Fig. 25C*) gave a series of Y ions identifying a ganglioside with NeuAc-Hex-(NeuAc-)HexNAc-Hex-Hex-Hex sequence, most likely the ganglioside disialoglobopentaosylceramide ($(Neu5Ac)_2$-Gb_5). However, since fraction 6 also contained the Neu5AcGD1a ganglioside (the strongest binder of LT-IIc-B) it could not be used for testing of LT-IIc-B binding to the disialoglobopentaosylceramide. Further chromatographic steps were judged to not give the separation of these co-migrating gangliosides since there were only a few mg in fraction 6.

LC-ESI/MS of fraction 10 gave two major doubly charged molecular ions at *m/z* 1319 and *m/z* 1333, respectively (*Fig. 26*), corresponding to a ganglioside with two Neu5Ac, three HexNAc and five Hex and d18:1-h22:0 (*m/z* 1319) or d18:1-h24:0 (*m/z* 1333) ceramides. However, here the carbohydrate sequence could not be fully elucidated by MS^2 and MS^3. The binding of the unknown glycosphingolipid in the fraction 10 is inconclusive, however, further analyses are underway.

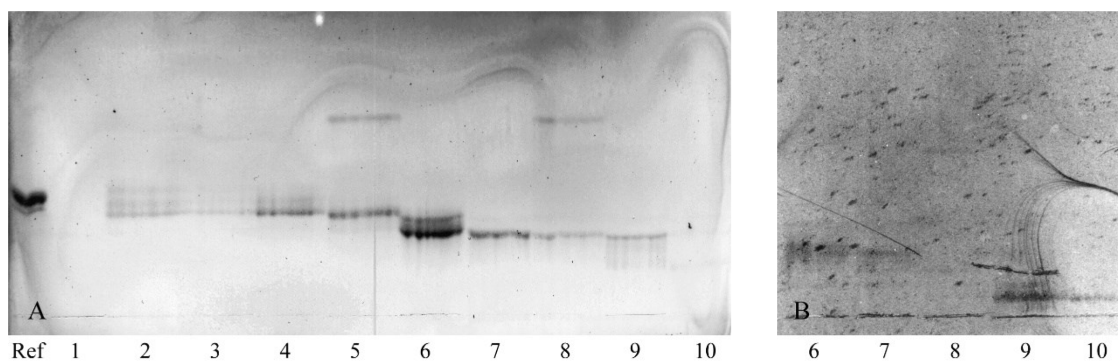

Fig. 24 HPTLC migration of the fractions after second column chromatography compared to their binding results*. TLC plate stained with anisaldehyde reagent (A), and fractions 6-10 on a TLC plate with bound LT-IIc-B are presented (B). Neu5AcGD3 was used as a reference. (8 µg per lane)*

FRACTION	GLYCOSPHINGOLIPID				AMOUNT
1	No glycolipid				12.1
2	Neu5AcGD3 (d18:1, h22)	Neu5AcGD3 (t18:0, h22)			1.40
3	Neu5AcGD3 (t18:0, h22)				0.30
4	Neu5AcGD3 (t18:0, h22)				3.30
5	Neu5AcGM3 (d18:1, 24)	Neu5AcGD3 (d18:1, h22)			1.50
6	Neu5AcGD1a (t18:1, h24)	Neu5AcGD3 (d18:1, h16)	Neu5AcGD1b (d18:1, h18)	**(Neu5Ac)$_2$-Gb$_5$Cer** (d18:1, h24)	2.50
7	Neu5AcGD1a (d18:1, h18)	Neu5AcGD1a (t18:1, h24)	Neu5AcGD3 (t18:0, h22)		0.30
8	Neu5AcGM3 (d18:1, 24)	Neu5AcGD1a (t18:1, h22)			0.60
9	Neu5AcGD1a (d18:1, h16)		**(Neu5Ac)$_2$-Gb$_5$Cer** (d18:1, 16)		0.60
10	GSLs not identified				0.70

Tab. 5 Composition of the second chromatography fractions based on MS. *(amounts in mg) (t – phytosphingosine, d – dihydrosphingosine, h – fatty acid containing hydroxy group)*

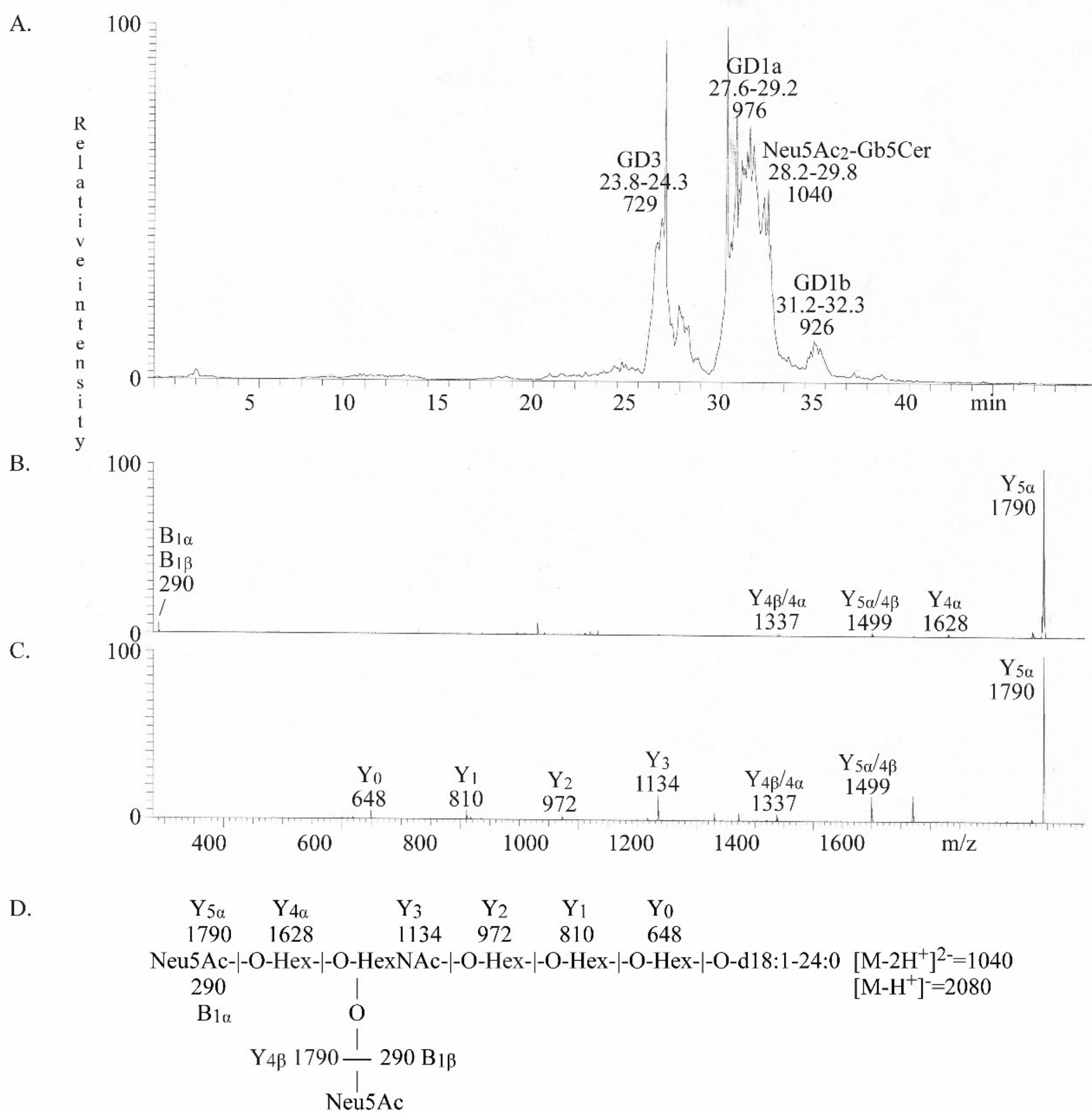

Fig. 25 MS spectrum obtained from fraction 6 after second column chromatography.

Fig. A contains the base peak LC-MS chromatogram of the fraction.

Fig. B shows the MS^2 mass spectrum of the molecular ion at m/z=1040 from Fig. A.

Fig. C shows the MS^3 of the ion $Y_{5\alpha}$ at m/z=1790 from Fig. B.

Fig. D shows fragmentation diagram of the $(Neu5Ac)_2$-Gb5Cer ganglioside.

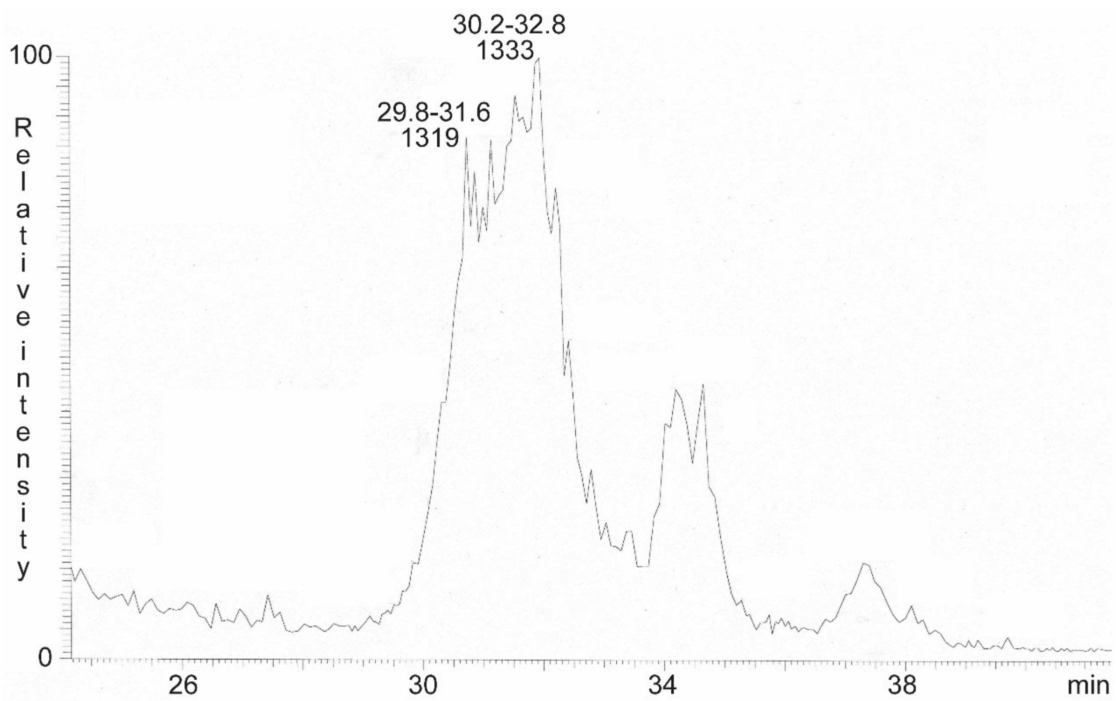

Fig. 26 The base peak chromatogram obtained from LC-ESI/MS of the fraction 10.

VI. DISCUSSION

In this thesis the carbohydrate recognition of LT-IIc-B was investigated. We found that LT-IIc-B preferentially bind to gangliosides with a terminal Neu5Gc/Neu5Acα3Gal sequence as the ganglioside Neu5AcGD1a, Neu5Acα3nLc$_4$Cer and Neu5Acα3nLc$_6$Cer. The Neu5AcGM3 and NeuGcGM3 gangliosides were also recognized to some extent.

Thus, even though LT-IIb and LT-IIc enterotoxins were discovered to be distinct in point of B subunits homology, their carbohydrate-binding preferences are not totally different. While B subunits of LT-IIb does not bind the GM1, GM2 and GM3 gangliosides[36, 99], the B subunits of LT-IIc does not bind GM1 and GM2 but binds relatively strongly to GM3. The same optimal binding sequence Neu5Acα3Galβ4GalNAc as for LT-IIc was reported for LT-IIb in 1988 by Fukuta et al.[36]. Furthermore, in a more recent study by Zalem et al.[99], the neolacto-series gangliosides we have found to be recognized by the B subunits LT-IIc were also recognised by the B subunits of LT-IIb, further suggesting strong similarities of not only the optimal binding sequence but as a consequence, of the carbohydrate binding sites of these B subunits. Hence, the only discrepancy is that LT-IIc, but not LT-IIb, binds to Neu5Acα3Galβ4 (like in GM3), which may suggest, that the differences in B subunits homology influence the binding site structure in a way allowing LT-IIc to be more promiscuous towards the gangliosides, not limited by a three-carbohydrate binding sequence. This hypothesis may be supported by ongoing co-crystallization studies of LT-IIc B subunits at the *Centre de Recherches sur les Macromolécules Végétales (The National Center for Scientific Research and Université Grenoble Alpes, Grenoble*, *France)*.

Interestingly, in the study of Zalem et al.[99] the LT-IIb B subunits were found to have two carbohydrate binding sites within one subunit, positioned close one to the other and binding the same ligand, allowing the whole B-fragment to bind 10 carbohydrates at once. This type of binding site distribution has never been observed for other B subunit proteins in the AB$_5$ cholera toxin (CT) family. However, similar properties were observed in Shiga-like toxins from *E. coli* with high structural although very feeble protein sequence homology with CT family.[100] The importance of this finding remains unclear. However, based on findings of Zalem et al. and previously described highly related structural preference of the GSLs, we hypothesise that this binding site

architecture may also be present in other LT-IIs B subunits. As mentioned above, co-crystallization efforts of LT-IIc-B are ongoing.

The indications of two-binding site finding uncover numerous questions needed to be answered. Which carbohydrates are preferred to bind to the sites? Is the preference of each site identical? So far, only Neu5Ac has been identified to bind to both.[99] May both sites be occupied by the ligands coming from the same carbohydrate chain? Only the GD1a carbohydrate sequence has been tested so far, although negatively. Does the intoxication of the cell require binding on all sites? Site-directed mutagenesis exchanging amino acids in the two carbohydrate binding sites of LT-IIb B subunits have been initiated with the intention to test GSL binding of the recombinant B subunits. Further testing could answer these questions and thus contribute to the better understanding of the pathogenesis of LT-II caused diarrhoea in the small intestine.

Several human intestinal gangliosides have been described, the most notable are GM3, GD1a, GT1b, Neu5Ac-α3-neolactotetraosylceramide and Neu5Ac-α3-neolactohexaosylceramide[99, 101] and as all of these are recognized by LT-IIc, and some by LT-IIb, the common GSLs could be therefore presumed as responsible for LT-II enterotoxin-caused diarrhoea. Further testing regarding these findings may allow more targeted approach in the development of an effective countermeasure.

In contrast to our results, a recent study by Berenson et al., reported the binding of LT-IIc-B to the GM1 ganglioside.[2] However, a distinct GM1 species was used in this study. The binding GM1 contained C24 fatty acid and it came from the murine macrophages, whereas the GM1 used during our research was a standard human neuronal GM1 with C18. The influence of the fatty acid lengths on binding capabilities of the LT-IIc-B needs to be investigated further, to establish the relevance of the ceramide composition on binding capabilities.

All the cholera toxin family toxins have been found to possess strong immunomodulatory properties, enhancing both humoral and cellular immunity to a co-administered protein antigen, so-called adjuvant effect. The mechanism involves direct interactions between the enterotoxins and white blood cells gangliosides[102, 103], however, the exact mechanism is not yet fully understood.

Several studies on mice had shown adjuvant effects of CT, LT-I, LT-IIa, and LT-IIb, suggesting the same findings also for the LT-IIc.[37, 104] In a recent study, this prediction has turned out to be true. However, in addition to LT-IIc being as strong adjuvant as LT-IIb and having similarly strong capacity to induce a strong antigen memory response, LT-IIc exhibits some properties, that differ from those of LT-IIb and LT-IIa. In mice, LT-IIc effect on pro-inflammatory cytokine production is significantly higher, than the effect of LT-IIb. The cytokine composition suggests balanced activation of both CD4+ T cell subtypes, Th1 and Th2.[105] Furthermore, rapid and robust effect of LT-IIc on CD8+ T cells had been observed, while for LT-IIb a slower and gradual response was noted.[106] All of the mentioned discrepancies in immunomodulatory effects have been attributed to the B subunits ganglioside specificity, although the findings of Hu et al.[1] indicate the B subunit involvement to be inconclusive.

The involvement of B subunits' specificity in the immunomodulatory discrepancies may be supported by the findings of Nagafuku et al.[103] In mice, GM3 and GD1a gangliosides (among others) have been found necessary for the activation of the CD4+ T cells. Therefore, when exposed to LT-IIc, a stronger response of CD4+ T cells may be expected as LT-IIb binds only to GD1a, whereas LT-IIc binds to both and could, therefore, influence the activation more. However, the effects of LT-II enterotoxins' on T cell activation are still being researched.

The ganglioside composition of human leukocytes has been extensively characterized.[107-111] With the exception of the GM3, these gangliosides have one or several *N*-acetyllactosamine moieties, where one or more of the *N*-acetylglucosamines may be substituted with α3-linked fucose(s), additionally, there are gangliosides with both α6- or α3-linked Neu5Ac. Thus the Neu5Acα3neolacto binding capacity of both LT-IIc and LT-IIb might be involved in the interaction of these enterotoxins with human leukocytes.

Further research of GSL composition of human leucocytes and description of the exact mechanism involved in the adjuvant effect exerted by the enterotoxins, followed by *in vitro* and *in vivo* experiments on humans could contribute to the formulation of more potent recombinant vaccines as the ones available today.

The next step in LT-IIc binding specificity elucidation will be obtaining the 3D structures from crystallographic analysis of the subunits, and of the holotoxin, followed by 3D structures after co-crystallization of the LT-IIc B subunits with a suitable

carbohydrate sequence or preferably with a whole GSL, *e.g.* GD1a. Moreover, binding studies with the one-point mutations in the B subunits will be done, further demonstrating B subunit binding effects towards the carbohydrates and results of these experiments will hopefully lead to better understanding of the B subunit-carbohydrate binding interactions. Identification and characterization of the carbohydrate binding site inside the B subunit as well as elucidation of the parts of the carbohydrates, that are responsible for the interactions are crucial in providing a basis for the development of the compounds with enhanced inhibitory effects.

Similar work may had been seen during the development of neuraminidase inhibitors used for influenza virus treatment, where the sialic acid had been identified as the critical substrate for neuraminidase enzyme, necessary for the pathogenesis. Thus, the chemically modified sialic acid was developed to block the binding site of the neuraminidase of the virus and therefore, to prevent the spread of the virus in the respiratory tract.[112]

VII. CONCLUSIONS

The carbohydrate binding capacities of the novel enterotoxin LT-IIc have been investigated in this thesis. By binding LT-IIc-B with several glycolipids, most of them not investigated in the previous studies, we have identified the probable optimal binding carbohydrate sequence. Furthermore, we have put some basic foundations for inhibitory effects of the carbohydrates on the binding of LT-IIc-B.

According to the obtained results, following structure-binding relationships between B subunits of LT-IIc and the carbohydrate chains on GSLs can be established:

- Sialic acid is necessary for the binding, two sialic acids in a row interrupt it,
- Carboxylic group of the sialic acid needs to be intact,
- Terminal Siaα3Galβ3/4 is the minimal binding sequence,
- Terminal Siaα3Galβ3GalNAc or Siaα3Galβ4GlcNAc are the optimal binding sequences,
- Branching and length of the glycan play an important role but is not fully understood yet.

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