BENZON SYMPOSIUM No. 54

GLYCOSYLATION:
OPPORTUNITIES IN DRIG DEVELOPMENT

JUNE 11-14, 2007, COPENHAGEN, DENMARK

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Abstracts - MONDAY, June 11, 2007

FUNCTIONAL GLYCOMICS

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The human glycome is comprised of numerous glycan structures, totaling >100,000 by some estimates. The elucidation of the glycome poses enormous analytical challenges. Yet glycan structure is encoded by ~300 genes responsible for the non-template driven synthesis of glycan chains of glycoproteins, glycolipids and glycopolymers. These elaborate a finite set of structural elements (or motifs) that are largely known, and can be synthesized using current chemical and chemo-enzymatic methods. Functional glycomics has emerged as a term for studying the biological roles of glycans by focusing on mammalian and pathogen glycan binding proteins (GBPs) that recognize structural motifs of glycans as ligands, and mediate diverse aspects of biology. Microarrays containing natural and synthetic glycan structures are finding wide utility for assessing the specificity of GBPs for their ligands. This in turn has stimulated development of tools to understand the roles of ligands in GBP function. The siglecs are a representative family of GBPs, whose 13 members are differentially expressed on the majority of leukocyte cell types, and recognize sialic acid containing glycans. Exemplary is CD22 (Siglec-2), a regulator of B cell receptor signaling whose activity is modulated by its interaction with glycan ligands containing the sequence Neu5Acα2-6Galβ1-4GlcNAc (26SM), a structural motif abundantly expressed on B cells and other immune cells. Synthetic multivalent sialosides and high affinity analogs of 26SM have been instrumental in probing the organization of CD22 on the cell surface and the roles of ligands in CD22 function. Elements of this basic approach are being applied across the functional glycomics landscape to elucidate the biology of glycan binding proteins (Supported by NIGMS and NIAID).

TARGETING CARBOHYDRATE-RECOGNISING PROTEINS AS DRUG DISCOVERY TARGETS

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It has become evident that a number of sialic acid recognising proteins play key roles in the progression of a variety of significant diseases [1-3]. For example, influenza virus successfully uses a membrane-bound haemagglutinin and sialidase to effect tremendous infection [4]. Rotavirus has a membrane-associated lectin VP8* that also recognises sialic acids [1]. A number of these sialic acid-recognising proteins have been successfully crystallised and their structures solved. These crystal structures now provide a wealth of information for drug discovery. As a part of our interest in understanding sialic acid-recognising proteins in disease processes and as drug discovery targets we have used NMR spectroscopy, x-ray crystallography, computational methods, synthesis and biological assays to investigate some of these proteins [see for example 4-7]. From these studies we have designed a number of sialic acid-based and sialylmimetic ligands that may provide a basis for the discovery of high affinity compounds. In this lecture aspects of our research effort into the exploration of sialic acid-recognising proteins as drug discovery targets with a particular emphasis on virus-associated sialic acid recognizing proteins will be presented.

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SYNTHETIC HEPARIN: FROM FONDAPARINUX AND IDRAPARINUX TO A NOVEL CARBOCARRIER TECHNOLOGY FOR THERAPEUTIC PROTEINS

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A unique pentasaccharide domain in heparin provides the active principle causing activation of antithrombin III (AT-III), a coagulation factor inhibitor which is present in relatively high concentrations in blood. A synthetic counterpart of this unique sulphated pentasaccharide was prepared and further clinically developed to deliver the new anti-thrombotic drug fondaparinux (Arixtra®) in 2002. The drug shows superior anti-thrombotic activity and brings about AT-III mediated anti-factor Xa activity exclusively. A more potent analogue, idraparinux, could be prepared that is easier to synthesize and which, because of its long half life (130 hrs in man) in circulation, needs only once a week administration. The long residence time of idraparinux is explained by the strong and specific interaction of the pentasaccharide with its target AT-III, thereby preventing rapid renal clearance of the small, polar pentasaccharide. Following structure based design novel conjugates with thrombin binding entities were prepared. Not only novel multifunctional conjugates with specific anti-factor Xa and anti-thrombin activities can be prepared, but also the half-life in man can be tuned (up to half lifes of 130 hrs for conjugates with the long acting pentasaccharide idraparinux). In this lecture it will also be shown that conjugation of various therapeutic peptides and proteins with specific AT-III binding pentasaccharides results in significant prolongation of plasma half-life of the conjugated moiety. This so called '*carbocarrier*' technology is exemplified by the construction of a novel truly long acting insulin-pentasaccharide conjugate.

References: *Angewandte Chemie Int. Ed. 43, 3118 (2004); Drug Discovery Today 10, 769-779 (2005).*

DEVELOPMENT OF HAEMOPHILUS INFLUENZAE TYPE B CONJUGATE VACCINE USING A SYNTHETIC ANTIGEN

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Conjugate vaccines represent an important step forward in the fight again infectious diseases. Alternatives to existing technologies are continuously needed in order to increase the supply for fulfilling the massive needs of vaccines throughout the world. The possibility of reproducing the structure of protein or polysaccharide antigens by chemical synthesis was demonstrated in many cases. However, the development of bacterial vaccines using these synthetic antigens was interfered by many issues. We developed a process for the chemical synthesis of *Haemophilus influenzae* type b oligosaccharides[1] as a base for a new conjugated vaccine. After complex preclinical and technological development that includes clinical testing in the target population we demonstrated that the vaccine containing fully synthetic oligosaccharides representing a fragment of the bacterial capsular polysaccharide is as effective as their natural counterpart[2]. The vaccine was introduced in Cuba since 2004 and is now part of the National immunization program and it is also introduced in several other countries. The specific circumstances allowing successful development of a vaccine having a synthetic carbohydrate antigen will be analyzed.

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APPROACHES TOWARDS GLYCOCONJUGATE VACCINES AGAINST *NEISSERIA MENINGITIDIS* BASED ON SYNTHETIC OLIGOSACCHARIDE STRUCTURES

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Carbohydrate structures, in the form of capsular polysaccharides (CPSs) or lipopolysaccharides (LPSs), are important surface antigens of bacteria and accordingly of interest for serotyping of and as vaccines against bacteria. The successful introduction of glycoconjugate vaccines, i.e., saccharide structures conjugated to a carrier protein has dramatically increased the interest in this type of vaccines. There are now three types of efficacious commercial glycoconjugate vaccines, against *Haemophilus influenzae* type b (Hib), *Neisseria menigitidis* type C (MenC) and *Streptococcus pneumoniae* (seven serogroups), based on partly hydrolyzed native capsular polysaccharide structures.

However, the use of native bacterial polysaccharides is sometimes combined with various problems, e.g., heterogeneity, instability, toxicity or molecular mimicry of these structures. For example, with *N. menigitidis* bacteria, serogroup B (MenB) is a severe problem since its CPS structure is identical to a human carbohydrate structure. Another issue is the poor stability of MenA vaccines due to the inherent instability of the anomeric phosphate diester linkages of the serogroup A CPS. An interesting alternative is then synthetic part structures or analogues. Owing to the fast progress in oligosaccharide synthesis during the last years the synthesis of these often most complex structures has become feasible. For the Hib vaccine there is now already a commercial vaccine based on chemically synthesized oligosaccharide structures.
We will present our approach towards glycoconjugate vaccine candidates based on synthetic carbohydrate structures against infections caused by *Neisseria meningitidis*, both stabilized analogues, including anomeric C-phosphonate analogues, of the serogroup A CPS as well as inner core LPS structures to target mainly serogroup B, for which the CPS structures can't be used in vaccines due to the molecular mimicry.

STRUCTURE AND INHIBITION OF ENZYMES THAT ASSEMBLE GRAM-NEGATIVE ENDOTOXIN, A POTENT SACCHAROLIPID ACTIVATOR OF INNATE IMMUNITY

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The outer surface of Gram-negative bacteria is covered with a remarkable, macro-molecular saccharolipid known as lipopolysaccharide (LPS), the hydrophobic anchor of which is lipid A. In *Escherichia coli*, lipid A consists of a hexa-acylated disaccharide of glucosamine with phosphate groups at the 1 and 4' positions. Inhibition of any of the first seven enzymes that synthesize lipid A is lethal. These enzymes are expressed constitutively and are conserved in virtually all Gram-negative bacteria.
High-resolution cyrstal or NMR structures of the first two lipid A biosynthetic enzymes have been determined. UDP-*N*-acetylglucosamine (UDP-GlcNAc) acyltransferase (LpxA) catalyzes the first step, the transfer of the *R*-3-hydroxyacyl chain from *R*-3-hydroxyacyl acyl carrier protein (ACP) to the glucosamine 3-OH group of UDP-GlcNAc. The crystal structure of the *E. coli* LpxA homotrimer, determined previously at 2.6 Å in the absence of substrates or inhibitors, revealed that LpxA contains an unusual, left-handed parallel β-helix fold. We have now solved crystal structures of *E. coli* LpxA at 1.7 Å with several bound lipid products, incluidng UDP-3-*O*-(R-3-C14)-GlcNAc, showing how the *E. coli* enzyme acquires its extraordinary ability to select for fourteen carbon fatty acyl chains. In addition, we have solved the structure of *Leptospira interrogans* LpxA at 2.0 Å, which shows absolute selectivity for the novel sugar nucleotide UDP-GlcNAc3N in which NH_2 replaces the GlcNAc 3-OH.
We have also determined the crystal structure of *E. coli* LpxA in complex with a penta-decapeptide and a dodecapetide inhibitor at 1.8 and 1.6 Å, respectively. Three peptides, each of which adopts a β-hairpin conformation, are bound per LpxA trimer. The peptides are located at the interfaces of adjacent subunits in the vicinity of the three active sites, as determined by site directed mutagenesis and x-ray analysis of the LpxA/UDP-3-*O*-acyl-GlcNAc complexes. Theses peptides are potent inhibitors of *E. coli* LpxA (K_i ~ 50 nM). The compact β-turn structure of the bound peptides may open new approaches to the rational design of LpxA inhibitors with antibiotic activity.

FROM CARBOHYDRATE LEADS TO DRUGS: WHY IS IT SO DIFFICULT?

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Investigations over the past 10 years have shown that carbohydrates possess an enormous potential as lead structures for drug discovery. However, only limited progress has been made in antagonizing the interaction of physiological carbohydrate ligands with their receptor proteins.
The reasons for this disappointingly slow progress are manifold. Firstly, physiological carbohydrate-lectin interactions are notoriously weak, i.e. in the μM to mM range. Secondly, the dynamics of the binding process of carbohydrates to lectins is still poorly understood. Thirdly, the development of drugs from carbohydrate leads suffers from complex syntheses, which hampers lead optimization and scale-up of potential drug candidates.
In addition to these drawbacks, which are related to the pharmacodynamic properties of carbohydrate-derived drugs, the pharmacokinetic hurdles, which are an inherent problem of carbohydrates and mimetics thereof, have been completely neglected.
In this lecture, the classical lead-to-candidate search for antagonists of the myelin-associated glycoprotein (MAG) is compared with a two-stage, combinatorial approach leading to high affinity ligands with drug-like properties. MAG has been identified as a sialic acid-binding immunoglobulin-like lectin (Siglec-4). Its role, as one of several myelin components inhibiting axonal regrowth after injury, has drawn a lot of attention. Schnaar [1] reported that a limited set of structurally related gangliosides, known to be expressed on myelinated neurons *in vivo*, are functional ligands of MAG. The recently reported ability to reverse MAG inhibition with monovalent glycosides [2] encourages the search for high affinity ligands.

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GALECTINS AS DRUG TARGETS IN CANCER AND INFLAMMATION

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Animal experiments suggest rate limiting roles of galectins in cancer and inflammation, but their mechanisms of action remain largely unknown, even if a wide variety of possibilities have been proposed based on experiments at the cellular level. This encouraged us to further investigate the role of their fine specificity at the cellular level, and to develop inhibitors as future therapeutics and research tools. The galectin specificity for natural and artificial ligands can be fairly well summarized by a model involving five subsites A-E, where site C is the most conserved and binds galactose whereas the others vary among the galectins. The best studied, galectins-1 and -3 prefer LacNAc in site C-D, but the N-terminal CRDs of bi-CRD galectins prefer lactose as found in glycolipids. Interactions in site A-B refine specificity further, and galectin-8, for example, has a uniquely high affinity (K_d ~100 nM) for NeuAcα2-3Lactose. Despite these differences galectins-1,-3, and -8 all bind N-linked glycans at the cell surface and the fine specificity is not needed, as combined interaction with second best ligands of moderate affinity appears to be enough. However, after uptake of the galectin by endocytosis, its intracellular targeting is determined by the fine specificity. This opens a new way to think about galectin function, and ties in well with results from others on intracellular targeting and endocytosis. Using models of ligand interaction in sites B-D we have also developed potent galectin inhibitors with varying degrees of specificity for one galectin over another.

HELICOBACTER PYLORI ADHESINS AND GLYCAN RECEPTORS

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Helicobacter pylori infection is the main cause of gastritis and peptic ulcer disease and major risk factor for gastric cancer. The high prevalence of *H. pylori* infection worldwide and emergence of antibiotic resistance makes a vaccine against *H. pylori* highly warranted. The ABO blood group antigen binding BabA adhesin is a virulence factor prevalent in patients with severe gastric disease. Native BabA was purified to homogeneity from *H. pylori* and tested as a vaccine candidate in Leb (transgenic fucosyltransferase) mice, by intranasal immunization together with the mucosal adjuvant, CTA1-DD. Immunization with BabA provided both prophylactic and therapeutic immunity against *H. pylori*, i.e. cure of infection. In addition, immunization of Rhesus macaques conferred protective immunity. Taken together, these results show the therapeutic potential in bacterial lectins/ adhesins as vaccine candidates against persistent infection and alternatives to antibiotic treatment. Interestingly, immune responses can be rather individual, and we analyzed Rhesus macaques for influence of secretor phenotype on inflammatory responses. In both humans and macaques, the ABO/Lewis blood group antigens expressed in gastro-intestinal epithelia and secretions of positive secretor individuals are used as binding sites for BabA mediated *H. pylori* adherence. A majority of macaques included in the study were of weak-secretor phenotype, a secretor phenotype common in South-East Asian populations. Experimental long-term *H. pylori* infection transiently increased mucosal sialylation and reciprocally decreased mucosal fucosylation. Gastritis, mucosal sialylation, and *H. pylori* infection density were all lower in weak-secretors. These results demonstrate the role of secretor phenotypes in infection, inflammation, and susceptibility for disease and suggest that the ABO antigens are part of our innate immune system.

SOLID-PHASE CHEMICAL TOOLS FOR GLYCOMICS: GLYCOCHIPS AND NANOPARTICLES

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Many of the milestone technologies that have been so important for unraveling the structures and biology of nucleotides and proteins (such as rapid sequencing methods, *in vitro* synthesis, and cloning) cannot be readily applied to carbohydrates. Carbohydrates are not directly encoded but are constructed by the concerted activity of synthesizing and modifying enzymes. This indirect linkage between genome and glycome presents a barrier to unraveling the

complex biology of glycans. Thus there is a pressing need to develop glycan microarrays, glycochips, which can be used to analyze the occurrence and interactions of carbohydrates in a high-throughput fashion.
Here we present new methods for construction of covalent glycan microarrays, extending our previously reported linkers for chemoselective, oriented immobilization of carbohydrates. We focused on chip-sized surfaces for economy of reagents and sensitivity, and tested a range of materials. This was complemented with 'bead arrays' of PEGA resin, i.e. glycans and peptides anchored to PEGA as substrates for glycosyl transferases. We describe first applications using lectins, antibodies, and carbohydrate binding domains.

GLYCOBLOTTING-BASED CLINICAL GLYCOMICS

Nishimura S-I, Miura Y, Shinohara Y, Kurogochi M, Furukawa J, Kita Y, Shimaoka H & Amano M; Advanced Chemical Biology, Graduate School of Advanced Life Science, Hokkaido University, Japan

Global glycomics of human whole serum glycoproteins appears as an innovative and comprehensive approach for the discovery of surrogate non-invasive biomarkers for various diseases. We have demonstrated the versatility of *glycoblotting* technology [Nishimura, S. –I., et al, (2005) High-throughput protein glycomics: Combined use of chemoselective glycoblotting and MALDI-TOF/TOF mass spectrometry. *Angew. Chem. Int. Ed. Engl. 44*, 91-96] in the enrichment of whole oligosaccharides liberated from glycoproteins and subsequent large-scale structural characterization by mass spectrometry. Recently, our extensive efforts allowed for the optimized protocol of quantitative and reproducible glycoform analysis by means of the glycoblotting method. This protocol needs only 5-20 micro liter of human serum for the quantitative profiling of 30-40 kinds of major glycoforms within 5-8 hours.
In the present communication, we report a standard strategy for high throughput and large-scale human serum glycomic analysis in automated manner. The feasibility of this technique in the clinical glycomic/glycoproteomic approach toward discovery research of new biomarkers will be demonstrated.

Poster No. I-1

PROBABLE TRANSITION STATES FOR NEIGHBORING GROUP GLYCOSYLATION REACTIONS

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Based on Density Functional Theory (DFT) Quantum Mechanical (QM) calculations of proposed intermediates and Transition States (TS) for 2-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucopyranosyl (**1**) and mannopyranosyl (**2**) oxacarbenium ions reacting with methanol as nucleophile, a complete model of the neighboring group participation glycosylation can now be formulated. Neutral α-configured donors are shown to smoothly ionize to a $^4H_3$ conformer for both **1** and **2** by a series of stepwise elongations of the C-1--X bond using DFT-QM. However, β-configured donors are shown to ionize with a pre-ionization on or off conformational change for both **1** and **2**. For **1** this leads via a $^1S_3$ conformer to a similar to α $^4H_3$ conformer. Once ionized the oxacarbenium ions can interconvert to their other low energy conformations $^5S_1$ for **1** and ^3E for 2 via pathways involving pseudorotation as determined by constrained ab initio molecular dynamics. These monocyclic ions could in principle react with methanol via facial selective complexes with considerable hydroxonium ion character. Further studies of **1** and **2** suggest that neighboring group ions are formed by rotation about C-2--O-2 with barriers between 22 and 39 kJ mol^{-1} with the maximum at CH-2-C-2--O-2-C(=O) torsion angle of near 90. These bicyclic ions are typically around 60 kJ mol^{-1} more stable then the monocyclic ones. These bicyclic ions can form complexes with methanol at the former carbonyl carbon or C-1. These two species are in facile equilibrium with almost no barrier between them. In order to proceed to glycosides from these complexes the C-1--O(methanol) bond must shorten, C-1 must rehybridize and the hydroxyl proton must transfer. Probable TS for neighboring group assisted glycosylation of both **1** and **2** were found that involve C-2--O2 bond rotation with the CH-2-C-2--O-2-C(=O) torsion angle of near 90. As well, the hydroxylic proton is involved in intramolecular hydrogen bonding. These two previously little recognized factors suggest methods to control the stereoselectivity and reactivity in glycosylation reactions.

Poster No. I-2

CONFORMATIONAL ARMING OF GLUCOSYL DONORS

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Thio-glucosides protected with bulky silylethers are shown to be very reactive glucosyl donors due to a conformational change that places equatorial OR groups in an axial position. Gluco-, galacto-, manno-, and rhamno glucosyl donors were prepared and used in glucosylation reactions with armed thio-glycosides as acceptors. All the donors were activated using standard NIS/TfOH conditions at very low temperature (-80 °C to -90 °C). The stereo-

selectivity was good to excellent, with gluco- and galactosides giving β-saccharides as major products while manno-, and rhamnosides gave α disaccharides.

Poster No. I-3

MICROWAVE CAN OPEN A DOOR TO DEVELOP NEW GLYCOSYLATIONS

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Glycosylation has been well studied especially over the past quarter century. During this same period from 1986, microwave irradiation became popularly applied in chemical synthesis. It has been mainly used as an effective heating tool and, as a result, has allowed for higher yield and/or shorter reaction times. We have since revealed that microwave is not only an effective heating tool but also allows for control over other factors in chemical synthesis. Based on these premises, we here report on new glycosylation concepts in glycosylation; 1) glycosylation with methyl glycoside donors and 2) oligosaccharide synthesis with microwave irradiation at low temperatures.

1) In general, alkyl glycosides are not suitable as glycosyl donors because they require harsh acidic conditions to break the glycosyl bonds. In order to form new glycosyl bonds, therefore, glycosyl donors should have efficient leaving groups at their anomeric centers. Although methyl glycosides are often used as protecting groups for the anomeric center, it is difficult to use them as glycosyl donors. Nevertheless this background, we have discovered a new glycosylation system that, combined with microwave irradiation, leads to the formation of new glycosylated compounds using methyl glucopyranosides as donors in high yield.

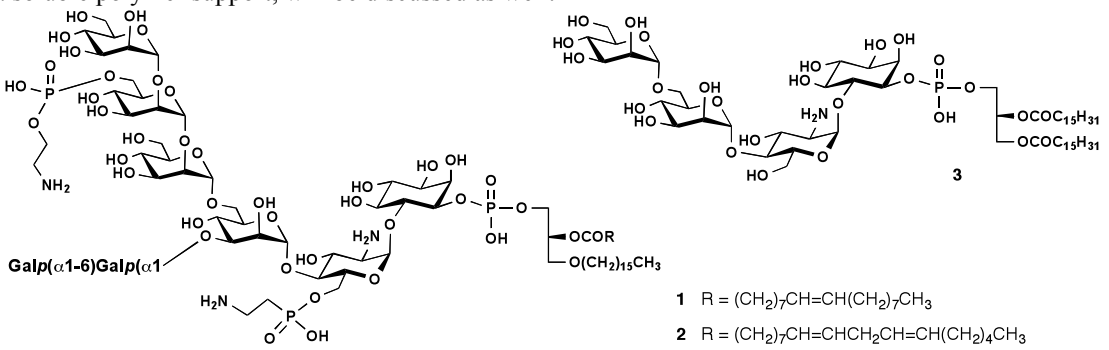
2) To synthesize Le^X trisaccharides, a galactosyl donor followed by a fucosyl donor are introduced to a glucosamine acceptor because of considering that the reactivity of the 4-OH on glucosamine is low; the fucosyl bond is relatively weak under acidic conditions. In addition, when the glucosamine acceptor is protected by a thioalkyl group on the anomeric position, the lone pair on the thio atom could be attacked by a donor cation and to lead to the formation of subproducts. Despite knowledge of this, we have found that microwave irradiation at low temperatures has demonstrated that the possibility of glycosylation of a galactose moiety after the introduction of a fucose derivative onto the 3-OH worked well in high yield and with fewer subproducts.

Poster No. I-4

GPI ANCHORS: RECENT SYNTHETIC DEVELOPMENTS

Yashunsky DV, Tee JA & Nikolaev AV; College of Life Sciences, University of Dundee, United Kingdom

Glycosylphosphatidylinositols (GPIs) are a class of natural glycosylphospholipids that anchor proteins and glycoproteins (through their C-terminus) as well as phosphoglycans (through the reducing end of the chain) to the membrane of eukaryotic cell. Since the first full assignment of a GPI structure in (M.A.J. Ferguson et al., *Science* **1988**, *239*, 753) a number of GPI anchors have been characterized. The discovered role of GPIs as mediators of regulatory processes (including insulin-mediated signal transduction, cellular proliferation and cell-cell recognition) makes the chemical preparation of the compounds and their analogues of great interest. The lecture will embrace recent advances in the preparation of GPI anchors from *Trypanosoma cruzi* trypomastigote mucins (compounds **1** and **2**). The synthesis of a GPI compound from *Trypanosoma brucei* (**3**), which was prepared using MPEG (M = 5000) as a soluble polymer support, will be discussed as well.



Poster No. I-5

CHEMICAL SYNTHESIS OF GPI COMPOUNDS, SUBSTRATE ANALOGUES FOR A NOVEL BIOSYNTHETIC ENZYME IN *TRYPANOSOMA BRUCEI*

Yashunsky D & Nikolaev AV; College of Life Science, University of Dundee, United Kingdom

The abundance of the GPI-anchored variant surface glycoprotein in *Trypanosoma brucei* has made this organism extremely useful for the study of the GPI biosynthesis. The GPI biochemical pathway was validated chemically and genetically as a drug target for African sleeping sickness (M.A.J. Ferguson, *PNAS*, **2000**, *97*, 10673). The core structure of the GPI is known (**1a**) and the main features of its biosynthesis pathway were elucidated earlier using a cell-free system technique. We synthesized a set of glycosyl phospholipids, as substrates/potential inhibitors for the trypanosome GPI pathway mannosyl transferase III (MT-III). Compounds **2a** (natural acceptor substrate for the enzyme) and **2b** (its analogue with modified lipid moiety) were used to establish a biochemical assay for MT-III in a cell-free system. The preparation of deoxygenated, fluorinated or aminated structural analogues **3b-9b** and results of their testing as acceptor substrates for MT-III will be discussed.

$NH_2C_2H_4$-OPO_3H-6Man(α1→6)Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**1a**)
Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**2a,2b**) 3-fluoro-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**6b**)
2-deoxy-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**3b**) 4-deoxy-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**7b**)
2-amino-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**4b**) 6-deoxy-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**8b**)
3-deoxy-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**5b**) 6-amino-Man(α1→6)Man(α1→4)$GlcNH_2$(α)PI (**9b**)
PI = 6-*myo*-Ino1-OPO_3H-1Gro(2,3-di-O-acyl) (**a**) or 6-*myo*-Ino1-OPO_3H-$C_{18}H_{37}$-*n* (**b**)

Poster No. I-6

CONVENIENT SYNTHESIS OF SHORT OLIGOSACCHARIDE FRAGMENTS OF BACTERIAL POLYSACCHARIDES: THE EXAMPLE OF SHIGELLA FLEXNERI 3A

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Shigellosis, or bacillary dysentery, is a devastating disease of the most impoverished caused by Gram negative bacteria named *Shigella*. The *Shigella* is classified in four species. *Shigella flexneri* is the major cause of the endemic form of the disease in developing countries. In addition to the lipopolysaccharide being an important virulence factor for these bacteria, the corresponding O-specific polysaccharide part (O-SP) is the major target of the protective humoral response. Interestingly, the repeating units of most *S. flexneri* O-SPs are branched pentasaccharides which share a common linear tetrasaccharide backbone (**ABCD**). Diversity and serotype-specificity rely on branched α-D-Glucosyl (**E**) and O-Acetyl (*Ac*) "decorations". Looking forward to a better understanding of the serotype-specificity of the immune protection resulting from natural infection, we have undertaken the synthesis of oligosaccharides representative of frame-shifted fragments of the O-SP of various *S. flexneri,* among which serotypes 2a, 3a and 5a.
This contribution will report on the efficient linear and, whenever appropriate, convergent chemical synthesis of di- to pentasaccharide fragments of *S. flexneri* 3a O-SP, defined by the branched pentasaccharide **(E)AB(*Ac*)CD** repeating unit. Studies on targets bearing residue **A** or residue **B** at their reducing end will be investigated. Focus will be on the design, synthesis, and use of appropriate **D** donors as well as on the input of solvents involved in the glycosylation step.

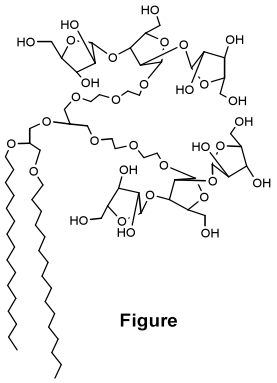
E **A** **B** **C** **D**
2)-[α-D-Glc*p*-(1→3)]-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-[2*Ac*]-α-L-Rha*p*-(1→3)-β-D-GlcNAc*p*(1→

Poster No. I-7

SYNTHESIS AND BIOLOGICAL EVALUATION OF BIVALENT ARABINOFURANOSYL GLYCOLIPIDS AS INHIBITORS IN MYCOBACTERIAL GROWTH ASSAYS

Jayaraman N, Naresh K, Mukherjee R & Chatterji D; Department of Organic Chemistry and Molecular Biophysics Unit, Indian Institute of Science, India

Lipoarabinomannans (LAMs) are present abundantly in envelops of several *mycobacterium* strains, such as *mycobacterium tuberculosis*, *mycobacterium laprae* and other *mycobacterial* opportunistic strains. Tuberculosis (TB) continues to be a leading infection and overcoming the multi-drug resistant to *mycobacterium tuberculosis* strains is essential in expanding effective therapies. Pathogenicities of LAMs involve arabinan motifs that are exposed on the surface of the *mycobacterium* cell wall. The arabinofuranosides are further capped with dimannopyranosides to afford Man_4Ara_6 and



Figure

Man_2Ara_4 components. We have undertaken an effort to synthesize a variety of branched arabinomannan oligosaccharides, identify the structural and conformational features of these synthetic oligosaccharides, as it pertains to their ability to bind target receptor proteins. This exercise is anticipated to lead in the development of small molecular weight multivalent sugar ligands as inhibitors of mycobacterial strains. Synthesis of oligovalent arabinofuranosyl trisaccharides attached with lipidic chains was accomplished (*e.g.* **Figure 1**), starting from individual components of the sugar moiety, the long alkyl chain moiety and glycerol which acts as the branch point of the oligovalent glycolipids. We are currently studying the effect of the oligovalent glycolipids as inhibitors in varied mycobacterium smegmatis involved assays. The results of synthesis and the biological studies of these oligovalent glycolipids, targeting specifically the mycobacterial pathogens, will be presented in the Symposium.

Poster No. I-8

SYNTHESIS OF NON-IONOGENIC ANTAGONISTS OF *E. COLI* LIPID A

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Lipid A is a phospholipodisaccharidic unit of lipopolysaccharides (LPS, endotoxin), which constitute the exterior monolayer of the outer membrane in Gram-negative bacteria. It is fully responsible for the endotoxic activity of LPS. The syndromes most commonly connected with endotoxin are severe sepsis and septic shock, which are systemic complications of many diseases. On the other hand, lipid A is a highly potent stimulator of immune system.
Due to the lysis of Gram-negative bacteria released LPS, they bind to a LPS-binding protein circulating in the blood and this complex binds to a receptor molecule found on the surface of immune cells called macrophages. This is thought to promote the ability of the toll-like receptor TLR-4 to respond to the LPS, triggering the macrophage to release various defense regulatory chemicals called cytokines. The cytokines then bind to cytokine receptors on target cells and initiate inflammation. Depending on the level of the released cytokines, the action of the agonistic *E. coli* lipid A can lead up to severe sepsis and septic shock.
The contribution introduces the results of our approach to the search for non-ionogenic, antagonistic lipodisaccharides that could interfere with the pathways processing lipid A and become the targets for anti-sepsis drug development. Thus, we have synthesized several antagonistic lipid A mimics, in which several linkages have been substituted with enzymatically non-hydrolysable bonds.
Acknowledgments: The work was supported by the APVT-51-039802 and VEGA-2/6129/26 grants.

Poster No. I-9

SYNTHESIS OF NOVEL MANNOSYL DERIVATIVES OF PEPTIDOGLYCAN MONOMER

Golub R[a], Peroković VP[a], Tomašić J[b] & Tomić S[a]; [a]Department of Chemistry, Faculty of Science, University of Zagreb, Croatia; [b]Institute of Immunology, University of Zagreb, Croatia

Lectins, carbohydrate-binding proteins, are very specific for monosaccharides such as mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine. The formation of glycoconjugates of biologically active compounds with monosaccharides, for which specific lectins exhibit high affinity, may enhance the biological activity of compounds.
We report on the synthesis of mannosyl derivatives of peptidoglycan monomer in order to study the influence of the mannose moiety on peptidoglycan's activity. Peptidoglycan monomer (PGM) is a natural disaccharide pentapeptide (GlcNAc-MurNAc-L-Ala-D-*iso*Gln-*meso*DAP(ωNH_2)-D-Ala-D-Ala) originating from *Brevibacterium divaricatum*. In several experimental models *in vivo* and *in vitro* PGM exhibited strong immunomodulating, antitumor and antimetastatic properties. In this derivative PGM is connected to mannose through an *O*-glycosidically bonded chiral linker. The key step in the preparation of the glycoconjugate was the condensation of the unprotected PGM with *N*-hydroxysuccinimide ester of *O*-mannopyranosyloxy carboxylic acid. In the process an amide bond was obtained with the free amino group of *meso*-diaminopimelic acid in parent PGM molecule.
Both anomers of the mannose conjugate of PGM were prepared and fully characterized. They are water-soluble and non-pyrogenic substances. Further studies, including experiments in animal models, are in progress.

Poster No. I-10

TOWARDS GALACTOSE AND MANNOSE DERIVED SMALL INHIBITORS OF GALECTINS

Tejler J, Salameh BA, Skogman F, Leffler H, & Nilsson UJ.; Organic Chemistry, Lund University, Sweden

In order to investigate roles of the galactose-binding galectin family of proteins, which are important in cancer growth and immune regulation, efficient inhibitors are essential. However, the use of natural oligosaccharide ligands as

inhibitors is hampered by their difficult synthesis, sensitivity to hydrolysis, and high polarity. Two strategies to circumvent these disadvantages by synthetic modifications of monosaccharides will be presented.
First, a fragment-based approach to the development of simple galactose-based monosaccharide inhibitors of galectin-3 will be discussed. Derivatization of galactose with structures optimized for interaction with two subsites neighboring the galectin CRD proved successful and gave monosaccharide derivatives superior to natural oligosaccharide ligands. Hence, aromatic 3-deoxy-3-triazol-1-yl-*O*-β-D-galactopyranosyl aldoximes provided highly selective and high affinity (K_d down to 11 μM) small monosaccharide based inhibitors of galectin-3. Molecular modeling suggests that pinching of an arg-residue by the galactose C3-triazole and the anomeric aldoxime leads to the observed high affinity and selectivity.
An alternative strategy based on using galactose-mimicking structures to simplify synthesis and possibly improve efficiency of novel galectin inhibitors will also be presented. The structural similarity between galactose and mannose, where the mannose HO-2 mimics the key galactose HO-4 and mannose C-1 functionalities mimic affinity-enhancing galactose C-3 structures, was hence successfully exploited. Easier synthetic access to C1 in mannose, as compared to C3 in galactose for attachment of affinity-enhancing structures rendered an important synthetic advantage. For example, a 1*H*-[1,2,3]-triazol-1-yl mannoside had for galectin-9N a K_d value of 540 μM, which compared favorably with its galactoside counterpart (K_d=670 μM) and with LacNAc (K_d=500 μM). Construction of a human galectin-9N homology model and modeling of its complexes suggests that the mannosides are indeed mimicking the galactose binding.

Poster No. I-11

ARGININE BINDING MOTIFS: DESIGN AND SYNTHESIS OF GALACTOSE-DERIVED ARGININE TWEEZERS AS GALECTIN INHIBITORS

Öberg CT[1], Leffler H[2] & Nilsson UJ[1]; [1]Organic Chemistry, Lund University, Sweden, [2]Dept. Laboratory Medicine, Lund University, Sweden

The galectins, a family of galactose-binding proteins, have been implicated in many biological events, notably inflammation, immunity and cancer progression,[1] which motivates the development of galectin inhibitors. We have recently reported the discovery of inhibitors with high affinity for galectins.[2] These inhibitors have been designed based on galectin-ligand crystal structures and particular success have been achieved by exploiting π--cation interactions between aromatic inhibitor substituents and arginine residues lining the CRDs.
Herein, we wish to report an inhibitor design that takes this one step further by adding suitably located anionic substituents that together with an aromatic substituent ”pinches” an arginine residue. Thus, a collection of arginine “tweezers” has been synthesized and evaluated as galectin inhibitors, which revealed an increased affinity for galectin-3. Molecular modeling corroborated the hypothesis of arginine pinching by the inhibitor aromatic and anionic substituents. These promising results, together with the fact that arginine is ubiquitously found on the surface of proteins, demonstrate that designed arginine tweezers provide a viable route towards efficient galectin inhibitors.
[1] H. Leffler editor, Glycoconjugate J., 19, 433 (2004).
[2] I. Cumpstey, A. Sundin, H. Leffler, U. J. Nilsson, Angew. Chem. Int. Ed., 44, 5110 (2005).

Poster No. I-12

ASSESSING THE INHIBITORY POTENCY OF GALECTIN LIGANDS IDENTIFIED FROM COMBINATORIAL GLYCOPEPTIDE LIBRARIES USING SURFACE PLASMON RESONANCE SPECTROSCOPY

Maljaars CEP,[a*] André S,[b] Halkes KM,[a] Gabius H-J[b]& Kamerling JP[a]; [a] Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, The Netherlands; [b] Institute of Physiological Chemistry, Faculty of Veterinary Medicine, Ludwig-Maximilians University, Germany. [*] Current address: NV Organon, Department of Medicinal Chemistry, The Netherlands.

Galectins are involved in several biological processes, such as cell-cell and cell-matrix adhesion, cell migration, and regulation of cell growth and apoptosis, with relevance to inflammation and tumor spread. Since galectin-1 (h-Gal-1) and -3 (h-Gal-3) are considered to be valuable diagnostic targets, it is of great interest to characterize their interaction mechanism. To obtain synthetic mimics that can be used for interaction studies, glycopeptide ligands were identified from the screening of three glycopeptide libraries. The screening of two combinatorial solid-phase (glyco)peptide libraries, containing Gal(β1-O)Thr, and Gal(β1-S)Cys and Gal(β1-N)Asn, respectively, with fluorescently labeled h-Gal-1 and h-Gal-3, mainly yielded peptide lead structures, which predominantly contained Cha, Phe, and Ile in the central part. In contrast, the screening of a library containing Lac(β1-O)Thr, predominantly resulted in glycopeptide hits, mainly containing Arg and Lac(β1-O)Thr.

Surface plasmon resonance-based inhibition assays were performed to measure the ability of some selected and resynthesized (glyco)peptide hits to inhibit galectin binding to immobilized asialofetuin. The h-Gal-1-specific O-lactosylated glycopeptides were 1.2 - 27 fold more potent than lactose, and all h-Gal-3-specific lead structures were 10 - 65.8-fold better than lactose. Two out of 11 h-Gal-1-specific (O-/N-galactosylated glyco)peptides showed a higher inhibitory activity than lactose, whereas the other structures showed an altered binding event, which may be explained by aggregation of the galectin.

Poster No. I-13

IDENTIFICATION OF INHIBITORS OF O-GLCNAC TRANSFERASE FROM A URIDINE-BASED LIBRARY

Leavy T, Boyce M & Bertozzi CR; University of California, Berkeley. California, USA

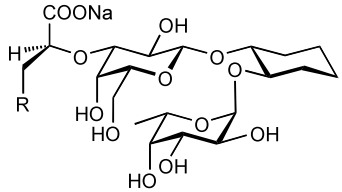
Glycosylation of intracellular proteins by *O*-GlcNAc has emerged as a widespread posttranslational modification. This unique form of glycosylation has been implicated in numerous cellular processes but a detailed understanding of its overall functional significance is lacking. Small molecule specific inhibitors of *O*-GlcNAc glycosylation could serve as valuable tools to probe the biological function of this modification. While a number of inhibitors that are active *in vitro* have been identified to date, physiologically relevant inhibitors that are active in cell culture have not yet been reported. Previously, our lab generated a 1338-member uridine-based library that has aided in the identification of a number of cell permeable inhibitors for various enzyme systems. In this study, a high-throughput assay was developed in order to screen the uridine library against OGT. Library screening revealed two structurally related uridine analogs that were resynthesized for hit verification and have subsequently emerged as the most potent inhibitors of OGT reported to date. These compounds show competitive inhibition with UDP-GlcNAc, and are selective for OGT over other UDP-recognizing enzymes. SAR studies of a panel of structurally related compounds are currently in process to probe the specific components of the inhibitors that are required for binding. Additionally, cell based studies are currently underway in order to investigate the potency of these compounds in cell culture.

Poster No. I-14

PREORGANIZATION OF THE CARBOXYLIC ACID OF SIALYL LEWIS^x MIMETICS IS ESSENTIAL FOR BINDING TO E-SELECTIN

Titz A[1], Alker AM[2], Porro M[1], Hennig M[2], Francotte ER[3], Schwardt O[1] & Ernst B[1]; [1]Institute of Molecular Pharmacy, University of Basel, [2]F.Hoffmann-La Roche Ltd., X-ray Crystallography; [3]Novartis Institutes for Biomedical Research, Separations, Switzerland

The selectins play a key role in the inflammatory process, i.e. the recruitment of leukocytes from blood vessels into inflamed tissues. Excessive infiltration of leukocytes can cause acute or chronic reactions, as observed in reperfusion injury, stroke or rheumatoid arthritis. Therefore, the antagonism of selectins is a valuable pharmaceutical approach. The tetrasaccharide epitope sialyl Lewis^x (sLe^x) is present in all physiological selectin ligands and served as lead structure in our search for antagonists. It has been shown that preorganizing the pharmacophores of sLe^x in the bioactive conformation [1] contributes substantially to the affinity of E-selectin antagonists [2].



For the preorganization of the carboxylic acid, the *(S)*-configuration of C-2 of the neuraminic acid mimetics, as well as the substituents *R* are decisive elements. In order to further investigate the contribution of the substituent *R* to the preorganization, a series of derivatives was synthesized and biologically evaluated.

[1] Scheffler, L. *et al. Angew. Chem. Int. Ed.* 1995, *34*, 1841. Rinnbauer, M. *et al. Glycobiology* 2003, *13*, 435.
[2] Kolb, H. C.; Ernst, B. *Chem. Eur. J.* 1997*, 3*, 1571.

Poster No. I-15

PREORGANIZATION OF E-SELECTIN ANTAGONISTS IN THE BIOACTIVE CONFORMATION

Schwizer D[1)], Kato A[1)], Meyer C[1)], Patton J[2)], Cutting B[1)], Schwardt O[1)], Magnani JL[2)] & Ernst B[1)]; [1)]Institute of Molecular Pharmacy, University of Basel, Switzerland, [2)]Glycomimetics, Rockville, USA

The activity of E-selectin antagonists benefits from the preorganization of the ligand in the bioactive conformation [1]. We have demonstrated that the optimal spatial presentation of the pharmacophores to the receptor site can be enforced by steric factors [2]. Along these lines, potent E-selectin antagonists based on novel Glc*N*Ac mimetics were developed. Biological evaluation in a static E-selectin binding assay showed substantial improvement compared to ligands missing the steric compression. The conformational flexibility vs. preorganization was further evaluated by computational techniques and NMR studies.

[1] B. Ernst, H. C. Kolb, O. Schwardt in *The Organic Chemistry of Sugars*, (Eds.: D. E. Levy, P. Fügedi), CRC Press/Taylor & Francis, Boca Raton, **2006**, pp. 828-862.

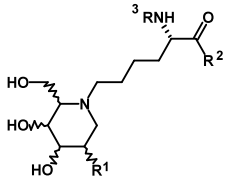
[2] G. Thoma, J. L. Magnani, J. T. Patton, B. Ernst, W. Jahnke, *Angew. Chem. Int. Ed.* **2001**, *40*, 1941.

Poster No. I-16

IMINOSUGAR-CONTAINING NEOGLYCOPEPTIDES

Stütz AE, Kartusch C, Steiner AJ & Wrodnigg TM; Glycogroup, Graz University of Technology, Austria

Conjugates between sugars and amino acids are of general interest in combinatorial chemistry and modern drug discovery and design [1]. Intramolecular reductive amination of various ketoaldohexoses [2] and pentodialdoses, respectively, with L-lysine containing peptides gives rise to a new class of neoglycopeptides, bearing *N*-alkylated iminosugar moieties of different configurations, covalently attached to the peptide backbone via a very stable tertiary amine. Examples will be presented and the potential of the method for peptide and protein modification will be outlined [3].



R^1 = OH or NHAc
R^2 = amino acid or Oalkyl
R^3 = amino acid or protective group

Acknowledgment: We thank the Austrian Wissenschaftsfonds for financial support, FWF P 18998-N17.

[1] Gruner SAW, Locardi E, Lohof E, Kessler H *Chem. Rev.* **2002**, 102, 491 – 514.

[2] Baxter EW, Reitz AB *J. Org. Chem.* **1994**, 59, 3175 – 3185.

[3] Stütz AE, Steiner AJ, Wrodnigg TM *European Patent Application*, EP 06019545, **2006**.

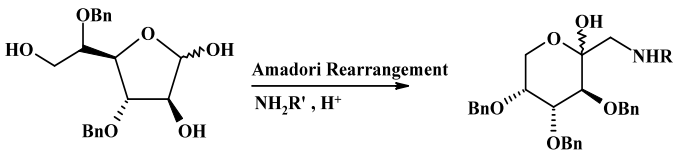
Poster No. I-17

THE AMADORI REARRANGEMENT OF PARTIALLY PROTECTED ALDOSES AS MODEL REACTION TOWARDS PROTEIN MODIFICATION

Kartusch C, Steiner AJ, Stütz AE & Wrodnigg TM; Glycogroup, Technical University Graz, Austria;

The decoration of proteins with carbohydrate motifs can introduce enormous diversity concerning structural as well as biological behavior. Therefore, protein glycosylation has become a very important research field [1], although synthetic methods are still demanding and time consuming.

We are interested in the investigation of the Amadori rearrangement as a tool for site selective protein modification. Having in mind that strain release by ring expansion during the Amadori rearrangement accounts for excellent yields of the product [2], 3,5-di-*O*-benzyl-α,β-D-glucofuranose **1** is thought to be an excellent model compound in order to investigate the optimal reaction conditions for the rearrangement (**2**).



1 **2**

Acknowledgment: We thank the Austrian Wissenschaftsonds for financial support, FWF P 18998-N17.

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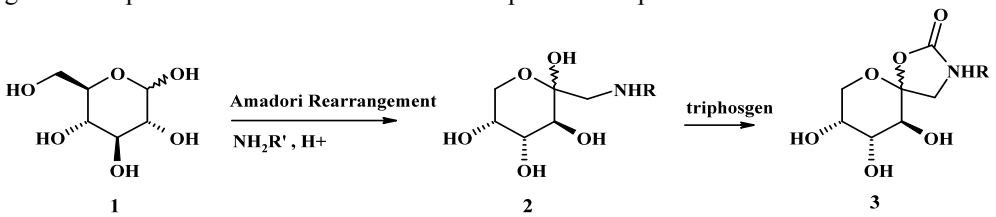
Poster No. I-18

THE AMADORI REARRANGEMENT AS KEY REACTION FOR PROTEIN MODIFICATION

Wrodnigg TM , Kartusch C, Steiner AJ & Stütz AE; Glycogroup, Technical University Graz, Austria

Glycoconjugates are major determinants on cell surfaces and have been recognized as immensely important molecules in Glycoscience. They are believed to exhibit great potential for new therapeutic strategies [1]. New concepts for reliable syntheses of pure samples of glycoconjugated proteins are clearly needed in order to promote this research field.

We are interested in the use of the Amadori rearrangement for protein modification. Applied to suitable aldoses, the rearrangement product presents a "C-glycosidic" conjugation (**2**). The generated hemiketal function can be stabilized by formation of a cyclic carbamate (**3**) between the anomeric hydroxyl group and the amino function resulting from the rearrangement. Experimental details of different examples will be presented.



Acknowledgment: We thank the Austrian Wissenschaftsfonds for financial support, FWF P 18998-N17.

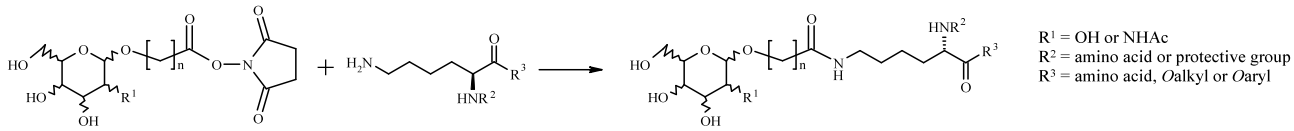
[1] a) Buskas T, Ingale S, Boons G-J *Glycobiology* **2006**, 16 (8), 113R – 136R b) Pratt MR, Bertozzi CR *Chem. Soc. Rev.* **2005**, 34, 56 – 68.

Poster No. 1-19

GLYCOCONJUGATION EMPLOYING CARBOHYDRATE BEARING *N*-HYDROXY-SUCCINIMIDE ESTERS

Steiner AJ, Kartusch C, Stütz AE & Wrodnigg TM; Glycogroup, Graz University of Technology, Austria

Glycosylation serves as a means of changing the properties of peptides and proteins. Carbohydrate moieties can influence the secondary structure, solubility and biological half-life of a peptide and may also enhance delivery, targeting the aglycon to specific cells or tissues [1]. Modification of the primary amino sites (*N*-terminal or lysine residues) of biologically active proteins employing carbohydrate building blocks has been investigated in the context of posttranslational protein glycosylation [2]. Our approach relies on the coupling of stable *N*-hydroxysuccinimide esters [3] featuring various carbohydrate moieties to the N_ε-amino functions of L-lysine residues in oligopeptides.



Acknowledgment: We thank the Austrian Wissenschaftsfonds for financial support, FWF P 18998-N17.

[1] Seitz O, Wong CH (Ed.) *Carbohydrate based Drug Discovery*, Wiley-VCH, Weinheim, **2003**, 169 – 214.

[2] see for example: a) Suzuki K, Susaki H, Okuno S, Yamada H, Watanabe HK, Sugiyama Y *J. Pharmacol. Exp. Ther.* **1999**, 288, 888 – 897. b) Baudys M, Uchio T, Hovgaard L, Zhu EF, Avramoglou T, Jozefowicz M, Rihova B, Park JY, Lee HK, Kim SW *J. Cont. Rel.* **1995**, 36, 151 – 157.

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Poster No. I-20

GLYCOCONJUGATES FROM *STREPTOCOCCUS PNEUMONIAE* SEROTYPES 6B, 19F AND 23F. EVALUATION OF DIFFERENT METHODS AND CHARACTERIZATION OF THE PRODUCTS

Peña L & Chang J; Center for the Study of Synthetic Antigens, Faculty of Chemistry, Universidad de la Habana, Cuba

Streptococcus pneumoniae is one of the major causative agents of pneumonia, meningitis, acute otitis media, bacteremia and sepsis; mainly in children less to 5 years old. Capsular polysaccharide (Psn) from the bacteria conjugated to a carrier protein has proven to be a successful vaccine to prevent the infection in children.

In the present work after generation of polysaccharide fragment by partial hydrolysis of the capsular polysaccharide of S. pneumoniae 23F, 19F and 6B we study two strategies for conjugation:

A- we adapted the method developed for synthetic Hib oligosaccharides[1]

B- Activation through periodic oxidation and conjugation by reductive amination.

The process of fragmentation and activation of capsular polysaccharide was followed by NMR spectroscopy techniques in order to characterize the resulting products for every reaction performed. All glycoconjugates were characterized by physico-chemical and immunological techniques.

1 V. Verez, V. Fernández, E. Hardy, M.E. Toledo, R. Roy, et al. Synthetic capsular polysaccharide antigen against Hamophilus influenzae type b, Science, 2004, 305, 522-525.

Poster No. I-21

FROM DISULFIDE PROTEINS TO THIOETHER PROTEINS: NEW SYNTHESES AND USES OF THIOLS

Bernardes GJL, Gamblin DP, Kasteren S van & Davis BG; Department of Chemistry, University of Oxford, United Kingdom

Synthetic protein modification opens the possibility of obtaining pure post-translational modified protein conjugates. This has the potential to unravel the complex biological effects different post-translational modifications (PTM) can have. [1]

Glycosylation is a major PTM and it has been estimated to occur in 50% of proteins from eukaryotic cells. We have recently described a one-pot method consisting of direct thionation followed by thiol-mediated chemoselective ligation which can be used for site-selective protein glycosylation. This procedure, which uses Lawesson's reagent, has been shown to be fully compatible with unprotected sugars, the products of which can be directly used in a selenenylsulfide protein glycosylation strategy.[2]

The covalent attachment of prenyl anchors to proteins is also a major PTM. A ready two-step procedure has been developed to directly convert allylic alcohols into the corresponding thiols, the products of which have been successfully conjugated in a site selective manner to a protein. These results demonstrate the first examples of chemical site-selective protein prenylation and confirm the dramatic effect of prenylation in the hydrophobicity of the protein, further suggesting that prenylation can be used to modulate the function of the protein.

We are now investigating the conversion of disulfide- to thioether-linked glycoconjugates by using amino-phosphines in a unique reaction in which a single sulfur atom is lost from a disulfide glyconjugate. The first results show that this procedure is compatible with a variety of different sugars, including GlcNAc, and also with different amino acid residues. We are currently undertaking studies on protein systems.

[1] B. G. Davis, *Science* **2004**, *303*, 480. [2] G. J. L. Bernardes *et al.*, *Angew. Chem. Int. Ed.* **2006**, *45*, 4007.

Poster No. I-22

METAL MEDIATED PROTEIN MODIFICATION: GLYCOPROTEINS BY ORGANOMETALLICS

Chalker J & Davis BG; Chemical Biology, University of Oxford, United Kingdom

Organometallic chemistry has revolutionized the capacity for selective bond construction in modern organic synthesis. In particular, Mizoroki-Heck, Suzuki, and Sonogashira cross-couplings have matured to functionally tolerant reactions that can be run in aqueous media. However, adapting these transformations for protein modification is complicated by a narrow pH window, fragile tertiary structure, and the presence of lewis basic side chains that coordinate metallic species. Pursuing such transformations on proteins, we describe progress in site-selective di-iodination of tyrosine residues and subsequent metal mediated modification with carbohydrate derived coupling partners. This strategy is a new approach to divalent glycoproteins by tyrosine-selective protein modification.

Poster No. I-23

INVESTIGATION OF 5'-THIO-UDP-GAL AS A DONOR SUBSTRATE FOR GALACTOSYLTRANSFERASES

Mannerstedt K, Palcic MM & Hindsgaul O; Carlsberg Laboratory, Denmark

The surface of animal cells is covered with different carbohydrate structures such as glycoproteins and glycolipids. These structures are known to be involved in divers and important biological processes acting as receptors for growth factors, toxins, viruses, as well as binding to other cells. The metabolism of cell-surface glycolipids involves their endocytosis and recycling back through internal organelles where they are either catabolized by glycosidases or extended by glycosyltransferases before they head back again to the cell-surface. Deficiencies in any of the enzymes involved in this metabolism can lead to severe disease. We are therefore developing tools to facilitate the study of the metabolism of cell-surface glycolipids.

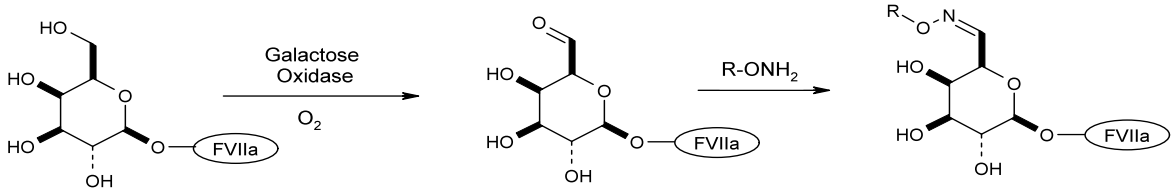
We describe here an investigation of the suitability of a series of galactosyltransferases for the preparative synthesis of metabolically-stabile glycolipid analogs. Synthetic 5´-thio-UDP-galactose, where the galactose residue has a sulfur atom in the ring instead of the natural oxygen atom, is evaluated as a donor substrate with different glycolipid-related acceptors, to yield galactoside-analogs having sulfur in the ring. Such thio-glycosides are expected to be much more resistant to glycosidases compared to the corresponding oxygen compounds, thus diminishing their lysosomal degradation during recycling. It is hoped that this increased stability will permit a more facile analysis of intracellular glycosyltransferase activities.

Poster No. I-24

GLYCOENGINERING OF RECOMBINANT FVIIa USING GALACTOSE OXIDASE

Behrens C, Garibay PW & Zundel M; Protein and Peptide Chemistry, Novo Nordisk A/S, Denmark

Recombinant blood factor VIIa (rFVIIa) has recently been proved useful for prophylactic treatment of severe hemophilia patients with inhibitors [1], and it is likely that prophylactic rFVIIa treatment besides reducing the number of bleeding episodes also may prevent or reduce the development of chronic hemophilic arthropathies in such patients [2]. However, with a short circulating half-life of only 2h, rFVIIa is not ideal for long term prophylactic treatment, as both high doses and frequent injections are required for maintaining pharmacological relevant plasma levels. Thus rFVIIa analogues with prolonged in vivo activities could be of interest in prophylactic treatments. Given the complexity of FVIIa, we have focused on chemistries with high site-specificity, and have examined the use of galactose oxidase to selectively modify rFVIIa. Galactose oxidase is a copper enzyme that catalyzes the two-electron oxidation of galactose or N-acetylgalactose to their corresponding 6-aldo derivatives. Galactose oxidase offers a mild and highly selective method for introducing aldehyde functionalities into glycoproteins. Here we present work on glycoengineered FVIIa using a chemoenzymatic process comprising of 1) trimming of sialic acids with neuraminidase, 2) oxidation of exposed galactose residues with galactose oxidase and 3) oxidation with hydroxylamine. The protocol has been used to prepare glycopegylated FVIIa derivatives.



[1] Young, G.; McDaniel, M. and Nugent, D.J. Prophylactic recombinant factor VIIa in haemophilia patients with inhibitors. Haemophilia 2005; 11: 203.

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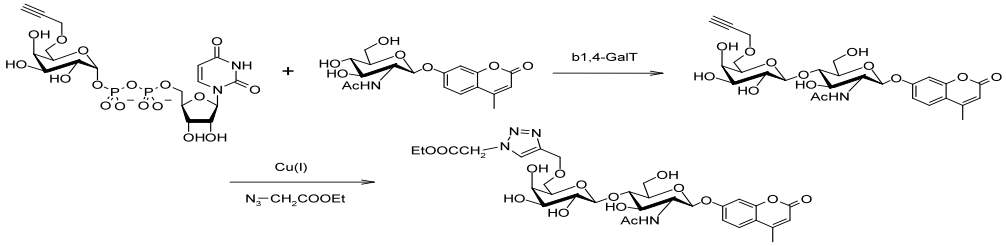
Poster No. I-25

TOOLS FOR CHEMOENZYMATIC MODIFICATION OF GLYCOPROTEINS

Zundel M & Behrens C; Protein and Peptide Chemistry, Novo Nordisk A/S, Denmark

It is often desirable to improve the pharmacokinetic properties of a therapeutic protein, for example by conjugating protracting moieties such as PEG polymers or albumin binders. We show here that when the protein of interest is a glycoprotein, one can take advantage of the N-glycan moieties to introduce a modification on the protein according to the following scheme: 1) initial trimming of the N-glycan tree to the GlcNAc level using sialidase and galactosidase 2) β1,4 galT catalyzed introduction of a synthetic galactose-UDP derivative [1] carrying a non-biogenic reactive handle and 3) coupling of the modifying group.

The feasibility of the two last steps of this scheme has been established on a model system using GlcNAc-UM as the acceptor, and 6-O-propargyl-galUDP as the donor in the transgalactosidation step [2]:



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Poster No. I-26

TARGETING OF GLYCOPROTEINS BY GALACTOSYLTRANSFERASE-MEDIATED-LABELING (GALTMEDLAB)

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The paper presents our work on the chemo-enzymatic synthesis of novel modified nucleotide sugars and their potential use in glycobiology and biomedical research. It is well documented in literature that changes in the glycosylation pattern of glycoproteins are related to diseases like rheumatoid arthritis (IgG), IgA nephropathy (IgAN)

or Tn-syndrome (cancer). The aim of our current project is the development of a method for the specific labeling of the involved glycan structures by the glycosyltransferase-catalyzed transfer of modified donor substrates.
The combination of the enzymatic oxidation of UDP-Gal(NAc) using galactose oxidase with the chemical modification using biotin-ε-amido-caproylhydrazide (BACH) in one-pot led to the efficient synthesis of UDP-6-biotinyl-Gal(NAc) (UDP-Gal(NAc)-biotin) in 100 mg scale. So far, the galactosyltransferases hβ3Gal-T5, hβ4Gal-T1, hβ4Gal-T4, mα3Gal-T, and core1-β3Gal-T from *Drosophila melanogaster* were identified to transfer the Gal-biotin-label onto the neoglycoconjugate BSA$(GlcNAc)_{17}$, ovalbumin, IgG or human IgA1, respectively, by utilizing their intrinsic acceptor specificity and their acceptance of UDP-6-biotinyl-Gal as donor substrate. The galactosyltransferase-mediated-labeling (GalTMedLab) with UDP-Gal-biotin was further applied to determine under-galactosylation of N-glycans on IgG from different species (rat, mouse, horse, human, chicken) and on IgG from patients with RA. For IgAN we can demonstrate that GalTMedLab has the potential to become the first diagnostic serum assay for this disease.
Acknowledgement: We thank Prof. Dr. Henrik Clausen (Copenhagen), Dr. Tilo Schwientek and Prof. Dr. Georg Hanisch (Cologne) as well as Prof. Dr. E. G. Berger (Zurich) for providing GT enzymes and GT genes.

Poster No. I-27

CHEMICAL AND CELL BIOLOGICAL APPROACHES TO STUDY *O*-LINKED BETA-*N*-ACETYLGLUCOSAMINE

Boyce M & Bertozzi CR; Department of Chemistry, University of California at Berkerley, USA

The reversible attachment of monomeric beta-*N*-acetylglucosamine (GlcNAc) to serine and threonine hydroxyls of nuclear and cytoplasmic proteins comprises a ubiquitous signaling paradigm in mammalian cells. This regulated protein "*O*-GlcNAcylation" has been implicated in critical biological processes, including cellular energy metabolism, cell migration and cell cycle control. In addition, *O*-GlcNAc transferase (OGT), the enzyme responsible for protein *O*-GlcNAcylation, is essential for cell viability and may be dysregulated in clinically important human diseases, including diabetes. However, the identities and functions of the proteins that are *O*-GlcNAcylated in response to biological cues remain largely unknown, and the regulation of OGT activity and substrate choice in response to cellular signals is poorly understood. We have taken chemical and genetic approaches to study the role of OGT and *O*-GlcNAcylation in model cell systems. First, we have exploited a metabolic engineering approach previously developed in our lab to label native OGT substrates with unnatural azide-bearing GlcNAc analogs. This system permits the *in vivo* labeling and subsequent *in vitro* purification and identification of endogenous OGT substrates. We are using this approach to identify OGT substrates that are *O*-GlcNAcylated in response to cell migration cues and to test the potential physiological significance of these modifications using functional migration assays. Second, we are developing model systems for studying the cellular functions of OGT activity, including the construction of a cell culture system in which OGT protein levels are under the control of a small molecule. We are using this system to dissect the functional role of OGT and its substrates on basic processes such as cell cycle regulation and transcriptional control. Taken together, our results will help to elucidate the physiological significance of OGT and protein *O*-GlcNAcylation in mammalian cell biology.

Poster No. I-28

BREAKING IMMUNE SELF-TOLERANCE OF CANCER-ASSOCIATED CARBOHYDRATE ANTIGENS FOR CANCER IMMUNOTHERAPY

Miller I[1], Prescher J[1] & Bertozzi C[1-3]; Departments of [1]Chemistry and [2]Molecular and Cell Biology and the [3]Howard Hughes Medical Institute, University of California at Berkeley, Berkeley, CA, USA

Cancer cells often show aberrant glycosylation of surface molecules, resulting in the display of carbohydrate epitopes never or rarely seen on the cell surfaces of healthy tissues. These epitopes, known as tumor-associated carbohydrate antigens (TACAs), have received significant attention for their promise as components of anti-cancer vaccines. While clinical trials have shown that the body is capable of producing a weak antibody response to these molecules, they not yet been shown to be an effective tool in the treatment of cancer. One explanation for their relative ineffectiveness is that the body recognizes these molecules as "self," and so is incapable of mounting a strong immune response against them. The cancer vaccine strategy outlined here seeks to break the body's immune self-tolerance of TACA vaccines by first chemically modifying the carbohydrates to make them foreign. The immune response against the modified TACAs can be directed against tumors by providing the tumor with correspondingly modified carbohydrate building blocks that are tolerated by the body's glycosylation machinery, resulting in the display of the modified carbohydrate antigen on the surface of the tumor. Specifically, disaccharide TACA sialyl Tn has been modified at one or both monomer positions with an azido group appended to the *N*-acetyl substituent. By providing azido-modified precursors, cancer cells can be induced to display the modified, non-self TACA. This work describes the synthesis of these unnatural antigens and initial findings on the strength and specificity of the immune response they generate.

Poster No. I-29

HIERARCHICAL ASSEMBLY OF MODEL CELL SURFACES: SYNTHESIS OF MUCIN MIMETIC POLYMERS AND THEIR DISPLAY ON SUPPORTED BILAYERS

Rabuka D[1], Parthasarathy R[2], Chen X[1], Groves JT[1] & Bertozzi CR[1]; [1]University of California, Berkeley. California, USA [2]University of Oregon, Eugene, Oregon, USA

Molecular level analysis of cell surface phenomena could benefit from model systems comprising structurally-defined components. We have begun to work toward the bottom-up assembly of model cell surfaces – the synthesis of mucin mimetics and their incorporation into artificial membranes. Natural mucins are densely glycosylated O-linked glycoproteins that serve numerous functions on cell surfaces. Their large size and extensive glycosylation makes the synthesis of these biopolymers impractical. We have designed synthetically tractable glycosylated polymers that possess rod-like extended conformations similar to natural mucins. The glycosylated polymers were end-functionalized with lipid groups and embedded into supported lipid bilayers where they interact with protein receptors in a structure-dependent manner. Furthermore, their dynamic behavior in synthetic membranes mirrored that of natural biomolecules. This system provides a unique framework with which to study the behavior of mucin-like macromolecules in a controlled, cell surface-mimetic environment. We have begun to probe the biophysical characteristics of the mucin mimetic polymers using fluorescence interference contrast microscopy, which provides nanometer-scale topographic information about objects near a reflective interface. We are able to measure the orientation of the mucin mimics relative to the membrane and have begun to quantitatively explore the mechanisms that control the spatial conformation of cell surface proteins. Of particular interest are the roles of macromolecular density and elaboration with higher order glycans, including charged monosaccharides, in governing molecular orientation.

Poster No. I-30

SOLID-PHASE CHEMICAL TOOLS FOR GLYCOMICS: NANOPARTICLES

Thygesen MB & Jensen KJ; Dept. of Natural Sciences, University of Copenhagen, Faculty of Life Sciences, Denmark

Quantum Dot (QD) and Gold (Au) nanoparticles are likely to become key tools for glycobiology, due to their unique spectroscopic properties. QD's, which are made of semi-conductor material, show strong, size-dependent fluorescence and exceptional stability against photobleaching. Applications are likely to include bio-imaging (*e.g. in vivo* studies of glycan receptors), sensors of multivalent glycan binding interactions, and as a general tool for systems biology. Functionalized nanoparticles are central to the emerging field of glyconanobioscience. However, a crucial element for the application of nanoparticles in glycomics is the spacer (linker) between the glycan and the nanoparticle, as it ideally (i) should enable highly efficient and chemoselective anchoring of the glycan to the nanoparticle, (ii) should allow correct display of the glycan and allow for interaction between the glycan and proteins, and (iii) should confer suitable physico-chemical properties to the glyco-nanoparticle. Hence, the linker should have build-in chemistry for chemoselective reactions and modulate the properties of the particle to make it biocompatible.

Here we present new and general methods for the bio-compatible functionalization of nanoparticles based on bifunctional spacers, which allow chemoselective, oriented, covalent anchoring of carbohydrates. Well-defined PEG chains were functionalized with combinations of aminooxy, thiol, or azido groups, which were introduced by Mitsunobu chemistry. Phth and Fmoc protected aminooxy groups were used for the crucial quantification of loading, *i.e.* ligand density.

Poster No. I-31

ASSESSING THE TERMINAL GLYCOSYLATION OF GLYCOPROTEINS BY NAKED EYE

Sørensen MD, Martins R & Hindsgaul O; Carbohydrate Chemistry, Carlsberg Laboratory, Denmark

We describe a simple new tool that can provide information on the *terminal* glycosylation state of a glycoprotein. The naked eye can be used as a sensor making sophisticated equipment unnecessary. One immediate area of application would be in the analysis of recombinant glycoprotein therapeutics.

The method begins by exposing a glycoprotein to an exo-glycosidase of known specificity. Any released monosaccharide is then covalently captured on hydroxylamine-functionalized glass beads where it is detected by complex formation with a novel tetramethylrhodamine-functionalized aryl boronate reagent (TMR-B).

The TMR-B stains the glass beads red in proportion to the amount of monosaccharide that was captured. The red color derived from exposure to micromolar concentrations of monosaccharide can easily be visualized by naked eye, and the intensity can be used to estimate the amount of monosaccharide that was present in relation to a set of standards. Incubation of the stained glass beads with glycerol releases the TMR-B back into solution allowing more precise spectroscopic quantization.

The method has been demonstrated for the estimation of terminal β-galactosylation of asialo-fetuin, but is not restricted to hexoses like galactose, as captured fucose, sialic acid and N-acetylglucosamine also form complexes with TMR-B.

Poster No. I-32

DESIGN AND SYNTHESIS OF OLIGOBORONIC ACIDS FOR THE RECOGNITION OF COMPLEX OLIGOSACCHARIDES

Takahashi D & Hindsgaul O; Carbohydrate Chemistry, Carlsberg Laboratory, Denmark

Cell surface carbohydrates structures, as part of glycoconjugates such as glycolipids and glycoproteins, play a central role in cell surface recognition. Therefore, new tools to facilitate the structural analyses of the oligosaccharides and elucidation of structure-activity relationships are highly desirable. Carbohydrate-binding proteins such as lectins and antibodies have been very widely used in such studies.

We propose the development of small-molecules that have carbohydrate-recognition properties equivalent to those of proteins. Boronic acids are known to bind diol moieties through reversible boronate formation under physiological conditions. Consequently, boronic acid compounds have been widely used as artificial receptor for sugars like glucose and fructose. But small molecule involving boronic acids have not yet been reported to bind to a defined oligosaccharide strongly and specifically in neutral water.

Herein, we report the synthesis of oligoboronic acids designed to bind to non-reducing oligosaccharides. Our design of these small carbohydrate-recognizing molecules incorporates three major components: 1) complex formation between a boronic acid moiety and pyranoside diols, 2) the construction of an appropriate three-dimensional scaffold bearing two or more boronate moieties and 3) inclusion of fluorescent tag or biotin to permit high sensitivity detection. To this end, we designed a tri-boronic acid attached to a tetra-cyclic amine (1,4,7,10-tetraazacyclododecane) scaffold tagged with a fluorescein moiety and accomplished its synthesis. We report the carbohydrate binding properties of this molecule, and those of simpler diboronic acid analogs.

This work was supported by the Danish Agency for Science, Technology and Innovation and the Japan Society for the Promotion of Science.

Poster No. I-33

NEW METHODS IN THE STRUCTURE ANALYSIS OF COMPLEX BACTERIAL OLIGOSACCHARIDES

Petersen, BO, Nyberg, NT, Sørensen, OW & Duus JØ; Carlsberg Laboratory, Denmark

The understanding of bacterial oligo- and polysaccharide function and biosynthesis require detailed description of the structure, starting with the primary structure and when possible conformation and dynamic descriptions. Here NMR spectroscopy has a central role combined with chemical analysis and mass-spectrometry.

The methods for primary structure determination of simpler oligosaccharides are well established, but often the task of NMR assignment is complicated either by the heterogeneity of the sample or the size of the non-repeating structure even using fairly high magnetic fields.

Here will be presented our contributions to the toolbox of NMR experiments towards the structure determination of rather large oligosaccharides with an example of an oligosaccharide with more than 30 carbohydrate residues. The experiments are extensions to the H2BC experiment developed by us and has proven very useful for carbohydrate assignment.

Another complication for the understanding of bacterial oligo- and polysaccharide structure-functions is the potential problem of chemical or enzymatic modifications during isolation and purification. Here the use of HR-MAS NMR of intact bacterial cells can clarify whether the purified carbohydrates are identical to the structures at the surface of intact bacteria.

Poster No. I-34

DIFFERENCES AND SIMILARITIES OF SUGAR KINASES STUDIED BY STD NMR SPECTROSCOPY

Blume A[1,2], Benie AJ[2], Fitzen M[2], Berger M[3], Hinderlich S[3], Schmidt RR[4], Reutter W[3] & Peters T[2]

[1]Carlsberg Laboratory, Denmark; [2]University of Lübeck, Institute of Chemistry, Germany; [3]Charité-Universitätsmedizin Berlin, Institut für Biochemie und Molekularbiologie, Germany; [4]University Konstanz, Fachbereich Chemie, Germany

Sugars are used by cells as a source of carbon or energy, but they also play a key role in many biological processes such as cell migration, cell signaling, or cell-cell recognition. The first step in sugar metabolism after transport into the cell is phosphorylation to prepare them for further chemical reactions, either catabolic or anabolic. This phosphorylation reaction is catalyzed by specific sugar kinases.

Using saturation transfer difference NMR spectroscopy (STD NMR) we characterized the interactions of three sugar kinases with a number of different ligands under physiological conditions, i.e. in aqueous solution. STD NMR has the potential, to gain insight into so far not known mechanistic aspects of enzymes, and the characterization of ligand binding epitopes is a potential key for the design of compounds for use as inhibitors.

Three sugar kinases from the hexokinase family were investigated in this study. Hexokinase is the first enzyme in the glycolytic pathway, catalyzing the transfer of the γ-phosphate group from ATP to the 6-hydroxyl group of glucose to form glucose-6-phosphate and ADP. *N*-acetylglucosamine (GlcNAc) kinase converts GlcNAc into GlcNAc-6-phosphate, which then can enter either a catabolic pathway ultimately leading to the formation of fructose-6-phosphate, or it can enter an anabolic pathway leading to the formation of UDP-GlcNAc. *N*-actylmannosamine

(ManNAc) kinase is part of the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase and phosphorylates ManNAc in the six positions to yield ManNAc-6-phosphate and ADP.
A detailed understanding of sugar kinases is particularly important in the context of many diseases related with sugar kinases. A fundamental knowledge of substrate binding to sugar kinases in solution, and the knowledge of mechanistic aspects of the enzymatic reaction are important in furthering our understanding of these enzymes and to obtain a more detailed insight into similarities and differences between members of one family.

Poster No. I-35

STUDY BY NUCLEAR MAGNETIC RESONANCE OF THE INTERACTION OF CYCLOPHILIN B WITH HEPARIN

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The cyclophilin B (CyPB) is a prolyl *cis*/*trans* isomerase (PPIase) found in biological fluids and able to bind to cell surface of T lymphocytes and macrophages. CyPB induces migration and adhesion of these leukocytes, by way of activation of β1 integrins, suggesting that CyPB might be involved in the recruitment of inflammatory leukocytes. CyPB interacts with two types of binding sites: The first one is formed at least by the CD147 receptor, the other one are cell surface Heparan Sulphate ProteoGlycane (HSPG). Interactions of CyPB with CD147 and HSPG induce the activation of ERK1/2MAPK and generate calcium signaling.
Modifications by site-directed mutagenesis in either CD147 (Pro^{180} and Gly^{181}) or the catalytic site of CycB abolish the cell responses induced by both proteins, suggesting that the PPIase activity of cyclophilins is required to induce cell responses *via* CD147. The binding region of CyPB to Heparan sulfate has been delineated by site-directed mutagenesis and proposed to contain the peptides $^{3}KKK^{5}$ in the N-terminal region of the protein and in addition the region containing $^{14}YFD^{16}$. On the other hand, the HS motif recognized by CyPB is an octasaccharide of which an unsubstituted glucosamine is the main specification.
The interaction of the CyPB protein with heparine DP8, DP12 and DP14 has been investigated by Nuclear Magnetic Resonance. The interaction results in the perturbation of the chemical shift of the resonances from amino acid in the binding region. The region delineated includes the N-terminal unstructured region of the protein and the C-terminus. However, the $^{14}YFD^{16}$ is not found in the binding region. The previous mutagenesis probably resulted in an unfavorable protein conformation for binding. Impact of the heparin binding on the PPIase activity of CyPB with a CD147 peptide was explored. We indeed observed a stimulated activity of the CyPB in presence of DP12. This is supported by the data on protein/heparin interaction that shows an extension of the chemical shift perturbations upon binding towards the binding site, including for example the Tryptophane. The hypothesis that PPIase activity of CyPB would be dependent on the binding to specific heparan sulphate motifs is an original mechanism of regulation of signal transduction. This will be further explored by using an IgG-like domain of CD147 as substrate for CyPB, in the presence or not of Heparine.

BENZON SYMPOSIUM No. 54

GLYCOSYLATION:
OPPORTUNITIES IN DRIG DEVELOPMENT

JUNE 11-14, 2007, COPENHAGEN, DENMARK

Organizing committee:
Ole Hindsgaul (Copenhagen), Henrik Clausen (Copenhagen),
Monica M. Palcic (Copenhagen) and Povl Krogsgaard-Larsen (Copenhagen)

Abstracts - TUESDAY, June 12, 2007

O-GLCNAC CYCLING ON REGULATORY PROTEINS: A NUTRIENT/STRESS SENSOR REGULATING TRANSCRIPTION, TRANSLATION AND SIGNALLING

Hart GW, Slawson C, Housley M, Whelan S, Wang Z, Sakabe K, Cheung W, Butkinaree P, Park K, Shimoji S, Zeidan Q, Dias W & Bullen J; Biological Chemistry, John Hopkins University School of Medicine, USA

O-GlcNAc is an abundant modification of nucleocytoplasmic proteins that cycles rapidly in response to stimuli, metabolism and nutrients. O-GlcNAc has a dynamic interplay with phosphorylation, often competing with it. During the past twenty-four years, O-GlcNAc has been documented on over five hundred proteins from virtually every functional class. O-GlcNAc occurs in all metazoans and the on internal proteins of many viruses. O-GlcNAc is required for life at the single cell level in mammals. O-GlcNAc cycling is controlled by two highly conserved enzymes, O-GlcNAc Transferase (OGT) and O-GlcNAcase (OGase). OGT is regulated by tyrosine phosphorylation, availability of its donor substrate, UDP-GlcNAc and by its specific transient interactions with myriad targeting subunits. OGase appears to also be highly regulated by transient protein:protein interactions, but much less is known.
Recent data support the hypothesis that O-GlcNAcylation is an important nutrient stress sensor that modulates signaling cascades, transcription and translation. Increased O-GlcNAcylation is a underlying mechanism for both insulin-resistance (type II diabetes) and for glucose toxicity (particularly abnormal transcription associated with hyperglycemia). Furthermore, it is becoming clear that dysregulation of O-GlcNAc plays a role in neurodegenerative diseases associated with aging (eg. Alzheimer's Disease) and in the activities and turnover of oncoproteins or tumor suppressors. Compounds that selectively disrupt the protein:protein interactions targeting the O-GlcNAc cycling enzymes, should have great potential as therapeutic agents.
Supported by NIH grants HD13563, CA42486, DK61671, DK71280, and NIH contract N01-HV-28180. Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody. Terms of this arrangement are managed by JHU.

ROLES FOR O-FUCOSE GLYCANS IN NOTCH SIGNALLING

Stanley P, Ge C, Stahl M, Uemura K, Shi S & Hou X; Department of Cell Biology, Albert Einstein College of Medicine, USA

Notch receptors contain 29-36 epidermal growth factor-like (EGF) repeats, many of which are potentially modified with *O*-fucose at the conserved consensus site $C_2X_{4-5}S/TC_3$ by protein *O*-fucosyltransferase 1 (Pofut1). Fringe, a β(1,3) *N*-acetylglucosaminyltransferase, adds a GlcNAc to *O*-fucose attached to Notch EGF repeats. The disaccharide can be further elongated by other glycosyltransferases. Pofut1 is essential for Notch signaling, while Fringe potentiates signaling by Delta1 and inhibits Jagged1-induced signaling. In order to understand mechanisms by which *O*-fucose glycans control Notch signaling, we have investigated ligand-dependent and ligand-independent Notch signaling and ligand binding in embryonic stem (ES) cells lacking Pofut1, CHO cells with RNAi-targeted Pofut1 and Lec13 CHO cells that have low GDP-fucose. Cells lacking Pofut1 or GDP-fucose responded poorly to ligand-induced Notch signaling. However, Notch receptors were equivalently present at the cell surface in the absence of Pofut1 or O-fucose on Notch. Lec13 cells were rescued for both ligand binding and Notch signaling by the addition of Fuc but not Gal. We conclude that neither Pofut1 nor GDP-Fuc are essential for Notch receptors to traffick to the cell surface in non-polarized mammalian cells, but that O-fucose on Notch is necessary for optimal ligand binding and Notch signaling. We have also found that O-fucose in the ligand binding site of Notch1 is necessary for optimal ligand binding and Notch1 signaling. Mice homozygous for a point mutation that abrogates the O-fucose site in Notch1 EGF12 (T466A) grow more slowly than littermates and have a marked defect in T cell development. By contrast, Notch1 T466S

acquires O-fucose and is also reverted for ligand-induced Notch signaling. Thus O-fucose in the ligand binding domain of Notch1 is necessary for optimal signaling to occur.
Supported by NCI grant RO1 95022

GLYCOSYLATION IN BIOPHARMACEUTICALS

Harris RJ; Analytical Development, Genentech, Inc., USA

Nearly all of Genentech's licensed biopharmaceuticals are glycoproteins whose oligosaccharides may confer desirable or undesirable properties. This presentation will discuss glycosylation as it relates to clearance, cell line selection, process development, analytical testing, and commercial-scale production.

Terminal saccharides can mediate clearance via the asialoglycoprotein and mannose receptors. For a TNFR-IgG molecule produced in CHO cells, clearance from primates was mediated by terminal GlcNAc moieties on the N-linked complex-type structures, with little contribution by terminal Gal. For two therapeutic antibodies, clearance from mice or humans was independent of Fc oligosaccharide type. The impact of O-linked oligosaccharide sialylation on clearance of a receptor-Fc fusion protein was also studied.

Production in CHO cells has glycosylation advantages relative to myeloma and HEK/293 cell lines. Adding galactose to cultures can increase galactosylation of antibodies, which may confer higher *in vitro* complement-dependent cytotoxicity. Higher afucosylated forms correlate with increased *in vitro* ADCC, with greater effects in the low percentage afucosylated range. Analytical methods have been developed to rapidly screen N-glycans, especially for recombinant antibodies, which allow us to measure minor forms at sensitive levels. Glucose in mammalian cell cultures leads to glycation that may either be distributed across a large number of lysine side-chains or enriched at one accessible site.

Unexpected variability can occur at the commercial manufacturing scale. For example, beta-galactosidase activity was observed in a low-pH purification pool for TNK-tPA, and an EndoH-like activity was also inferred when a culture medium contaminated with bacteria was used to produce an antibody. Regulatory authorities can consider glycosylation an indicator of cellular metabolism, thereby insisting on a degree of consistency that can present challenges for batch release testing or when demonstrating comparability.

MODIFIYING GLYCANS TO IMPROVE PERFORMANCE OF GLYCOPROTEIN THERAPEUTICS

Zopf D, DeFrees S, Clausen H, Bayer R, Sjoberg E, Bowe C & Johnson K; Drug Development, Neose Technologies Inc, USA

Hundreds of recombinantly expressed glycoproteins are approved as drugs or are in development for a wide variety of therapeutic applications. Their potencies primarily rely upon correctly folded polypeptide domains capable of binding to cognate receptors, but important pharmaceutical properties such as polypeptide conformational stability, half-life, solubility, and immunogenicity often are determined by the presence of attached glycan chains with specific structural features. Given the important modulatory roles that carbohydrates may play, we reasoned that introducing certain modifications on glycan chains might extend the range of benefits conferred by native sugar structures. By developing a library of recombinant glycosyltransferases we have created a technology platform that improves therapeutic performance of glycoprotein drugs by *in vitro* addition of native or substituted sugars. For example, significant improvements in half-life occur when soluble polymers such as polyethyleneglycol (PEG) are appended to glycan chains as substituents linked to sialic acid. This process, termed GlycoPEGylation™, employs any of several sialyltransferases plus a modified CMP-NeuAc donor synthesized with PEG covalently linked to sialic acid. Acceptors can include N- or O-glycans introduced on a protein by a eukaryotic host expressor cell. Alternatively, to take advantage of the economy of protein expression in *E. coli*, polypeptide O-GalNAc transferases can initiate *in vitro* O-glycosylation of unglycosylated polypeptides containing one or more amino acid acceptor motifs, thereby creating sites for GlycoPEGylation. Introducing PEG selectively on glycans avoids chemical modification of the natural polypeptide backbone and thereby preserves bioactivity. This lecture will illustrate application of industrialized pilot scale GlycoPEGylation technology to create long-acting recombinant human protein therapeutics, including some drugs currently in early stage clinical trials: erythropoietin, G-CSF, and Factor VIIa.

HEPARAN SULFATE-PROTEIN INTERACTIONS: ARE THEY SPECIFIC ?

Lindahl U; Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden

The polysaccharide, heparan sulfate (HS) is composed of alternating units of hexuronic acid and glucosamine, and these sugar residues are variously sulfate-substituted at different positions. Proteoglycans carrying HS chains are ubiquitously expressed at cell surfaces and in the extracellular matrix. The structures of these chains are highly variable, yet under strict biosynthetic control. Due to their high negative charge, HS chains interact with a variety of proteins, including growth factors/morphogens and their receptors, chemokines, and extracellular-matrix proteins. These largely ionic interactions serve to regulate key events in embryonic development and in homeostasis.

Questions regarding the degree of specificity in HS-protein interactions currently attract much interest. This aspect has been investigated using chemo-enzymatically generated oligosaccharide libraries in formation of ternary complexes with various fibroblast growth factors and their receptors. A different approach utilizes phenotype analysis of mice deficient in selected enzymes involved in HS biosynthesis, thus with structurally perturbed HS. The results suggest, unexpectedly, that many HS-protein interactions of key importance during embryonic development depend more on the overall organization and sulfation of protein-binding domains within the HS chain than on their fine structure. These findings have implications on the potential generation of HS-based drugs.

SHEDDING LIGHT ON GLYCANS

Bertozzi CR; Department of Chemistry and Molecular & Cell Biology, University of California at Berkerley, USA

A majority of cell surface and secreted proteins are posttranslationally modified by the addition of glycans. These complex structures can provide information regarding the state of health of cells or organisms. Indeed, changes in the structures of protein- and cell surface-associated glycans are a hallmark of many cancers, chronically inflamed tissues, and microbial infections. The ability to probe glycosylation in living systems may therefore reveal new biomarkers of disease and provide new avenues for diagnostic imaging. We are developing chemical technologies for visualizing glycans in living systems. As products of secondary metabolism, glycans are constructed from simple monosaccharide precursors. We exploit these metabolic pathways to incorporate bioorthogonal chemical reporters into glycans. The chemical reporters comprise small functional groups, such as the azide, that can be detected by covalent reaction with highly selective probes. New chemical reactions have been developed for this purpose, such as the Staudinger ligation with phosphines and strain-promoted [3+2] cycloaddition with functionalized cyclooctynes. Applications of the technique to the identification of cancer-associated glycan biomarkers and noninvasive imaging of are currently being pursued.

THE USE OF HUMANIZED YEAST TO EXPLORE THE GLYCO-DESIGN SPACE – A NOVEL DISCOVERY AND MANUFACTURING PLATFORM FOR BIOTHERAPEUTICS

Gerngross TU; Department of Chemistry and Biological Sciences, Darthmouth College and GlycoFi Inc., USA

Recent advances in the Glycobiology field have helped to establish a relationship between therapeutic protein function and glycosylation structures. Most of these studies rely on the comparison of mixed glycoforms, which complicate the clear interpretation of distinct structure activity relationships. We describe the use of combinatorial genetic libraries to engineer yeast cells that perform entirely human-like glycosylation with exceptional fidelity and uniformity. The use of these libraries to elucidate structure function relationships of glycoproteins and the ability to manufacture complex glycoproteins with unprecedented control over glycosylation will be discussed.

SYNTHESIS OF COMPLEX OLIGOSACCHARIDES USING METABOLICALLY ENGINEERED BACTERIA

Samain E, Fierfort N, Randriantsoa M, Drouillard S & Priem B; CERMAV, CNRS, France

The synthesis of oligosaccharides by metabolically engineered living bacterial cells has recently emerged as a powerful new method for the large-scale production of biologically important complex sugars, which are otherwise difficult to obtain by conventional synthetic methods or by extraction from natural sources.

This "living factory" approach is based on the use of whole *Escherichia coli* cells that express the appropriate recombinant glycosyltransferase genes while providing the necessary sugar nucleotide precursors. Sugar transporters, such as the galactose or the lactose permease, are used to efficiently internalize simple exogenous sugars which serve as primary acceptors for recombinant glycosyltransferases present in the bacterial cytoplasm. To prevent degradation of the primary acceptors and of the glycosylated products, mutants devoid of the appropriate catabolic activities are used.

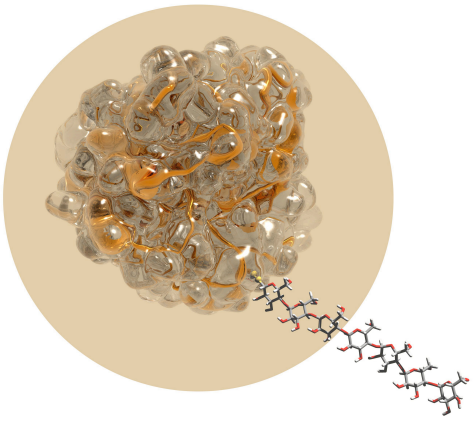
By co-expressing several glycosyltransferase genes in strains metabolically engineered to produce the necessary sugar nucleotides, a wide range of bioactive structures has been produced by this method, including most of the ABH and Lewis histo-blood group antigens and the carbohydrate portion of several glycosphingolipids such as the Ganglioside GM1 and the Globo-H antigen.

All these molecules can be economically produced with a 1-10 $g.l^{-1}$ yield by cultivating the bacteria at high cell density on inexpensive substrates. The fermentation process is simple and can be scaled up for the production of complex oligosaccharides on a multi-kg or even multi-tons scale.

SUGAR & ENZYMES: EXPLORING AND EXPLOITING PROTEIN-CARBOHYDRATE INTERACTIONS

Davis BG; Department of Chemistry, University of Oxford, United Kingdom

Sugars are critical biological markers that modulate the properties of proteins. Our work studies the interplay of proteins and sugars. This lecture will discuss recent developments our laboratory in two areas: (i) glycoprotein & glycoconjugate synthesis – the use of glycosylation to modulate function; and (ii) carbohydrate-processing enzyme mechanism – the engineering, study and use of glycosidases & glycosyltransferases and the synthesis of probes of their mechanism.



(i) Glycoprotein & glycoconjugate synthesis: Precisely glycosylated enzymes & proteins can be used in • preparative biocatalysis • drug delivery • selective protein degradation. New classes of glycoconjugate, *glycodendriprotein* and *glycoviruses* act as powerful nanomolar inhibitors of bacterial interactions or gene delivery vehicles, respectively. Antioxidant glycopolymers enhance cellular lifetimes and enhance function. Mimics of post-translationally modified proteins can be used as powerful probes of *in vivo* function.

(ii) Carbohydrate-Processing Enzymes: Glycosidases and glycosyltransferases are powerful synthetic catalysts. Mutants and novel glycomimetic syntheses illuminate substrate selection and catalytic mechanisms.

CARBOHYDRATE ACTIVE ENZYMES

Henrissat B; CNRS, Bat. AFMB, UMR6098, Case 932, Universites Aix-Marseille I & II, France

We have named “carbohydrate-active enzymes” the enzymes that build (glycosyltransferases) and cleave (glycosidases and polysaccharide lyases) glycosidic bonds. Although these enzymes are potential targets for the development of new drugs in the field of Glycobiology, many, and especially glycosyltransferases, prove extremely difficult to investigate experimentally. It is therefore desirable to make the best possible use of the few experimental data that appear in the literature. During the last 15 years, we have developped a classification system for these enzymes in families based on amino acid sequence similarities. We have made this classification system available to the community through the CAZy database server (http://www.cazy.org/CAZY) since September 1998. The CAZy database is continuously updated and, as of December 2006, we have analyzed the carbohydrate-active enzyme content of more than 430 genomes (eukaryotic or prokaryotic), many of which are of pathogens. This conference will present our current work on this classification framework and will show how CAZy helps relating experimental glycobiology with structural enzymology and functional genomics data.

A NOVEL HUMAN CELLULAR TOOLBOX FOR EXPRESSION OF GLYCO-OPTIMISED FULLY HUMAN GLYCOPROTEINS

Goletz S; GLYCOTOPE GmbH, Germany

Most pharmaceutical proteins are expressed in bacteria, yeast or CHO cells resulting in proteins lacking glycosylation or carrying glycans which largely differ from human carbohydrate chains in various aspects including sialylation. GlycoExpress^{TM} is a novel glycoprotein expression technology based on a toolbox of glycoengineered human cell lines. It enables the generation of proteins with fully human carbohydrate chains and a controlled optimization of the sialylation degree for large increases in bioactivity, improved pharmaceutical properties and new patent protection. The basis is two human glycoengineered cell lines SialoFlex and SialoMax. The parental cells for both cell lines were selected based on a large GlycoProfiling screening program comparing the mRNA expression profile of relevant enzymes, enzymatic activity, and expression of carbohydrate determinants, allowing the identification of those cells with the largest sialylation potential in all human sialylation pathways including those not existing in rodents (alpha 2-6 sialylation) and lack of NeuGc. Using GlycoEngineering SialoMax was further improved to the maximal sialylation potential in all pathways and SialoFlex was designed to be deficient in a critical component of the sialylation precursor pathway. By controlling certain media components proteins with defined gradual degrees of sialylation ranging from virtually 0% to 100% can be produced in a reproducible robust process. The toolbox allows the screening of the optimal human glycosylation and sialylation forms of proteins. The biotechnological features are favorable to current systems: high speed serum-free system with yields superior to CHO, suspension system with superior easy single cell cloning, transfection and cloning efficiencies, robustness and genetic stability. In case studies we show for example the optimization of the human growth factor GM-CSF with a fully human glycosylation and optimized sialylation resulting in a ~500 fold higher activity and several fold improved bioavailability in combination with an optimized high sialylation degree with alpha 2-3 and alpha 2-6 sialylation when compared to current GM-CSF

products. In another case studies high yield serum free expression clones of the therapeutic antibody were generated with a manifold increased ADCC due to its optimized human glycosylation. With this technology new generations of biomolecules can be identified and generated with a new patent protection and largely improved pharmaceutical properties in respect to bioactivity, pharmacokinetics, immunogenicity, dosage and/or side effects.

Poster No. II-1

ANALYSIS AND PREDICTION OF MAMMALIAN PROTEIN GLYCATION

Johansen MB[1], Kiemer L[2] & Brunak S[1]; [1]Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, Denmark, [2]Molecular Genetics Group, Department of Biology, Tor Vergata University of Rome, Italy

Glycation is a nonenzymatic process in which proteins react with reducing sugar molecules and thereby impair the function and change the characteristics of the proteins. Glycation is involved in diabetes and aging where accumulation of glycation products causes side effects. In our study (Johansen *et al*., Glycobiology, 16:844-853, 2006), we statistically investigate glycation of ε amino groups of lysines and also train a sequence-based predictor. The statistical analysis suggests that acidic amino acids, mainly glutamate, and lysine residues catalyze the glycation of nearby lysines. The catalytic acidic amino acids are found mainly C-terminally from the glycation site, whereas the basic lysine residues are found mainly N-terminally. The predictor was used to predict the glycation potential for all proteins in the human proteome. Interestingly we find that proteins involved in regulation of protein kinase activity and DNA replication have a high glycation potential. The fact that these proteins are involved in cancer could suggest a link between glycation and cancer. Our analysis of the glycation potential of the proteins in the human proteome further suggests that proteins associated with the extracellular matrix have a very low glycation potential. This could be a result of an evolutionally pressure disfavoring highly glycated proteins. This hypothesis is further strengthened by the fact that extracellular matrix proteins have a long biological half life thus exposing them to glycation for a longer time. The predictor was made by combining 60 artificial neural networks in a balloting procedure. The cross-validated Matthews correlation coefficient for the predictor is 0.58 which is quite impressive given the relatively small amount of experimental data available.

The method is made available at http://www.cbs.dtu.dk/services/NetGlycate-1.0.

Poster No. II-2

PREDICTION OF GLYCOSYLATION SITES IN PROTEINS

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Protein glycosylation is more abundant and structurally diverse than all other types of post-translational modifications combined. Glycosylation is known to affect protein folding, localization and trafficking, protein solubility, antigenicity, biological activity and half-life, as well as cell-cell interactions. The goal of our research is to provide tools for prediction of glycosylation sites from amino acid sequence alone, for as many different types of glycosylation as possible. These prediction tools are useful when: i) predicting protein function
ii) characterizing a new protein iii) interpreting mass spectrometry results iv) predicting protein structure
v) understanding and designing protein antigenicity vi) engineering of new glycosylation sites vii) abolishing undesirable glycosylation sites.

At our website, www.cbs.dtu.dk/services, we currently provide predictors for N-glycosylation (NetNGlyc), mucin-type O-glycosylation (NetOGlyc) [**1**], O-β-GlcNAc-glycosylation in cytoplasmic and nuclear proteins (YinOYang) and O-α-GlcNAc-glycosylation in a lower eukaryote (*Dictyostelium discoideum*, DictyOGlyc). In our poster, we will present new work:

- A predictor of C-mannosylation sites, NetCGlyc, which correctly predicts 100% of the positive sites and 98% of the negative sites.
- Ongoing work on a predictor of proteoglycan attachment sites in *C. elegans* and comparisons between recognition sequences in *C.elegans* and mammals. [**2**]

[**1**] Julenius K, Molgaard A, Gupta R, Brunak, S; Glycobiology (2005)15(2):153-64
[**2**] Wang H, Julenius K, Hryhorenko J, Hagen FK; *submitted to JBC*

Poster No. II-3

TRANSFERASE SPECIFIC PREDICTION OF T1, T2 AND T3 O-GALNAC GLYCOSYLATION SITES USING BIOINFORMATICS

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Mucin type O-linked glycosylation (O-GalNAc glycolysation) is carried out by a family of quite similar enzymes called UDP-Nacetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferases (ppGalNAc Ts). Some 20 members of this family exist in humans (T1 through T20), each featuring somewhat unique substrate specificities and working in tandem when glycosylating a substrate. A neural networks based method (NetOGlyc) for prediction of mammalian O-GalNAc glycosylation sites given amino acid sequence, has previously been published [julenius2005]. It was trained on a database of proteins with known glycosylation sites, and its predictions are thus not transferase specific. This study was concerned with devising a method (NetOGlycT) for predicting glycosylation sites specific to T1, T2 and T3 of the 20 transferases. It too was based on a neural network approach and trained on a limited amount of *in vitro* glycosylation data originating from experiments in which individual peptides were exposed to each of the 3 transferases, and glycosylation were subsequently determined by mass spectrometry. It was anticipated and shown that the prediction performance of NetOGlycT was better than NetOGlyc when looking at isolated glycosylation sites. It was also anticipated and shown that NetOGlyc would maintain the upper hand with regards to mucin-like sequences in which the individual glycosylation sites are closely spaced.

Poster No. II-4

ADAPTIVE EVOLUTION OF MUSTARD FAMILY MYROSINASES THAT ARE INVOLVED IN PLANT DEFENSE SYSTEM

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Glucosinolate-myrosinase system is a defense system of Brassicaceae (Mustard family) including Arabidopsis. Myrosinases degrade glucosinolates, and the degraded products become repellents for generalists, recognition cues for specialists, and mediate tritrophic interactions. Therefore, this is a key system for organism-organism interactions in the ecosystem. Phylogenetic analysis of this study inferred the Brassicaceae myrosinases have experienced adaptive evolution. Genes under adaptive evolution show significantly accelerated substitution rates beyond neutral evolution. Further sequence and structural analyses revealed that amino acids under adaptive selection, diversification of N-glycosylation pattern, and insertions/deletions were localized on the rim of the catalytic cave of the myrosinase molecule. Molecular dynamics simulation of the myrosinase molecule revealed that hyper dynamic amino acid residue was also located on the rim. Molecular dynamics is a computer simulation that calculates three-dimensional molecular motion by solving Newton's equations. These evidences conformed to disturbance of interactions with other molecules. It suggests that the myrosinases were under selective pressure of interaction with inhibitory molecules of other organisms. The diversification of N-glycosylation pattern found in myrosinases is very similar to that found in HIV-1 gp120 (evolution of glycan shield). This seems to be a general evolutionary strategy of proteins involved in organism-organism interactions. The hyper dynamic amino acid residue found in this study can increase fitness of the source plant by disturbing interactions with inhibitory molecules.

Poster No. II-5

MOLECULAR MODELING INSIGHT INTO THE CATALYTIC MECHANISM OF GNT-I

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Understanding the structural origin of the catalytic efficiency of enzymes is a fundamental goal and challenge in biological science. Theoretical studies of enzyme-catalyzed reactions have recently received much attention because they provide detailed information at the microscopic level and may guide design of inhibitors.

This work is the first application of QM/MM methodology to investigate the reaction mechanism of inverting glycosyltransferases, and more particularly of *N*-acetylglucosaminyltransferase I (GnT-I). A theoretical model of a Michaelis complex was built using the X-ray structure of GnT-I in complex with the donor having geometrical features consistent with kinetic studies. The calculated model identified a concerted S_N2-type of transition state with D291 as the catalytic base for the reaction in the enzyme active site. The TS model features nearly simultaneous nucleophilic addition and dissociation steps accompanied by the transfer of the nucleophile proton H_b2 to the catalytic base D291. The structure of the TS model is characterized by the O_b2-C1 and C1-O1 bond distances of 1.912 Å and 2.542 Å, respectively. The activation energy for the proposed reaction mechanism was estimated to be ~19 kcal mol^{-1}. The calculated α-deuterium kinetic isotope effect of 1.060 is consistent with the proposed reaction mechanism. Theoretical results also identified interactions between the H_b6 and β-phosphate oxygen of the UDP and a low-barrier hydrogen bond between the nucleophile and the catalytic base D291. This modeling study provided

detailed insight into the mechanism of the GlcNAc transfer catalyzed by GnT-I and the TS structure, which is the first step in design of transition-state analogue inhibitors.

This work was supported by the grant the Slovak Science and Technology Assistance Agency under contract No. APVT-51-004204.

Poster No. II-6

CHARACTERISATION OF LIGAND BINDING PROPERTIES AND SUBSTRATE SPECIFICITY OF *L. MAJOR* UDP-GLUCOSE PYROPHOSPHORYLASE BY NMR SPECTROSCOPY

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Leishmania are protozoan parasites that cause diseases ranging from self-healing cutaneous lesions to lethal visceral forms. In *Leishmania major* (*L. major*) various glycoconjugates are essential for survival and proliferation in the sand fly vector and mammalian host. The biosynthesis of glycoconjugates depends on the availability of activated nucleotide sugars. The UDP-glucose pyrophosphorylase (UGP) represents a key position in the activation of glucose and galactose, both major components of *Leishmania* glycoconjugates. UGP catalyses the synthesis of UDP-glucose from glucose-1-phosphate and UTP. Formation of UDP-glucose is a prerequisite for the synthesis of UDP-galactose. Using a gene deletion approach we identified UGP to exert an important function for *L. major* virulence.

To gain a first insight into the catalytic mechanism and thus provide a starting point for the design of specific inhibitors, we characterized the protein-ligand interactions of *L. major* UGP by saturation transfer difference (STD-) NMR. Using this technique, we could directly prove that the enzyme follows an ordered bi-bi reaction mechanism with UTP preceding binding of glucose-1-phosphate. Interestingly, UDP and UMP were not recognized by the enzyme and did not facilitate binding of glucose-1 phosphate. This demonstrates the relevance of the gamma-phosphate group for binding of UTP and for induction of the presumed change in conformation, which then allows entry of glucose-1 phosphate. Though the nucleotide part is important for binding, the substrate specificity is influenced by the sugar moiety. Epimerization of position 4 of the hexose is sufficient to prevent binding, as demonstrated by the absence of binding of the C4-epimer UDP-galactose. Thus the correct orientation of the C4-hydroxyl group mediates substrate specificity of *L. major* UGP.

Poster No. II-7

CRYSTALLOGRAPHIC STRUCTURES OF THE CARBOHYDRATE-BINDING DOMAIN VP8*, CRITICAL TO ROTAVIRUS HOST-CELL RECOGNITION: STRAINS CRW-8, RRV AND WA THAT INFECT PIG, MONKEY AND HUMAN

Blanchard H, Yu X, Kraschnefski, MJ, Scott SA & von Itzstein M; Institute for Glycomics, Griffith University, Queensland, Australia

Rotavirus infection results in high incidence of gastroenteritis in the young, leading to death of approximately 500,000 children annually, worldwide. There is urgent need for development of a rotavirus-specific drug. Rotaviruses only infect mature enterocytes on intestinal villi suggesting existence of a specific host cell-receptor, however attachment of the virion is a complex process with multiple cell surface interactions, some being species-specific. Virons bind cell surface glycoconjugates but the exact nature of the critical chemical components is undefined, and involvement of sialic acid is controversial. Host-cell attachment occurs via the outer capsid spike protein that contains a carbohydrate-binding domain VP8*, critical in the recognition of cell-surface glycoconjugates. We are investigating rotavirus host-cell recognition and attachment, particularly in relation to carbohydrate-binding properties of VP8*, ultimately directing this information into design of potential drugs. We have determined crystal structures of VP8* of strains CRW-8, RRV and Wa that infect pigs, monkey and humans respectively. Our preliminary reports described the first crystallographic information on VP8* from a strain that infects humans, and that appears to demonstrate sialic-acid independent infection. These structures of VP8* from strains considered to be sialic-acid dependent (CRW-8) and independent (Wa) [1] as well as our crystal structures of Rhesus Rotavirus (RRV) wild-type and an $Arg_{101}Ala$ mutant enable us to investigate aspects of proposed sialic-acid dependency. This work along with our STD-NMR and infectivity inhibition studies to assess the binding and effect of ligands on infectivity, has given important information that is being directed into our structure-based drug-design program.

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Poster No. II-8

STRUCTURAL STUDIES OF HUMAN MUC2 GEL-FORMING MUCIN

Mackenzie J#, Grahn E#, Bäckström M§, Tompson E§, Hansson G§ & Krengel U#; #Department of Chemistry, University of Oslo, Norway §Department of Medical Biochemistry, University of Gothenburg, Sweden

Mucins are highly glycosylated proteins protecting the mucosal surfaces of the body. Their heavily O-glycosylated mucin domains, rich in the amino acids Ser, Thr and Pro, characterize all mucins. MUC2 is the main gel-forming mucin of the small intestines and is produced by the intestine goblet cells.

Mucins are very large structures, and the final translation product of MUC2 contains 5179 amino acids. The protein consists of five different regions: two mucin domains (one large and one small) and three different cysteine-rich parts, located in the N-terminal, middle and C-terminal end of MUC2. Due to the extreme glycolysation, the mucin domains do not fold into any secondary structures, but are instead stretched out with the glyco-chains sticking out like a bottlebrush. The cysteine-rich parts of MUC2 are structurally more interesting. They are built up from many different domains, most with unknown function.

The aim of the project is to do structural studies of the MUC2 cysteine-rich parts in order to gain knowledge of their function. The middle part of MUC2 consists of a so-called CysD-domain. This domain has been expressed in hamster CHO-cells, purified and initial crystallization screens have been set up. The C-terminal D4-domain is currently being expressed, also in CHO-cells. The N-terminal part is known to contain a trypsin-resistant core, which is suggested to take part in trimerization of the protein. This core domain has been cloned in junction with a his-tag. If correct folding of can be achieved, expression and crystallization will be started. Trial studies to produce parts of MUC2 in *Pichia pastoris* is initiated, in order to facilitate the large production of protein needed for X-ray crystallography.

Poster No. II-9

CRYSTAL STRUCTURE OF A MUSHROOM LECTIN

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Lectins are proteins recognizing and binding to carbohydrate molecules without modifying them. Lectins exist in all kingdoms of life and have several different biological functions. Structurally lectins belong to several unrelated families and their specificities vary a lot. A lectin from the mushroom *Marasmius oreades* has attracted scientific interest for over 50 years because of its ability to agglutinate blood group B erythrocytes. In more recent years this lectin, called MOA, has been shown to also have specificity for Gal(1,3)Gal-terminated carbohydrates, epitopes that are present on cells from all mammals except for humans and monkeys, thus presenting a barrier for xenotransplantation of organs from other mammals into humans.

We have solved the three-dimensional crystal-structure of MOA in complex with the linear trisaccharide Gal(1,3)Gal(1,4)GlcNAc to 2.4 Å resolution. The structure shows that the protein forms a dimer. Each protomer forms two distinct domains. The N-terminal domain has a beta-trefoil fold found in many carbohydrate-binding proteins and has three potential carbohydrate-binding sites. The C-terminal domain has no obvious similarity to any other known protein-structure but has some features indicating that it may have a yet non-identified enzymatic function.

Poster No. II-10

STRUCTURAL AND FUNCTIONAL CONSEQUENCE ANALYSIS OF NATURALLY-OCCURRING MUTATIONS IN THE BLOOD GROUP B GALACTOSYLTRANSFERASE

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Four key amino-acid-changing polymorphisms differentiate the blood group *A* and *B* alleles. Multiple missense mutations are associated with weak expression of A and B antigens but the structural changes causing subgroups have not been studied. Individuals or families having serologically weak B antigen on their red cells were studied. Alleles were characterized by sequencing of exons 1-7 in the *ABO* gene. Single crystal x-ray diffraction, 3D-structure molecular modeling and enzyme kinetics showed the effects of the *B* allele mutations on the glycosyltransferases. Seven unrelated individuals with weak B phenotypes possessed seven different *B* alleles, five of which are new and result in substitution of highly conserved amino acids: M189V, I192T, F216I, D262N and A268T. One of these (F216I) was due to a hybrid allele resulting from recombination between *B* and O^{1v} alleles. The two other alleles were recently described in other ethnic groups and result in V175M and L232P. The first crystal-structure determination (A268T) of a subgroup glycosyltransferase and molecular modeling (F216I, D262N, L232P) indicated conformational changes in the enzyme that could explain the diminished enzyme activity. The effect of three mutations could not be visualized since they occur in a disordered loop.

The genetic background for B_{weak} phenotypes is very heterogeneous but usually arises through seemingly random missense mutations throughout the last *ABO* exon. However, the targeted amino-acid residues are well conserved

during evolution. Based on analysis of the resulting structural changes in the glycosyltransferase, the mutations are likely to disrupt molecular bonds of importance for enzymatic function.

Poster No. II-11

A HIGH THROUGHPUT SCREENING ASSAY FOR GLYCOSYLTRANSFERASES

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Directed evolution and saturation mutagenesis have emerged as powerful tools to improve biocatalysts as well as broaden our understanding of the underlying principles of substrate specificity and stereoselectivity. In contrast to rational protein design, directed evolution does not require knowledge of the three-dimensional structure for a given enzyme or about the relationship between structure, sequence and mechanism.

Relatively few studies on directed evolution and (or) saturation mutagenesis of glycosyltransferases have been reported. One of the reasons for this is the difficulty in developing inexpensive and feasible high-throughput assays for glycosyltransferases. An exception was reported recently for sialyltransferases (1). In this case the fluorescent labeled enzymatic product sialoside was trapped into *E.coli*. The cell population was sorted using a fluorescence-activated cell sorter leading to the identification of a mutant enzyme with 400 fold higher catalytic activity than wild-type enzyme.

In our study the blood group A and B glycosyltranferases have been used as model enzymes to develop a novel high throughput assay for glycosyltransferases. The assay is general and should easily be applicable also to other glycosyltransferases.

We have recently shown that amino acid M214 is crucial for the donor specificity of the blood group B galactosyltransferase (2). Saturation mutagenesis at this position has been performed and the novel enzyme variants screened with the newly developed assay.

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Poster No. II-12

CLONING, EXPRESSION AND PREPARATIVE USE OF A MUTATED, BIFUNCTIONAL α(2-3/2-8)-SIALYLTRANSFERASE FROM *CAMPYLOBACTER JEJUNI*

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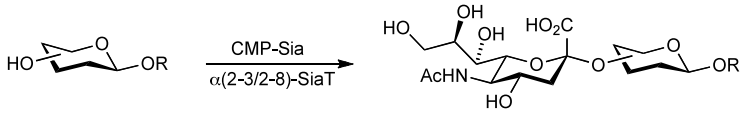
Carbohydrates from glycolipids and -proteins with terminal sialic acids are involved in a broad variety of biological recognition and adhesion phenomena [1]. Although numerous glycosylation methods are known, the chemical synthesis of complex oligosaccharides is still a cumbersome and time-consuming procedure. In particular, the formation of α(2-8)-linked sialic acids is one of the most difficult reactions in carbohydrate chemistry. Additionally, chemical sialidations suffer from poor stereoselectivity due to the lack of neighboring group participation. A convenient alternative is the use of enzymatic sialidations.



A bifunctional, recombinant α(2-3/2-8)-sialyltransferase (Δ32Cst-II) from *Campylobacter jejuni*, as well as a mutated variant, were cloned and over-expressed as His-tagged proteins in *E. coli* [2]. The potential of the enzymes for mono- and bis-sialylations of Type I and II substrates on a preparative scale was explored.

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Poster No. II-13

PROMISING SIALYLATION TOOLS: SIALYLTRANSFERASES FROM MARINE BACTERIA

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Sialic acids are present in a variety of glycoproteins and glycolipids, often at the non-reducing termini of carbohydrate chains. It has been demonstrated that sialic acids play very important roles in various biological and physiological events. Ample supply of sialosides and sialyl-glycoconjugates is indispensable in the study of their biological functions in detail.

Sialylation is divided into two methodology, chemical sialylation and enzymatic sialylation. Although the chemical sialylation has certain advantages compared with enzymatic sialylation in respect of its high flexibility and wide applicability, the reaction processes are complicated in many cases because the chemical reactions requires multiple protection/ de-protection steps. On the other hand, enzymatic sialylation using sialyltransferases is a single step process with high position- / anomer-selectivity and reaction yield. Furthermore, transfer of sialic acids by sialyltransferases to appropriate substrates in the final step under a mild reaction condition can prepare these materials in quantity.

Our research group has been screening a large number of bacteria for novel glycosyltransferase activities. During the course of the study, we have isolated over 20 bacteria that produce sialyltransferase. Bacteria that likely were new species were found among them.

In this presentation, we present recent progress in the study of bacterial sialyltransferases, especially on alpha2,3- and alpha2,6-sialyltrasnferases obtained from marine bacteria in our laboratory, which are very useful tools for the syntheses of sialosides.

Poster No. II-14

PRODUCTION OF WELL-DEFINED O-LINKED GLYCOPROTEINS

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Well-defined mucin-type O-linked glycopeptides can be synthesized either chemically or chemo-enzymatically, and both methodologies are utilized for synthesis of short glycopeptides 10-25 amino acids in length possessing simple Tn or T glycan structures. Yet difficulties can be encountered with both methodologies for synthesis of longer O-linked glycopeptides possessing multiple and/or larger oligosaccharides. The recent availability of numerous well characterized glycosyltransferases, and advances in the field of recombinant technologies, have opened up for novel ways of generating well defined glycoproteins possessing specific oligosaccharides attached to specific positions within the protein sequence. We have taken advantage of these recent developments, and combined the use of purified well-characterized recombinant enzymes and bacterial produced recombinant proteins, for the production of well-defined O-linked glycoproteins. Medium scale amounts of recombinant protein (0.5-5mg) have been obtained using a robust bacterial expression system and a simple, fast and standardized procedure involving sequential affinity purification schemes of expressed recombinant proteins carrying small affinity tags. The recombinant expression platform is based on complementing approaches, enabling the production of short proteins (up to 33 amino acids) and of long proteins (larger than 33 amino acids). Using available purified recombinant GalNAc-transferases, core-1 galactosyl-transferase and sialyltransferases, well defined short and long glycoproteins have been produced, and representative examples of both are presented. We envision that this complete system can be expanded, by use of additional glycosyltransferases, and that this recombinant approach can be semi-automated, which will provide us with a system applicable to medium-scale production of well-defined O-linked glycoproteins.

Poster No. II-15

GENERALIZED *O*-LINKED PROTEIN GLYCOSYLATION IN THE HUMAN PATHOGEN *NEISSERIA GONORRHOEAE*

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Although *O*-linked protein glycosylation (O-PG) is being seen with increasing frequencies in prokaryotic and archaeal species, the systems involved are invariably dedicated to modification of single protein or sets of highly related proteins. The pilin subunit of the *N. gonorrhoeae* (*Ngo*) type IV pilus colonization factor undergoes O-PG. The basal diacetoamidotrideoxyhexose sugar of the oligosaccharide is identical to that seen in *N*-linked glycans present in over 25 proteins of *Campylobacter jejuni* (*Cje*), the zoonotic agent of gastrointestinal disease. Genetic analyses and complementation show that these two systems are remarkably conserved save for the use of a Ser-directed oligosaccharyltransferase (Otase) in the former and an Asn-directed Otase in the latter. Accordingly, the *Ngo* O-PG system utilizes *en bloc* transfer of oligosaccharide from a lipid-linked donor.

We have gone on to show that at least 8 other *Ngo* proteins undergo O-PG by the same pathway that modifies pilin. These proteins are all localized to the periplasmic space and function in a variety of interconnected, metabolic processes including both aerobic and anaerobic respiration and redox pathways. Moreover, these proteins all contain a highly conserved, amino acid sequence motif evocative of a sequon. The importance of these "sequon-like" elements to O-PG have been confirmed by glycan localization to short peptides encompassing the motifs by mass spectrometry and site-directed mutagenesis of residues within and flanking the motifs. As in the case of the N-linked *Cje* system, these studies may provide a tractable system of reduced complexity with relevance to related processes in eukaryotes. In addition to assessing the significance of the generalized O-PG system to *Ngo* biology, potential exploitation of this system for unique glycoengineering strategies is being investigated.

Poster No. II-16

THE PRESENCE OF GLYCAN AT FIRST N-GLYCOSYLATION SITE PREVENTS POFUT1 AGGREGATION

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Protein *O*-fucosyltransferase 1 (Pofut1) is an enzyme responsible for the addition of *O*-fucose to EGF-like repeats of several proteins including Notch receptors and their ligands. Pofut1-mediated *O*-fucosylation is essential in Notch signaling, folding and targeting to the cell surface. We showed that the active enzyme is ubiquitously present in embryo and adult bovine tissues. By comparing Pofut1 sequences available in databases, we observed that mammalian Pofut1 enzymes possess two putative *N*-glycosylation sites and that only the first is conserved among bilaterians. PNGase and EndoH digestions revealed that endogenous bovine Pofut1 and recombinant Pofut1 from COS-1 cells are glycoproteins comprising two high mannose or hybrid type *N*-glycans. To gain more insight regarding the significance of *N*-glycans on Pofut1, we substituted, by site-directed mutagenesis, bovine Pofut1 N^{65}, N^{163} or both, by L or Q. We demonstrated that the loss of *N*-glycan on N^{163} caused a slight decrease in Pofut1 activity. Loss of glycosylation at N^{65} resulted in aggregation of Pofut1, suggesting that *N*-glycosylation at this site is essential for proper folding of the enzyme. This study showed that more than being essential for protein optimal efficiency, *N*-glycosylation can be a key factor for glycoprotein folding and production.

Poster No. II-17

STUDY OF SUBCELLULAR LOCALIZATION AND TRANSCIPTIONNAL REGULATION OF HST6GAL II

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A cDNA encoding a second human β-galactoside α-2,6-sialyltransferase (hST6Gal II) was previously cloned and characterized. The catalytic domain of the enzyme shares 48 % identity with that of hST6Gal I and, as shown by phylogenetic analysis, the corresponding genes of both enzymes have evolved from a common ancestor by gene duplication. Enzymatic assays with recombinant enzymes have confirmed that hST6Gal II was able to transfer a sialic acid residue in 6-position to Gal residue of the type II disaccharide Galβ1–4GlcNAc, and have shown that hST6Gal II prefers free oligosaccharides whereas hST6Gal I acts preferentially on Galβ1 -4GlcNAc-R linked to a protein. Using a panel of Galβ1-4GlcNAcβ1-2Manα1-*O*-octyl analogues, it has been recently shown that hST6Gal II was at least 8-fold more active on GalNAcβ1-4GlcNAc (LacdiNAc) sequence than hST6Gal I, suggesting that hST6Gal II is the enzyme responsible of the synthesis of Sialyl-LacdiNAc structures. In order to compare the subcellular localization of both enzymes and to precisely determine *in vivo* their substrate specificity, several constructs encoding full-length N-terminal-tagged enzymes have been prepared and stably co-transfected in COS-7 cells. hST6Gal I and II showed a very similar subcellular localization in the Golgi area. Both enzymes also differ in their tissue-specific pattern of expression. Whereas hST6Gal I gene is ubiquitously expressed in most of human tissues, hST6Gal II shows a limited tissue-specific pattern of expression, mostly expressed in brain, thyroid and kidney. 5'-ends rapid amplification of SH-SY5Y neuroblastoma cells mRNA allowed us to characterize a series of specific transcripts, different from that previously described (1) and suggesting the use of alternate promoters. Further characterization of these specific transcripts would shed light on the regulation and the function of hST6Gal II in brain tissue.

Poster No. II-18

THE ARG^{110}-GLN MUTATION IN HUMAN FUC-TVII INACTIVATES THE ENZYME THROUGH RETAINING THE PROTEIN IN ER

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The *FUT4* and *FUT7* genes are necessary for the biosynthesis of the SLex antigen, the carbohydrate receptor structure common to the E-, P- and L-selectins. The importance of these fucosyltransferase genes, their gene products and their interactions with the different selectins for inflammatory responses and lymphocyte homing has been elegantly described using e.g. various single and double-knock out mice. So far only one mutation has been reported for these genes in humans (*FUT7* G329A; Arg^{110}-Gln) and enzyme studies of transiently transfected COS7 cells showed a markedly reduced activity for the mutated enzyme compared to the wild type enzyme.

We have now established HEK 293 cells stably transfected with new constructs of the mutated and wild type enzyme. Using the sialylated type 2 chain acceptor cell lysates with mutated enzyme showed about 10% the activity compared to the wild type (Vmax 25 vs 192 pmol/mg protein*min) although *FUT7* mRNA levels were similar. Western blots of cell lysates using a rabbit anti Fuc-TVII antiserum (kind gift from Dr JB Lowe) and a denaturizing SDS-PAGE

analysis identified the protein at about 39 KDa in both cell lines. The mutated enzyme showed a slightly smaller weight, probably due to defective glycosylation. In cells transfected with the mutated enzyme and subjected to sub cellular fractionation the protein staining appeared in the ER-fractions (mapped with calnexin antibodies) whereas in cells transfected with the wild type enzyme the protein appeared in the Golgi (mapped with Rab6 antibodies) as well as in ER-fractions. The enzymatic activity of the different sub fractions was assayed and the activity was seen only in the Golgi fractions of the wild type enzyme.
Sequence alignment of α1,3/4-fucosyltransferases, in all species reported, identified the Arg as a highly conserved amino acid, in a conserved motif, of significant importance for the processing of these enzymes.

Poster No. II-19
Cancelled

Poster No. II-20
SEQUENTIAL PERTURBATION OF GOLGI PH SUGGESTS ORGANIZATIONAL DIFFERENCES BETWEEN GLYCOSYLATION ENZYMES OF THE TGN AND THE GOLGI STACK
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Acidic pH in the Golgi apparatus in mammalian cells is crucial for various post translational modification steps such as glycosylation, sulphation and proteolysis, as well as for sorting of newly synthesized proteins and lipids into their final destinations in a cell. However, it is still unclear to what extent, if any, these different Golgi functions are coupled or how the distinct pathways involved are organized at the molecular level. Here we investigate the organization of the N-linked glycosylation pathway by sequentially interfering Golgi pH with chloroquine (CQ), a weak base. We find that only a 0.1-0.2 pH unit increase in Golgi pH (obtained with 10-20 μM CQ) was sufficient to perturb terminal sialylation of a secretory form of carcinoembryonic antigen (sCEA). Higher concentrations of the drug (40 μM or higher) was required to perturb galactosylation and cis-medial-Golgi glycosylation events, as well as secretion and Golgi structure. Defective sialylation was found to coincide with the redistribution of the alpha-2,3 sialyltransferase (ST3GAL3) from the trans-Golgi network (TGN) to endosomal/lysosomal compartments, while beta-1,4-galactosyltransferase (B4GALT1) and a medial-Golgi enzyme (alpha-mannosidase II) remained associated with the Golgi stack. Our results show that terminal sialylation can be segregated from the other glycosylation steps on the basis of its pH sensitivity and by the observed redistribution of the corresponding sialytransferase to the endosomal compartments. These observations suggest organizational differences in the glycosylation machinery between the Golgi stack and the TGN.

Poster No. II-21
IL-6 AND IL-8 INCREASE THE EXPRESSION OF GLYCOSYLTRANSFERASES AND SULFOTRANSFERASES INVOLVED IN THE BIOSYNTHESIS OF SIALYLATED AND/OR SULFATED LEWIS X EPITOPES IN THE HUMAN BRONCHIAL MUCOSA
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Several studies have shown that inflammation may affect glycosylation and sulfation of various glycoproteins, including mucins. We have studied the effect of IL-6 and IL-8, two pro-inflammatory cytokines present in increased amounts in broncho-alveolar fluids from cystic fibrosis (CF) patients, on the mRNA expression of fucosyl-, sialyl- and sulfotransferases of the human bronchial mucosa. These enzymes are involved in terminal glycosylation of O-linked mucin chains, including sialyl-Lewis^X (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc-R) and 6-sulfo-sialyl-Lewis^X (NeuAcα2-3Galβ1-4[Fucα1-3](HO_3S-6)GlcNAc-R) epitopes, which are found in increased amounts in bronchial mucins from CF patients and are preferential binding sites for *P. aeruginosa,* and may contribute to the chronicity of airway infection observed in this disease.
Fragments of macroscopically healthy human bronchial mucosa were incubated with IL-6 or IL-8 (20 ng/ml for 16 h). Real-time quantitative PCR was used to study variations in the transcriptional expression of the glycosyl- and sulfotransferases between control and treated explants, and Western blotting using different antibodies and lectins was performed to study variations in the glycosylation and sulfation of bronchial proteins. We show that IL-6 and IL-8 significantly increase the expression of different α1,3-fucosyltransferases (FUT11 and FUT3, respectively), α2,6- and α2,3-sialyltransferases (ST3Gal VI for IL-6 and ST6Gal II for both cytokines), and GlcNAc-6-0-sulfotransferase (CHST6) mRNA present in the human airway, as well as the amount of sialyl-Lewis^X and 6-sulfo-sialyl-Lewis^X epitopes present at the periphery of high molecular weight proteins from bronchial explants. These results show that IL-6 and IL-8 could be responsible for the increased levels of sialyl-Lewis^X and 6-sulfo-sialyl-Lewis^X epitopes on human airway mucins from patients with cystic fibrosis.

Poster No. II-22

MEGAKARYOCYTES PACKAGE AND DELIVER GOLGI-ASSOCIATED GLYCOSYLTRANSFERASES INTO PLATELETS AND PLATELET SURFACES USING GRANULES

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We recently presented evidence for the presence of a 4galactosyltransferase (β4GalT) on the surface of platelets that catalyzes the coupling of galactose in a β1,4 linkage to exposed N-acetylglucosamine residues on the N-linked glycans of GP1b, and that this reaction improved the circulation of chilled murine platelets. Since glycosyltransferases are generally limited to the intracellular compartments of the secretory pathway, we explored the surface expression by demonstrating that intact platelets serve as an enzyme source for galactosylation 2.8 μm beads coated with the acceptor substrate GlcNAc- β-R. We further studied the origin of this enzyme in megakaryocytes and its mechanism of delivery to platelets. We report that Golgi marker GM130 as well as a YFP tagged Golgi marker construct based on the polypeptide transferase GalNAc-T2 expressed in cultured mouse megakaryocytes transports from the cell body to nascent platelets in discrete packets. In immature megakaryocytes, the Golgi organizes, as expected, into perinuclear arrays. However, once proplatelet extension begins, the perinuclear apparatus disassembles, and Golgi-associated proteins transport through the proplatelets in vesicular/granular structures. Labeling with granule markers reveals correspondence of Golgi markers with predominantly dense granules. These findings suggest that Golgi membranes become packaged into granules, which deliver Golgi-associated glycosyltransferases into the nascent platelets and from there to the platelet surface.

Poster No. II-23

MOLECULAR MECHANISMS CONTROLLING B3GALT2 GENE EXPRESSION IN RAT CORTICAL NEURONS

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Activity-dependent activation of transcription factor CREB plays a pivotal role in synaptic plasticity and neuronal survival. Neuronal cells are rich in glycoconjugates, whose role is largely unknown. Activity-dependent transcriptional regulation of glycosyltransferase genes may modify synaptic glycoconjugates, thereby contributing to regulation of synaptic function. Previously, we identified a novel CREB binding site (CRE) in the promoter of the B3galt2 (UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2) gene. This gene is up-regulated in rat cortical neurons in response to CREB activation induced by membrane depolarization. In this study, we further investigated the role of CRE and CREB in regulation of the B3galt2 gene and the signaling pathways that are involved. Promoter assays were performed using HEK293 cells transfected with reporter constructs containing the B3galt2 promoter, with either wild-type or mutated CRE, together with expression constructs containing coding sequences of either the wild-type or a dominant-negative CREB. Our results suggest that both functional CRE and CREB are essential for the transcriptional regulation of the B3galt2 gene. Results obtained from rat cortical neuron cultures treated with protein kinase inhibitors during membrane depolarization suggested that multiple signaling pathways, including the Ca(2+)/calmodulin-dependent protein kinases (CaMKs) and the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways, are involved in the regulation of the B3galt2 genes. CaMK activity directly contributes to CREB activation and the CREB-dependent B3galt2 gene expression, whereas ERK1/2 activity may control B3galt2 gene expression through other transcription factors.

Poster No. II-24

GLYCOSPHINGOLIPIDOMICS OF PATHOGENIC AND MODEL FUNGI: IDENTIFICATION OF POTENTIAL THERAPEUTIC AND DIAGNOSTIC TARGETS IN FUNGAL DISEASE

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Studies performed by us and others have advanced sufficiently over the last few years to outline some general patterns of fungal glycosphingolipid (GSL) expression in terms of their structures and biosynthetic pathways. Ongoing work within our lab is directed towards development of improved methods for glycosphingolipidomic analysis, to provide information complementing that derived from ongoing and completed projects in genome and proteome sequencing and functional analysis. Major long term goals are the discovery of potential therapeutic and diagnostic targets in fungal GSL pathways, and development of clinical applications for this knowledge base. GSL structural studies have revealed general pathways that yield common linkage and sequence motifs shared by a wide variety of species, as well as more specific variations related to phylogeny and, in some cases, morphogenesis. The distribution of expressed structures can be related to hypothetical biosynthetic pathways, suggesting glycosyltransferase activities, and putative genes coding for them, that could be targeted for functional studies (by knockout or knockdown) and, ultimately, if

essential functions can be identified, for therapeutic inhibition. Potential vaccine and diagnostic applications are also suggested by observations of species-specific expression of antigenic fungal GSLs. In this presentation, some results from studies of key opportunistic mycopathogens and related model species (e.g., *Aspergillus fumigatus* and *A. nidulans*) will be described and compared. In addition, some ongoing methodological developments to improve coverage, throughput, and sensitivity for analysis of GSL expression patterns in, e.g., uncharacterized species, in mutant strains from functional genomic studies, and in drug treatment studies performed in culture, will be described. Taken into account in the design of these methods is the potential for congruence with future diagnostic applications.
*With apologies to my associates and collaborators in these studies, who are too numerous to list in the space provided.

Poster No. II-25

DEFINING A BIOLOGICALLY ACTIVE GLYCOSAMINOGLYCAN LIGAND FOR INTERLEUKIN-8

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The interaction of chemokines and glycosaminoglycans (GAGs) on the endothelial surface is a crucial molecular event in establishing a chemotactic gradient which ultimately leads to the functional presentation of chemokines to their high-affinity 7TM-GPC receptors on approaching leukocytes. We have recently shown that syndecan-2 is upregulated in inflammatory settings and that it is the proteoglycan co-receptor of IL-8 on human endothelial cells. Based on molecular modeling, biophysical investigations, and in vivo experiments we have identified the minimum and the optimum oligosaccharide chain length of the IL-8/GAG interaction to be a disaccharide and a tetrasaccharide, respectively. This means, that although GAG disaccharides with defined structures bound to the chemokine with significant affinity and specificity, in a mouse peritonitis model only tetrasaccharides were found to exhibit anti-inflammatory activity. In addition, we were able to characterize chemokine-specific sulfation patterns of the oligosaccharides by comparing IL-8 preferential GAG oligosaccharide binding to that of other chemokines. Currently, mass spectrometry is applied to directly sequence chemokine-bound specific GAG oligosaccharides.

Poster No. II-26

GLYCANS AND POLYPEPTIDE GALNAC-TRANSFERASES AS MARKERS FOR SPERMATOGENESIS AND SPERM-RELATED INFERTILITY

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Mucin-type O-glycans are found in high density on mucins as well as on glycoproteins, and they play numerous important biological roles, including modulation of protein structure and protein resistance, intracellular sorting of glycoproteins, cell-cell adhesion, microbial interactions and cell maturation. The initial step in mucin-type O-glycosylation involves the transfer of GalNAc from UDP-GalNAc to selected serine and threonine residues. A large family of homologous UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase isoforms (GalNAc-transferases) catalyze the transfer and these isoforms are differentially expressed in cells and have different specificities for acceptor peptide sequences. We have previously shown a very restricted and developmentally regulated pattern of O-glycosylation and GalNAc-transferase expression in testis with only Tn, T and GalNAc-T3 expression in the maturing germ cells and mature spermatozoa suggesting a specific function, which may be related to sperm maturation, oviduct attachment or to adhesion of spermatozoa to the zona pellucida of an egg. To test their value as markers for sperm infertility we have in the present study further characterized the sperm glycan and glycosyltransferase profile and correlated GalNAc-T3 expression with parameters of semen quality in sub fertile and healthy individuals using immunofluorescence and western blot. We found that sub-cellular compartments of mature spermatozoa can be distinguished by expression of carbohydrate markers i.e. the whole acrosome (the head), the equatorial part of the acrosome, the mitrochondia containing midpiece and the whole surface. Many different glycosyltransferases tested were not expressed. Expression of GalNac-T3 correlated with Tn. GalNAc-T3 labeled a significantly higher proportion of sperm that were selected by gradient (n=43) i.e. good quality sperm than the proportion of sperm from the total ejaculate (n=150) i.e. unselected sperm. The results suggest that GalNAc-T3 is a marker of good quality sperm, but we found no difference between sub fertile (n=75) and healthy individuals (n=65). Approx 20 % of all the subjects had only 10% GalNAc-T3 positive sperm and two of the individuals had no expression of GalNAc-T3 at all. We hypothesize that absence of GalNAc-T3 leads to impaired O-glycosylation which again influences sperm maturation and therefore also fertility.

Poster No. II-27

THERMODYNAMIC BINDING STUDIES OF THE TN-ANTIGENIC FORM OF PORCINE SUBMAXILLARY MUCIN AND ITS SMALLER FRAGMENTS TO α-GALNAC SPECIFIC LECTINS

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Isothermal titration microcalorimetry (ITC) and hemagglutination inhibition measurements demonstrate that a chemically and enzymatically prepared form of porcine submaxillary mucin containing ~2300 α-GalNAc residues (Tn-PSM) and that possesses a molecular mass of ~10^6 daltons binds with ~10^7-fold greater affinities to the soybean agglutinin (SBA) and the *Vatairea macrocarpa* lectin (VML), which are both GalNAc-specific lectins, than the corresponding monovalent carbohydrate GalNAcα1-*O*-serine. Furthermore, the enzymatically derived 81 amino acid tandem repeat domain of Tn-PSM containing 23 α-GalNAc residues showed ~10^4-fold enhanced affinities, while the enzymatically derived 40/41-mer cleavage product of the 81-mer showed ~10^3-fold enhanced affinities. The naturally decorated carbohydrate form of PSM showed ~10^7 and 10^6 enhanced affinities for VML and SBA, respectively. The results demonstrate that Tn-PSM shows picomolar binding to VML and SBA, while fully decorated PSM shows picomolar and nanomolar binding to VML and SBA, respectively. The shorter fragments of Tn-PSM show reduced affinities for both lectins. These results indicate that longer chain forms of PSM possess substantially higher avidities for SBA and VML than shorter chain forms of PSM. ITC data indicate that a fraction of α-GalNAc residues in Tn-PSA and its two smaller fragments are occupied by VML and SBA, and that a smaller fraction of epitopes is occupied in the fully decorated form of PSM. The findings have important implications for the mechanisms of binding of lectins to mucins, and the biological properties of mucins.

Poster No. II-28

LABELED NOROVIRUS VLP FOR VISUALIZATION OF THE SPECIFIC BINDING TO ABH HISTO-BLOOD GROUP CARBOHYDRATES

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Norovirus virus like particles (VLPs) exhibit strain-specific patterns in their binding to ABH histo-blood group antigens and as such represent unique tools for visualization of cell-surface carbohydrate antigens. VLPs also offer the possibility to specifically administer reagents and drugs to cells carrying or overexpressing binding epitopes. In a first step of tagging recombinant VLPs we have radiolabeled VLPs from three different Norovirus strains. The intermediate agent *N*-succinimidyl-3-tributylstannyl benzoate was labeled with 8 MBq ^{125}I ($T_{1/2}$ = 60 days). The ^{125}I-labeled succinimidyl ester was then added to 8 μg VLPs for conjugation to accessible lysine residues, and typically 1 MBq of ^{125}I-VLPs were recovered, which corresponds to roughly 15 iodines on each VLP. The ^{125}I-VLPs were used in glycosphingolipid (GSL) chromatogram-binding assays. Norwalk VLPs possessed secretor dependent binding to H type 1, Lewis b, A type 1 and A Lewis b but not to B type 1, B Lewis b or to the non-secretor dependent Type1 precursor or Lewis a GSLs. This is the first time noroviruses have been demonstrated to bind to GSLs, which are present in epithelial cells throughout the gastrointestinal tract, and GSLs could be a receptor responsible for norovirus infection.

The tagging of viruses through the use of succinimidyl esters or related bioconjugate techniques offers the possibility to introduce a multitude of reporter groups e.g. fluorescent tags or various radioisotopes to study viral adhesion and infection. The present study has set the stage for us to address the targeting of labeled noroviruses to cellular receptors and to monitor the time-dependent spread of radiolabeled virus within an organism using nuclear imaging techniques.

Poster No. II-29

ELUCIDATING THE BIOSYNTHESIS OF CHITIN FILAMENTS AND THEIR CONFIGURATION WITH SPECIFIC PROTEINS AND ELECTRON MICROSCOPY

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Chitin is the second most abundant natural polysaccharide with many applications and even more potentials, it is very important to understand more details on the biosynthesis of this significant polymer. To deepen the knowledge on its mode of synthesis, nano-particles corresponding to vesicles with chitin synthase I activity were purified by biochemical methods from a yeast mutant strain. These vesicles were used to explore the biogenesis of nascent polymer-chains from the substrate UDP *N*-acetylglucosamine in the presence or absence of the chitinase ChiO1 or the chitin-binding protein CHB1, previously proven to target very specifically only chitin in α-configuration.

As viewed by electron microscopy, nascent chitin filaments extrude from one pole of the purified vesicles and grow to larger networks. These were shown to be degradable by the chitinase and to be targeted by the α-chitin-binding protein CHB1. The obtained results allow the conclusion that chains within the emerging chitin filaments have an antiparallel

orientation already during early stages of biosynthesis. In addition, the studies showed that the isolated vesicles contain highly organized fibroid structures which bind the CHB1 protein and are degraded by the chitinase. These findings suggest that during biogenesis nascent chains turn alternately back and forward provoking polarity-changes, to build the observed fibroid coils that are most likely stabilized *via* hydrogen bounds and *van der* Waals interactions. The crystalline structure of α-chitin has a high tensile strength; it is present in the exoskeleton of various organisms and within the cell wall of many fungi including pathogens. The presented knowledge will be also useful to screen and develop drugs inhibiting α-chitin-biosynthesis.

Poster No. II-30

DISTINCT SUBSTRATE SPECIFICITIES OF MEMBERS OF A NOVEL BACTERIAL α3GALACTOSIDASE GENE FAMILY

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In search of α-galactosidases with highly improved kinetic properties for removal of the immunodominant αGal in the blood group B, we recently identified a novel prokaryotic α3galactosidase gene family with highly restricted substrate specificity and neutral pH optimum. One member of the family derived from *B. fragilis* was characterized in detail and shown to exhibit exquisite substrate specificity for the branched blood group B structure, while linear α3Gal terminated structures such as the immunodominant Galα1-3Galβ1-4GlcNAc xenotransplantation epitope did not serve as substrate. In this study we have further characterized other members of the identified gene family and show that two distinct subfamilies of genes found in *B. fragilis* and *thetaiotaomicron* strains have related but distinct substrate specificities. While members of one subfamily have exclusive specificity for the branched blood group B structures, members of the other subfamily have a novel substrate specificity that includes both the branched blood group B structure as well as the linear xenotransplantation epitope. The latter subfamily offer enzymes with highly improved performance in enzymatic removal of the immunodominant α3Gal xenotranplantation epitope.

Poster No. II-31

BIOCHEMICAL CHARACTERIZATION OF A PEPTIDOGLYCAN DE-O-ACETYLASE: A NOVEL ENZYME DISCOVERED IN *NEISSERIA GONORRHOEAE*

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Peptidoglycan (PG) represents the rigid structural element in bacterial cell walls and thus serves to withstand the internal turgor pressure of the cytoplasm. In a number of pathogenic bacteria, addition of an acetate group to the C_6 position of *N*-acetylmuramic acid of PG has been demonstrated to affect the activity of muramidases, like lysozyme, thereby protecting these cells from innate defensive mechanisms. In addition, the *O*-acetylation of PG has also been shown to preclude the action of endogenous lytic transglycosylases. This class of autolysins is responsible for the growth and development of the PG sacculus that surrounds bacterial cells, the insertion of pores and flagella, spore formation, and the general turnover of PG. Without the removal of *O*-linked acetate, these processes would be severely limited. Despite this central importance, there is little information regarding peptidoglycan O-acetylation and even less known about its de-acetylation.

The current research is the first to identify a potential cluster of *O*-acetylpeptidoglycan related genes encoded in the genomes of a variety of Gram-negative and Gram-positive bacteria, including a number of important human pathogens, such as species of *Neisseria, Helicobacter, Campylobacter*, and *Bacillus*. Based on results from activity assays involving HPLC-based organic acid analysis, NMR, and chromogenic detection methods, one of the genes from this cluster, *ape1a*, has been identified as the first known acetylpeptidoglycan esterase (Ape). Through a combination of three-dimensional modeling, site-directed mutagenesis, Michaelis-Menten kinetics and inhibition studies, three residues (Ser80/His369/Asp366) were identified as the catalytic triad of Ape1a, which is characteristic of the serine protease/esterase family. These studies have aided in understanding the mechanism by which bacteria maintain certain levels of O-acetylated peptidoglycan and how these levels affect cellular processes, like cell-wall restructuring and turnover. Furthermore, these results are significant given the increasing number of pathogenic bacteria that are known to perform this O-acetyl modification.

Poster No. II-32

CHARACTERIZATION OF THE CAPSULAR POLYSACCHARIDE MODIFYING O-ACETYLTRANSFERASE OF *ESCHERICHI COLI* K1

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Escherichia coli K1 (*E. coli* K1) is a leading pathogen in neonatal sepsis and meningitis associated with high rates of mortality and severe neurological sequelae. The major virulence factor, which is essential for serum resistance and

vital passage of the blood brain barrier, is the capsular polysaccharide composed of alpha-2,8-linked polysialic acid (polySia). In several K1 strains, phase-variable modification of the capsule by O-acetylation is observed. This modification is synthesized by an O-acetyltransferase (OatK1) which catalyzes the transfer of acetyl-groups from acetyl-CoA to polySia. The corresponding gene is part of a 40 kb prophage and contains variable numbers of hepta-nucleotide-repeats at the 3'-end. Only repeat numbers that are a multiple of 3 allow full-length translation (phase on) whereas other numbers result in truncated translation products (phase off).
To understand the biochemical basis of polySia O-acetylation, recombinant OatK1 was purified to homogeneity and enzymatic activity was monitored in a spectrophotometric and a radioactive assay. Product analysis revealed that only sialic acid oligomers with ≥14 residues were O-acetylated, demonstrating a high acceptor substrate specificity. Using computer-assisted modeling for OatK1, we predicted a 3D-structure composed of a central left-handed parallel beta-helix fold with one protruding loop which assembles into a catalytic trimer. Two amino acids critical for catalytic activity were identified and corresponding alanine substitutions resulted in a complete loss of activity while expression level and oligomeric state were not affected. To investigate the role of repeat-encoded N-terminal extensions of OatK1, variants with 0, 12, 24 and 36 hepta-nucleotide repeats were purified. Notably, all variants were fully active, demonstrating that repeat-encoded extensions are not essential for activity. However, the catalytic efficiency increased with increasing numbers of repeats, revealing a new mechanism for regulating OatK1 activity.

Poster No. II-33

MODIFYING GLUCANSUCRASE REGIOSELECTIVITY TO SYNTHESIZE NEW OLIGOSACCHARIDES AND GLYCOCONJUGATES

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The potential of carbohydrate active-enzymes to synthesize new oligosaccharides or glyco-conjugates from renewable resources is large and far from having been fully explored and exploited. Within the known transglycosidases, glucansucrases from GH families 13 and 70, which synthesize from sucrose and without any nucleotide activated sugars homopolymers of glucose are very attractive [1,2]. In the presence of hydroxylated acceptors, these enzymes transfer the glucosyl residue from sucrose donor to the acceptors at the cost of polymer formation to produce glucosylated acceptors. The efficiency of such a reaction is, of course, dependant from the acceptor recognition. Our work has been focused on the glucosylation of non-natural acceptors i) arabinose, to develop new compounds from this hemicellulosic agro-resources and ii)flavonoïds to improve the solubility of these drugs [3]. Using glucansucrases of different specificities led to a variety of novel structures with interesting yields going up to 50 %. In the case of flavonoïds, reaction in non-water organic solvents had to be set up to increase glucosylation yield. Rational and combinatorial engineering was applied to better adapt the selected glucansucrases to these acceptors.

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Poster No. II-34

ENZYMES INVOLVED IN PROTEIN DEGLYCATION

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Fructosamine 3-kinase (FN3K) phosphorylates both low-molecular-weight and protein-bound fructosamines. Fructosamine 3-phosphates are unstable, breaking down spontaneously to 3-deoxyglucosone, inorganic phosphate and the amino compound that originally reacted with glucose. FN3K is therefore a 'deglycating' enzyme. Recent results obtained with FN3K-deficient mice confirm that FN3K acts as a protein deglycating enzyme in tissues.
Unlike FN3K, FN3K-related protein (FN3K-RP) does not act on fructosamines, but it does phosphorylate ketoamines with a D configuration in C3 (ribulosamines, erythrulosamines and, with a lower affinity, psicosamines). The ketoamine 3-phosphates that are formed by FN3K-RP are also unstable and their spontaneous decomposition leads to the regeneration of a free amino group, indicating that FN3K-RP is also a protein repair enzyme. This role has been confirmed in human erythrocytes, which are rich in FN3K-RP. Remarkably, the single FN3K-FN3K-RP homologue that is present in fishes, plants and bacteria appears to be also a ribulosamine/erythrulosamine 3-kinase, indicating that the repair of ribulosamines or erythrulosamines may be more important than the removal of fructosamines.
Ribulosamine and erythrulosamines most likely arise through a reaction of proteins with ribose 5-phosphate and erythrose 4-phosphate, two extremely potent glycating agents. The ribulosamine 5-phosphates and erythrulosamine 4-phosphates that are formed in this way must be dephosphorylated by a phosphatase. Glucose 6-phosphate is also a potent glycating agent, and a phosphatase acting best on protein-bound fructosamine 6-phosphates has recently been

identified as MDP-1. In conclusion, protein deglycation appears to involve a whole set of enzymes. A key question for future investigations is why it is important to rid proteins from their sugar adducts rather than replace them with newly synthesized macromolecules.

BENZON SYMPOSIUM No. 54

GLYCOSYLATION:
OPPORTUNITIES IN DRIG DEVELOPMENT

JUNE 11-14, 2007, COPENHAGEN, DENMARK

Organizing committee:
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Abstracts - WEDNESDAY, June 13, 2007

AUTOMATED OLIGOSACCHARIDE SYNTHESIS

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Access to usable quantities of pure oligosaccharides is of great importance for biochemical, biophysical, and biological research as well as medical applications of carbohydrates. Described is an integrated system for the automated solid phase assembly of oligosaccharides including the identification of a defined set of monosacchharide building blocks and their synthesis on large scale. Protocols for the synthesis of complex oligosaccharides containing "difficult" linkages such as alpha galactosides and beta mannosides are described.

A new automated synthesizer has been developed and purification and work-up procedures will be discussed. This synthesizer was used to assemble examples of all classes of carboohydrates including O-linked, N-linked glycans as well as glycosaminoglycans by automation.

Automated synthesis of oligosacccharides served as platform for the procurement of synthetic oligosaccharides for carbohydrate microarrays, carbohydrate affinity columns, labeled carbohydrates for imaging studies and carbohydrates to be used for structural studies involving X-ray crystallography. These chemicaltools are now employed in biochemical and biological studies. Examples of the use of such chemical tools will be discussed.

Therapeutic and diagnostic applications of synthetic carbohydrates are currently being pursued.Vaccine candidates for malaria, leishmaniasis, anthrax, tuberculosis, HIV and other important infectious are currently under development. Diagnostic tests for malaria, avian flu and TB have been developed. Illustrative examples will be used to demonstrate the potential of carbohydrates for these applications.

CHALLENGES AND OPPORTUNITIES IN NATURAL PRODUCT GLYCOSYLATION

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In nature, the attachment of sugars to small molecules is often employed to mediate targeting, mechanism of action, and/or pharmacology. As an alternative to pathway engineering or classical chemical glycosylation strategies, we report merging three promiscuous enzymes (a sugar kinase - 'E_1', a nucleotidylyltransferase - 'E_2', and a glycosyltransferase – 'E_3') with upstream synthetic chemistry and downstream chemoselective ligation provides a powerful method to diversify, via the attachment of variant sugars ('glycorandomize'), complex natural products. The optimization of E_1-E_3 using enzyme evolution and structure-based enzyme engineering, the glycorandomization proof of concept, the recent advances based upon the reversibility of glycosyltransferase-catalyzed reactions, the evolution of 'flexible' glycosyltransferases and the potential for *in vivo* glycosylation hosts will be highlighted. In addition, recent developments pertaining to our development of a complementary chemical method ('neoglycorandomization') will be discussed. The impact of the (neo)glycorandomization upon the activity of a variety of natural products including enediynes (calicheamicin), alkaloids (rebeccamycin, AT2433, colchicine) nonribosomal peptides (vancomycin), and steroids (digitoxin) may also be presented.

PLATELET GLYCOSYLATION: THE "IN AND OUTS" OF PLATELET TRANSFUSION

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Until recently, the only well-established mechanisms affecting platelet survival were antibody-mediated platelet clearance, consumption of platelets by coagulation reactions and massive bleeding. An effort to address a practical problem, how to refrigerate platelets for transfusion, led to the definition of a previously unsuspected platelet

clearance mechanism. We demonstrated that the lectin domain of αMβ2 receptors on hepatic macrophages mediates rapid clearance of washed murine platelets transfused after chilling for 2 hours, recognizing exposed βN-Acetylglucosamine (βGlcNAc) residues of N-linked glycans on clustered platelet von Willebrand factor receptors and that the same receptors elicit phagocytosis of chilled human platelets by human macrophages *in vitro*. A platelet-associated galactosyltransferase catalyzes the covering of exposed βGlcNAc residues with galactose in the presence of UDP-galactose, thereby blocking clearance of chilled mouse platelets *in vivo* and phagocytosis of human platelets *in vitro*. These intriguing findings contradicted prior studies with human platelets showing that the adverse effects of 4°C storage became irreversible only after >18 of 4°C storage and also are inconsistent with the recognition system for exposed galactose residues through asialoglycoprotein (ASGP) receptors. We therefore performed viability studies using human and murine platelets stored for two days. Following two days of 4°C storage, the survivals of the galactosylated human and murine platelets were no different than the non-galactosylated 4°C stored platelets. We further found that hepatic clearance of long-term cold-stored (LTCS) mouse platelets occurs in hepatocytes. Streptavidin-POD staining revealed abundant LTCS biotinylated platelets in hepatocyte phagosomes. Furthermore, cells of the hepatocyte HepG2 line avidly ingest fluorescently-labeled LTCS human platelets, as evidenced by flow cytometry, fluorescent microscopy and time-lapsed video microscopy. Long-term cold-storage increases binding of the RCA I lectin to platelets by ~1.7-fold, implying that with long-term cold-storage galactose exposure increases sufficiently to induce recognition by ASGP receptors. These results define a new clearance mechanism, representing the first example of blood cell removal by a non-myeloid cell. Because human platelets also express a sialotransferase that adds sialic acid to galactose residues, we propose that a combination of sialylation and galactosylation, achievable by addition of sugar substrates alone, might accommodate long-term cold storage of platelets for transfusion.

CARBOHYDRATE-VIRUS INTERACTIONS BY NMR

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Several viruses attach to carbohydrate-receptors in order to infect mammalian cells. Yet, the molecular mechanisms of virus attachment are largely unknown. Recently, we have shown that it is possible to study the binding of low molecular weight ligands and even of larger receptor fragments to native viruses using STD NMR. This is possible because the lines of the viral coat proteins are rather broad and extend into spectral regions where one can safely saturate the virus signals without touching resonances of the ligand. These promising results led us to speculate that one should also be able to target the binding of carbohydrates to viral surfaces employing STD NMR. It is well established that influenza viruses attach to target cells via interactions between hemaglutinin on the viral surface and sialic acid residues on the surface of host cells. It is less well known that a number of other viruses such as the Norwalk virus (NV), the rabbit hemorrhagic disease virus (RHDV), or the Hepatitis A virus (HAV) specifically adhere to carbohydrate structures on the surface of host cells. In the case of NV and RHDV these structures are blood group determinants, whereas in the case of HAV mucin related structures are discussed, and it has been shown that HAV adhesion is sialic acid dependent. Although the biological significance of these interactions is not yet fully understood, it appears that STD NMR has a great potential for the investigations of these interactions. We have therefore investigated the binding of H-type II blood group antigen related saccharides to recombinant RHDV virus like particles (VLPs). STD spectra allow an epitope mapping of the saccharides at atomic resolution and show that α-L-fucose is the minimal fragment that the virus recognizes. The talk will also address general aspects of NMR techniques to study virus-carbohydrate systems. We believe that STD NMR will significantly assist the design of corresponding viral entry inhibitors in general by precisely defining the pharmacophoric groups that are essential for the interaction with the viral coat.

DIFFERENTIAL N-GLYCOSYLATION OF RHEPO IN GOAT MAMMARY GLAND CELL EXPRESSED IN CELL CULTURE OR IN THE MILK

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The production of pharmaceutical recombinant proteins in the milk of transgenic animals is steadily receiving more and more attention. Some examples are human lactoferrin in mice and cows, human α-glucosidase in mice and rabbits, human EPO in mice and rabbits, and human antithrombin in goats. Human lactoferrin expressed in cow milk, showed complex-type glycans as in natural lactoferrin. Dislike natural lactoferrin, suggestion of the occurrence of *N,N'*-diacetyllactosediamine units were reported. Similar substitution was observed in antithrombin produced in goat milk. Sialylation occurred with both the Neu5Ac and Neu5Gc.

Recently our group has demonstrated a high level expression of rhEPO in goat milk by adenoviral transduction of the mammary gland. However, rhEPO exhibited low sialic acid content and for instance low *in vivo* erythropoietic activity. To understand this behavior a cell line from the goat mammary gland was established (GMGE). Adenovirally transduced GMGE cells cultured *in vitro* produced rhEPO with an N-glycosylation profile that differs drastically from

the rEPO-milk. None of each displayed the sialic acid content neither the biological activity of the classical CHO-EPO. GMGE-EPO is polyfucosylated and more sialylated than EPO-milk with almost only Neu5Ac. Whereas the EPO-milk exhibited a 1:1 ratio of Neu5Gc:Neu5Ac.
The N-glycosylation pattern of rEPO expressed in the goat mammary gland is far from the hEPO and the CHO cell recombinant form. The mammary gland cells of the goat correctly synthesized the recombinant hormone but lack the glycosylation machinery or part of their glycosyltransferases/glycosidases repressed or down regulated during lactation to assemble the correct hormone glycoforms needed to display the adequate erythropoeitic activity.

DO CARBOHYDRATE COATED NANOPARTICLES HAVE ANTITUMOR THERAPEUTIC POTENTIAL?

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Altered gene expression in tumor cells relative to the normal phenotype results in the modification of many cellular properties. Several of these changes involve glycoprocessing enzymes that assemble the sugar chains on glycoproteins and glycolipids. These "new" sugar chains lend novel adhesion properties to the cell and can be recognized as "foreign" by the host immune system. We have synthesized some of these so-called tumor-associated carbohydrate antigens (TACA) and attached them in a multivalent fashion to nano-sized gold particles for use as potential anti cell-adhesive agents. In addition, we have synthesized nanoparticles coated with mucin glycopeptides as possible components of anti-tumor vaccines. Initial data in an *in vivo* breast tumor model suggested that one of these particles can inhibit lung metastasis in mice. Several *in vitro* tests have revealed some very interesting properties of these novel entities that are dependent on particle size and surface chemistry. The latest results on the properties of these exciting new agents as well as progress on the synthesis of glycomimetics and glycopeptidomimetics for attachment to nanoplatforms will be presented. Results on the preparation and properties of carbohydrate coated quantum dots to be used as novel tumor imaging agents will also be described.

CONGENITAL DISORDERS OF GLYCOSYLATION: A LOOKE INTO THE CRYSTAL BALL

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Inherited defects in N-glycan biosynthesis are called Congenital Disorders of Glycosylation (CDG). Ten years ago only two defective genes were known; now there are 25. They affect >600 patients worldwide, but their recent discovery and under appreciation of glycobiology suggests that many more patients and types of disorders will be identified. Patients span an extremely broad clinical spectrum. Symptoms range from mild to severe and can include dysmorphic features, psychomotor retardation, brain abnormalities, neuropathy, failure to thrive, liver and kidney disorders, coagulopathy and protein-losing enteropathy. The specific molecular basis of these symptoms remain unknown. The disorders are divided into two groups. The first (I) involves defects in the assembly and transfer of the dolichol-linked precursor glycan to proteins in the endoplasmic reticulum, and the second (II) involves glycan processing. Defects have been found in monosaccharide precursor activation/interconversion, glycosyltransferases, glycosidases, chaperones, and nucleotide sugar transporters. More recently, defects were identified in cytoplasmic proteins that traffic the glycosylation machinery around the Golgi. Mutations in 4 of the 8 subunits of Conserved Oligomeric Golgi (COG) complex alter biosynthesis of both N- and O-glycans. Disruption of glycosylation by defective trafficking greatly increases the number of possible CDG candidate genes.
Only two disorders have any therapy. Oral mannose supplements are effective for phosphomannose isomerase (PMI, Fructose-6-P→Man-6-P) -deficient patients (CDG-Ib). Some GDP-Fucose transporter-deficient patients (CDG-IIc) respond to fucose therapy. There is no therapy for most common type, CDG-Ia, caused by defective phosphomannomutase (PMM2, Man-6-P→Man-1-P), but we are exploring a novel approach to treat these patients. We have begun to develop a mouse model of CDG-Ib and used non-anticoagulant heparin to treat protein-losing enteropathy (PLE) in another mouse model. Other CDG-I patients get PLE and heparin may be a therapeutic option for them.
Support: R01 DK55695, Children's Hearts Fund, CDG Family Network.

DEFECTIVE GLYCOSYLATION IN MUSCULAR DISTROPHY

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O-Mannosylation is an uncommon type of glycosylation in mammals but is important in muscle and brain development. We have identified and characterized glycosyltransferases, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1) and protein *O*-mannosyltransferase 1 (POMT1) and its homolog, POMT2 are involved in *O*-mannosyl glycan synthesis. Then POMGnT1 is found to be responsible for muscle-eye-brain disease (MEB) and POMT1/2 are for Walker-Warburg syndrome (WWS). MEB and WWS are congenital muscular

dystrophies with brain malformation and structural eye abnormalities. Recent data suggest that aberrant glycosylation of α-dystroglycan is also the cause of other four muscular dystrophies. These all are named α-dystroglycanopathies because these are caused by incomplete-glycosylation of α-dystroglycan.
Here we focus on protein *O*-mannosylation that is catalyzed by POMT1/2. Recently, we show that POMT1 forms a complex with POMT2, and the complex possesses protein *O*-mannosyltransferase activity, indicating that POMT1 and POMT2 associate physically and functionally *in vivo*. All mutations found in the *POMT1* gene of patients with WWS lead to great reduction of protein *O*-mannosyltransferase activity, although all POMT1 mutants coprecipitated with POMT2. These results indicate that the mutant POMT1s found in WWS patients could form hetero-complexes with POMT2 but that such complexes are insufficient for enzymatic activity. Possible regulatory mechanism of *O*-mannosylation will be discussed.

ROLES FOR A NOVEL MOLECULE PRAT4A IN THE SUBCELLULAR DISTRIBUTION AND RESPONSIVENESS OF TOLL-LIKE RECEPTORS

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The Toll family of receptors plays an essential role in innate recognition of microbial products. Cell surface TLR dimers consisting of TLR4/MD-2, TLR1/TLR2, or TLR2/TLR6 recognize microbial membrane components such as lipopolysaccharide (LPS) or lipopeptides, whereas nucleic acid sensing TLRs reside in intracellular orgnella. Immune cells such as DCs or macrophages express multiple TLRs, which are likely to be concomitantly activated in response to a pathogen that contains a variety of TLR ligands. It is, therefore, important to understand a mechanism regulating responsiveness of multiple TLRs. In this regard, we are interested in a mechanism governing the subcellular distribution of TLRs, which correlates well with their activity and ligand-specificity. We recently identified a novel molecule PRAT4A (a protein associated with TLR4), which is associated with TLR4 and regulates its cell surface expression on the cell surface. PRAT4A gene silencing leads to the lack of cell surface, fully glycosylated TLR4. We here addressed, by PRAT4A gene silencing, roles for PRAT4A in responses to a variety of TLR ligands, and found that PRAT4A is required not only for TLR4 but also for other cell surface TLRs. PRAT4A suggests a mechanism coordinating responsiveness of cell surface TLRs.

STRUCTURAL, FUNCTIONAL AND CLINICAL ASPECTS OF THE MBL PATHWAY (LECTIN PATHWAY) OF COMPLEMENT ACTIVATION - AN INNATE IMMUNE DEFENSE SYSTEM

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Microbes are recognized by the innate immune defence through highly conserved pathogen-associated molecular patterns (PAMPs). PAMPs are to a large extend suitably spaced carbohydrate determinants recognized by pattern recognition receptors (PRRs) or, in the case of my presentation, soluble, humoral pattern recognition molecules (PRMs). Mannan-binding lectin (MBL) represents the most studied PRM, not least due to the clinical implications of inherited deficiency. MBL deficiency is the most prevalent immunodeficiency, and increased susceptibility to infections thus only become apparent when the immune defence is otherwise stressed. MBL is a C-type lectin, i.e., the carbohydrate recognition domain (CRD) is dependent on calcium for its function. It is an oligomer of three or more subunits, each build of three polypeptides forming a collagenous stalk ending in three globular CRDs. The weak affinity for various hexoses is thus transformed into a very high avidity through multiple interactions with target. MBL circulates in complexes with three proenzymes (serine proteases or MASPs), and binding to target somehow mediates their activation. MASP-2 then activates downstream components of the complement system, resulting in the destruction of the target and initiating inflammatory reactions. The roles of MASP-1 and MASP-3 are not resolved. Three other proteins, ficolins, present an overall structure similar to MBL, but with fibrinogen-like binding domains. The ficolins are also associated with MASPs. they are known as lectins, but they react with many different acetylated molecules. They show reaction with some bacteria, but, like MBL, they also seem to be involved in apoptosis. We have made recombinant MBL, which is now being prepared for phase II replacement trials. We have determined several mutations resulting in MASP-2 deficiency (less frequent than MBL deficiency), and clinical associations are just now showing up, indicating a role also for this protein in clinical practice. We are awaiting possible clinical correlates to ficolin deficiency.

THE GOLGI UDP-XYLOSE TRANSPORT IS ESSENTIAL FOR THE FORMATION OF O-LINKED XYL-XYL-GLC EPITOPES ON EPIDERMAL GROWTH FACTOR LIKE DOMAINS

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The transport of nucleotide sugars from the cytoplasm into the Golgi apparatus is mediated by specialized type III proteins, the nucleotide sugar transporters (NSTs). Transport assays carried out *in vitro* with Golgi vesicles from mammalian cells, showed specific uptake for a total of eight nucleotide sugars. When this study was started, NSTs

with transport activities for all but two nucleotide sugars (UDP-Xyl and UDP-Glc) had been cloned. Aiming at identifying these elusive NSTs, bioinformatic methods were used to display putative NST-sequences in the human genome. Ten open reading frames were identified, cloned, and heterologously expressed in yeast. Golgi vesicles isolated from yeast cells expressing the human gene SLC35B4 exhibited transport activity for UDP-Xyl and expression of the epitope-tagged SLC35B4 in mammalian cells identified it as a Golgi localized protein. Because decarboxylation of UDP-GlcA is known to produce UDP-Xyl inside the ER and Golgi lumen, our study identified a second way for the delivery of UDP-Xyl to the Golgi apparatus. Our next question therefore was, if the two transport systems are simply redundant or may be components of two independent pathways. Besides being the initiating sugar in proteoglycan biosynthesis, Xyl is found in a trisaccharide (Xyl-Xyl-Glc) that decorates functionally important proteins like Notch and the blood coagulation factors VII and IX. A cellular knock-down model has been generated using the siRNA technique and in parallel to wild type cells was used as recipient for Notch fragments that contained the relevant sequon for trisaccharide attachment. Mass spectrometric analyses of the recombinant Notch fragments demonstrated a drastic reduction of the Xyl-Xyl-Glc epitope in knock-down cells. Since experiments carried out with a fragment of Decorin did not indicate changes in the glycosylation pattern, our results suggest that the Golgi UDP-Xyl transporter is mainly required for the modification of epidermal growth factor like domains.

Poster No. III-1

INHIBITORY EFFECTS OF SYNTHETIC HETEROCYCLIC COMPOUNDS ON ANGIOGENESIS AS WELL AS THE MIGRATION AND INVASION OF A MOUSE OSTEOSARCOMA CELL LINE EXPRESSING CHONDROITIN SULFATE

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The possibility of enhancing the process of drug discovery through the use of non-peptide synthetic heterocyclic compounds, which serve as templates for investigating eventual drugs, has been established. The idea of targeting cell migration for the development of anti-cancer therapies has also attracted considerable interests. Here we investigated effects of newly synthesized 2-substituted-5-(4-methoxy)-1-oxa-3-aza-spiro[5,5]undecanes (**1-3**), 2-(chloro-phenyl)-3-*p*-tolyl-thiazolidin-4-one (**4**), (2-substituted)(4-(6-fluorobenzo[d]isoxazol-3-yl)piperidin-1 -yl)methanones (**5,7,8**) and 4,5-disubstituted-4*H*-1,2,4-triazole-3-thiols (**6,9-11**) on the invasion of murine osteosarcoma cells (LM8G7), which are highly progressive, metastasize to the liver, and express chondroitin sulfate on the cell surface. The above compounds are positively charged and expected to interact with chondroitin sulfate on cell surface. Two different assays were employed to evaluate our compounds: (i) An *in vitro* assay for quantifying the inhibitory effects on the cell invasion, and (ii) an inhibition assay for wound-healing using cell migration of LM8G7. Among the tested compounds, compound **4**, (4-(6-fluorobenzo[d]isoxazol-3-yl) piperidin-1-yl)(2-ethoxyphenyl)methanone (**5**), and 4-(2,6-difluorobenzylideneamino)-5-methyl-4*H*-1,2,4-triazole -3-thiol (**6**) strongly suppressed the invasion of LM8G7 cells into the matrigel invasion chamber. Further results from the wound-healing assay substantiated the observations in the invasion assay. For example, the compounds **4-6** completely inhibited the cell migration. The compound **4** exhibited evidently the anti-angiogenic activity by inhibiting the formation of blood vessels in the chorioallantoic membrane assay in fertilized eggs.

Poster No. III-2

IMMUNOMODULATORY DRUGS AFFECT GALECTIN-1 EXPRESSION

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Galectin-1, a β-galactoside binding lectin, a well known regulator of T-cell homeostasis and survival, was recently shown to be also involved in control of monocyte/macrophage physiology. While galectin-1 mainly acts as an anti-inflammatory molecule, another member of galectin family, galectin-3 is known as a strong pro-inflammatory signal. We have recently recognized galectin-3 as a new molecular target of immunomodulatory drugs in monocyte/macrophage-like cells. In this study we investigated the effects of immunomodulatory drugs (aspirin, indomethacin, hydrocortisone and dexamethasone), applied in therapeutic ranges on the expression of galectin-1 at gene and protein level in monocytic THP-1 cells and the cells activated by lipopolysaccharide from *E. coli* (LPS).

The targeted mRNA level was evaluated using quantitative RT-PCR technique and the expression of galectin-1 in cell homogenates was determined by western-immunoblot analysis. The results showed that immunomodulatory drugs affected the expression of galectin-1 on both, gene and protein level, and that the effects were dependent on drug type and applied concentration as well as time of the exposure. The modulatory effects of the applied drugs on galectin-1 expression were also observed in the cells activated by LPS. These findings represent important step in the understanding of the effects of immunomodulatory drugs on galectin-1 expression, as well as the role of this lectin in the physiology of monocytes.

Poster No. III-3

THE NOVEL ETHER LIPID INO-C2-PAF IS A POTENT INHIBITOR OF CELL PROLIFERATION AND MIGRATION

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In the search for novel anticancer drugs that do not interfere with DNA synthesis or influence the cytoskeleton we developed a new group of antiproliferative compounds, the glycosidated phospholipids. These compounds cause remodeling of the structure and function of plasma membranes. Recently, we described two members of this group that contain alpha-D-glucose in the *sn*2 position of the glycerol backbone of phosphatidylcholine and platelet-activating factor, giving rise to Glc-PC (1) and Glc-PAF (2), respectively. Glc-PC and Glc-PAF inhibit growth of HaCaT cells at non-toxic concentrations. The introduction of myo-inositol (on a C2-spacer), in place of alpha-D-glucose leads to Ino-C2-PAF. The inositol-containing PAF enhances the antiproliferative capacity (IC_{50}=1.8 µm) and reduces the cytotoxicity relative to Glc-PAF (LC_{50} = 15 µm). In non-malignant keratinocytes, Ino-C2-PAF (3) induces differentiation and to a minor degree apoptosis. Furthermore, Inositol-C2-PAF increases matrix-dependent adhesion and decreases cell migration. It turned out that surface expression of integrins, the receptors for components of the ECM is not altered; instead Ino-C2-PAF induces increased clustering of integrins and cytoskeletal rearrangements. Ino-C2-PAF therefore seems to be a promising candidate as a lead structure for the treatment of hyperproliferative and migration-based diseases.

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Poster No. III-4

"ENGINEERED HEPARINS": NOVEL ?ETA-SECRETASE INHIBITORS AS POTENTIAL THERAPEUTICS FOR ALZHEIMERS DISEASE

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Cleavage of amyloid β-protein precursor (APP) by the protease beta-secretase (BACE1) is a key step in Aβ peptide processing. We recently described a novel role for heparan sulfate polysaccharides in AD pathology as one of the first naturally occurring inhibitors of β-secretase (BACE1) (Scholefield et al., *J. Cell. Biol*, 163, 97-107, 2003). We are currently evaluating "engineered" (chemically modified) heparin analogues as novel BACE1 inhibitors *in vitro* and *in vivo*.

To this end we have developed and extensively tested a number of engineered heparin analogues for their ability to inhibit BACE1 and also their activity as anticoagulants and as inhibitors of other proteases related to BACE1 (Patey et al, *J Med Chem* 49, 6129-6132, 2006). Several lead compounds have been identified that are effective BACE1 inhibitors, but have negligible activity as anti-coagulants or on other proteases related to BACE1. They are effective at lowering Aβ production and do not show cytotoxicity in organotypic brain cultures. We have gone on to test these novel compounds in the Tg2576 transgenic mouse model of AD. Initial assessments have been made of the bioavailability, pharmacodynamics and toxicity of both high and low molecular weight types of these compounds *in vivo*. We have tested the efficacy of the compounds by measuring brain Aβ levels as well as by behavioral testing. These data provide crucial new insights into the *in vivo* efficacy of selective engineered heparins as BACE1 inhibitors, and could underpin the development of new therapeutic strategies for human AD and other neurodegenerative disorders.

Poster No. III-5

IDENTIFICATION OF A NOVEL MISSENSE MUTATION IN *POMGNT1* IN SWEDISH PATIENTS WITH CONGENITAL MUSCULAR DYSTROPHY

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Several forms of congenital muscle dystrophies (CMDs) are associated with defective glycosylation of α-dystroglycan. Mutations in the gene for protein O-mannose β1,2-N-acetylglucosaminyltransferase (*POMGnT1*) have been shown to be linked to Muscle Eye Brain disease (MEB). Limb Girdle Muscular Dystrophy 2I (LGMD 2I) is a milder variant of CMD. LGMD 2I is a common muscular dystrophy in Europe and caused by mutations in the fukutin-related-protein (*FKRP*) gene. We immunostained muscle biopsies from 16 patients diagnosed with CMD using an antibody directed against glycosylated α-dystroglycan. DNA from patients was screened for mutations in *FKRP*. Patients with low or no immunostaining of α-dystroglycan and no mutation in *FKRP* were further analyzed for mutations in *POMGnT1*, *LARGE*, *POMT1* and POMT2.
We identified one patient, who was homozygous for the previously published IVS17+1G>A mutation (iE17/E18 G>A). Family analysis revealed a CMD diseased brother homozygous for iE17/E18 G>A, two healthy siblings without any mutation and the two parents heterozygous for the mutation.
In one patient, now a 24 year old woman with CMD, ocular abnormalities, frontal brain dysmyelination and earlier seizures, we identified, besides the iE17/E18 G>A mutation, a novel 1738 C>T missense mutation (^{580}Arg>stop). One of her parents was heterozygous for the novel 1738 C>T mutation and showed no iE17/E18 G>A mutation. The patient was thus a compound heterozygote in the *POMGnT1* gene.
Several mutations in *POMGnT1* have been reported. The iE17/E18 G>A mutation appears to be the most common. In order to elucidate if the iE17/E18 G>A mutation, in compound heterozygote or homozygote form, is associated with severity of the clinical phenotypes in CMD patients, as reported for the LGMD 2I and *FKRP* mutation 826 C>A, more CMD patients needs to be analyzed for this and other mutations in relevant genes.

Poster No. III-6

A POSSIBLE ROLE OF FUNGAL GALECTINS IN THE DEFENSE AGAINST COMPETITORS, PREDATORS, PARASITES AND PATHOGENS

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Galectins or S-type lectins occur in the animal and fungal kingdom but are not found in the plant kingdom and the domain of prokaryotes. Within the fungal kingdom, they are restricted to the mushroom-forming homobasidiomycetes. Since homobasidiomycetes harbor, in contrast to animals, only few members of the galectin family, these fungi are suited to study the structure and function of these lectins at an organismic level.
The homobasidiomycete *Coprinopsis cinerea* contains two isogalectins, CGL1 and CGL2, as well as a homologous lectin, CGL3. All three proteins are strongly induced during sexual development and highly enriched in the mushroom. Despite this differential expression pattern, neither ectopic expression during vegetative growth nor silencing of the respective genes had any obvious effect on mushroom formation.
In search of an alternative function, we recently detected a pronounced toxicity of the galectin CGL2 towards the nematode *Caenorhabditis elegans*. The phenotype of the toxicity strikingly resembles the one of the nematotoxic crystal toxins from *Bacillus thuringiensis*. We are currently investigating the specificity and the mechanism of this CGL2-mediated nematotoxicity. The glycan might represent a promising antigen for a vaccination against parasitic nematodes. CGL3, on the other hand, represents the first prototype galectin whose specificity is altered by a mutation in the conserved galactose-coordinating tryptophan residue. The substitution by an arginine residue changes the coordination of the glycan resulting in a no significant binding of lactose or LacNAc but preferral of chitobiose and LacDiNAc. Preliminary data show that CGL3 reveals no toxicity towards *C. elegans* but is able to agglutinate specific *E. coli* strains.
Taken together, these results suggest, that the two isogalectins CGL1 and CGL2 as well as the homologous CGL3 lectin of *C. cinerea* are part of a lectin-mediated defense of the fungus against competitors, predators, parasites and pathogens. Interestingly, a similar role has recently been demonstrated for human Gal-3 suggesting that such a function of galectins is conserved between the animal and fungal kingdom.

Poster No. III-7

OVEREXPRESSION OF THE CYTOTOXIC T CELL GALNAC TRANSFERASE (GALGT2) IN SKELETAL MUSCLE INHIBITS MUSCULAR DYSTROPHY IN MULTIPLE MOUSE MODELS OF THE DISEASE

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The cytotoxic T cell (CT) GalNAc transferase (Galgt2) is a type II Golgi β1,4-N-acetylgalactosaminyltransferase that glycosylates select glycoproteins and glycolipids to create the CT carbohydrate antigen (GalNAcβ1,4[NeuAc or NeuGcα2,3]Galβ1,4GlcNAcβ-R). In most vertebrate skeletal muscles, Galgt2 and the CT carbohydrate structures it creates become localized to the neuromuscular synapse, the synapse made between the nerve terminals of motor neurons and skeletal myofibers (Martin et al. (1999) Mol. Cell. Neurosci. 13; 105-118). Overexpression of Galgt2 in

the skeletal muscles of transgenic mice causes the extrasynaptic expression of the CT carbohydrate and stimulates the extrasynaptic expression of other synaptic molecules. One of these is utrophin, a synaptic homologue of dystrophin (the gene mutated in Duchenne muscular dystrophy. DMD), whose overexpression can ameliorate DMD-related disease. As a likely result of the changed expression of such molecules, transgenic overexpression of Galgt2 in the mdx mouse model for DMD prevents the development of muscular dystrophy (Nguyen et al. (2002) Proc. Natl. Acad. Sci. 99;5616-5621). This is the case for almost the entire lifespan of the animal (Xu et al (2007) Neuromuscular Disorders, in press). The profound effect of Galgt2 on muscular dystrophy in transgenic mdx mice, where overexpression begins from embryonic stages, is complicated by its additional effects on muscle growth and neuromuscular structure. Here, we use Adeno-associated virus to show that postnatal overexpression of Galgt2 in mdx skeletal myofibers is equally effective in inhibiting muscular dystrophy, but that it does so without altering muscle growth or neuromuscular structure. Unlike embryonic overexpression, postnatal overexpression of Galgt2 did not reproducibly increase the expression of utrophin. Moreover, Galgt2 overexpression inhibited muscular dystrophy to the same extent in utrophin-deficient mdx muscles as it did in utrophin-expressing mdx muscles. Thus, Galgt2 is a molecular target for therapy in DMD that can be utilized in a manner that separates its clinical benefit from its effects on development, and its clinical benefit is distinct from that achieved by utrophin. These data are also consistent with our recent finding that Galgt2 overexpression is therapeutic in mouse models for other forms of muscular dystrophy, including laminin α2-deficient congenital muscular dystrophy (MDC1A). Gene therapy and pharmacological approaches are being undertaken to develop therapeutics for DMD and other forms of muscular dystrophy based on these findings.

Poster No. III-8

THE ACUTE-PHASE PROTEIN ALPHA-1-ACID GLYCOPROTEIN (AGP) INDUCES RISES IN CYTOSOLIC CA2+ IN NEUTROPHIL GRANULOCYTES VIA SIALIC ACID-BINDING IMMUNOGLOBULIN-LIKE LECTINS (SIGLECS)

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Several studies have indicated that the highly glycosylated acute-phase protein α_1-acid glycoprotein (AGP) is involved in modulating inflammation and immune responses. AGP binds to neutrophils and may have a role in modulating neutrophile functions, but the signaling mechanism(s) have not been elucidated. In this investigation, we found that AGP dose-dependently induced rapid rises in intracellular calcium $[Ca^{2+}]_i$ in anti-L-selectin-pretreated neutrophils. The concentration of AGP required for a detectable effect on neutrophils was approximately 500-fold lower than the concentration normally found in plasma. The generated $[Ca^{2+}]_i$ signal was dependent on activation of phospholipase-C and Src tyrosine kinases, indicating a receptor-mediated response.

Neuraminidase and mild periodate treated AGP was not able to generate a $[Ca^{2+}]_i$ signal in anti-L-selectin treated neutrophils indicating a dependency for sialic acid in the interaction. When neutrophile lysate were passed over an affinity column with immobilized AGP, a protein reactive with anti-Siglec-5 antibodies was bound to the column. Antibodies to Siglec-5 could also induce an L-selectin-dependent $[Ca^{2+}]_i$ signal in neutrophils and were antagonized dose-dependently by AGP. The effect of AGP could be mimicked with 3'sialyllactose- and 6'sialyllactose-BSA conjugates but not with Sialyl-Le^x-BSA conjugate, which is consistent with the binding specificity for Siglec-5. However less glycosylated plasma proteins such as transferrin or fibrinogen did not show the same effect. Since antibodies to Siglec-5 cross-reacts with the newly characterized Siglec-14, we conclude that AGP can generate calcium signals in neutrophils after L-selectin engagement via interaction with Siglec-5 and/or Siglec-14.

Poster No. III-9

CONSEQUENCES OF MUTATIONS IN UDP-GLCNAC 2-EPIMERASE/MANNAC KINASE FOR PATHOLOGY OF HEREDITARY INCLUSION BODY MYOPATHY

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Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult-onset, slowly progressive distal and proximal muscle weakness, and typical muscle pathology with cytoplasmic "rimmed vacuoles" and cytoplasmic or nuclear inclusions composed of tubular filaments. More than forty different missense mutations in the gene encoding UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), the key enzyme in the biosynthetic pathway of sialic acid, have been identified in quadriceps sparing HIBM cases diagnosed in several isolated families worldwide. In order to biochemically characterize the HIBM mutations of GNE; we recombinantly expressed GNE proteins with thirteen different mutations in both domains of the bifunctional enzyme. All mutant enzymes still displayed UDP-GlcNAc 2-epimerase as well as ManNAc kinase activities, but compared to the wild-type enzyme either one or both enzyme activities were reduced. The extent of reduction strongly differs among the mutants, ranging from 20% to 80%. We further analyzed GNE in patient-derived muscle cell lines and found

reduction of UDP-GlcNAc 2-epimerase activity between 30% and 70%. However, analysis of overall glyco-conjugate sialylation in these cells revealed no differences, although cells with GNE mutations which cause residual activities of less than 50% showed reduced expression of polysialic acid. It is therefore likely, that subtle changes in sialylation contribute to the pathological mechanism of HIBM. Otherwise, different mutations result in different enzymatic activities but not in different disease phenotypes, not suggesting a role of the enzyme function of GNE in the disease mechanism.

Poster No. III-10

ROLE OF POLYPEPTIDE GALNAC-TRANSFERASE T3 IN FGF23 SECRETION

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Mutations in the glycosyltransferase, polypeptide GalNAc-T3 gene, involved in initiation of O-glycosylation, were recently identified as a cause of the rare autosomal recessive metabolic disorder familial tumoral calcinosis (FTC)(OMIM 211900). FTC is associated with hyperphosphatemia and massive ectopic calcifications. Here, we demonstrate that the secretion of the phosphaturic factor FGF23 requires O-glycosylation, and that GalNAc-T3 selectively directs O-glycosylation in a subtilisin-like proprotein convertase recognition sequence motif, which blocks processing of FGF23. Furthermore, this O-glycosylation is depending on the lectin domain of GalNAc-T3.

The study suggests a novel posttranslational regulatory model of FGF23 involving competing O-glycosylation and protease processing to produce intact FGF23.

Poster No. III-11

PROAPOPTOTIC AND ANTIANGIOGENIC ACTIVITY OF IMIDAZOLE DERIVATIVES MEDIATED BY UPREGULATION OF *BAX* AND DOWN REGULATION OF VEGF

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The newly synthesized imidazole derivatives functions as a cations and have the potential ability to modulate gene expression and signal transduction. We investigated the effect of imidazole derivatives on the activation of apoptotic and anti-angiogenic pathways in Ehrlich Ascites Tumor (EAT) cells. Treatment with imidazole derivatives *in vivo* resulted in inhibition of proliferation of EAT cells and ascites formation. Further, we demonstrate that the induction of apoptosis in EAT cells showed nuclear condensation, DNA fragmentation and translocation of caspase-activated DNase (CAD) to nucleus upon imidazole derivatives treatment. Imidazole derivatives-induced apoptosis is mediated through upregulation of BAX, down regulation of Bcl-2 and activation of caspase-3. On the other hand, the decreased secretion of ascites by EAT cells is corroborated by reduction in VEGF secretion upon imidazole derivatives treatment. These imidazole derivatives were able to bind to the receptor binding site of the VEGF by interfering with the co-receptor activity of heparan sulafate preoteoglycans on tumor cells. Further, H&E staining of peritoneum sections in imidazole derivatives treated mice suggests its efficacy in acting as anti-angiogenic compound in EAT cells by inhibiting proliferation of endothelial cells in mouse peritoneum However, immunofluorescence studies of NF-κB and HIF-1α revealed that the inhibition of nuclear translocation of NF-κB p65 and HIF-1α, transcription factors required for VEGF gene expression, in imidazole derivatives treated EAT cells. These inhibitors could represent as promising candidates for anticancer therapies.

Poster No. III-12

DEPENDENCE OF NEUROTROPHIC FACTOR ACTIVATION OF TRK RECEPTOR AND NEUROPROTECTION ON CELLULAR SIALIDASE

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A direct link between receptor glycosylation and its subsequent activation following natural ligand binding has not been reported. We have previously shown that a highly purified recombinant *T. cruzi* trans-sialidase (TS) specifically hydrolyzes the sialyl α-2,3-linked β-galactosyl sugar residues of TrkA receptors on TrkA-expressing PC12 cells and colocalizes with TrkA internalization and phosphorylation (pTrk) in the absence of neurotrophic factor. We now discover that nerve growth factor (NGF) and brain derived growth factor (BDNF) activation of their respective Trk receptors are also critically dependent on cellular sialidase(s) activity. We identify that NGF and BDNF binding to Trk receptors on Trk-expressing cell lines and primary cortical neurons induces membrane sialidase which targets and desialylate the receptor and, consequently, initiates Trk dimerization and activation. Measurement of live cell sialidase

activity in culture was developed by assessing fluorescence of hydrolyzed neuramindase-specific substrate 4-MUNANA (2′-(4-Methylumbelliferyl)-α-DN-acetylneuraminic acid). Lectin inhibition assays and colocalization experiments show that NGF-induced membrane sialidase(s) rapidly and specifically targets sialyl α-2,3-linked β-galactosyl sugar residues of Trk receptors. Phosphorylation of Trk activation was determined by immunocytochemical assays with anti-pTrk antibody. Sialidase activity induced by NGF-treated TrkA-PC12 and BDNF-treated TrkB-nnr5 cells and primary hippocampal neurons is blocked by neuraminidase inhibitors Tamiflu, BCX1812 and BCX1827 but not by DANA. In the presence of these inhibitors, no pTrk can be detected, and neurotrophic factor induced neurite outgrowth and neuroprotective effects are significantly reduced in a dose-dependent manner. Our data establish a novel mode of Trk receptor activation following natural ligand interaction, and define a new function for cellular sialidase. Supported by NSERC, Harry Botterell Foundation for Neuroscience Research, ARC, and Garfield Kelly Cardiovascular Res and Dev Fund.

Poster No. III-13

GLYCOSYLATED PEPTIDES AS DIAGNOSTIC TOOLS FOR AUTOIMMUNE DISEASES: A CHEMICAL REVERSE APPROACH

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Growing evidences indicate that glycosylation is a post-translational modification that, either native or aberrant, plays a fundamental role in a large number of biological events. The hypothesis that aberrant glycosylations trigger autoantibodies in autoimmune diseases has been already demonstrated in Guillain–Barrè Syndrome (GBS). In fact, *Campylobacter jejuni* elicits an antibody response to specific glycoepitopes cross-reactive with host gangliosides. By an innovative "chemical reverse approach" we demonstrated for the first time that an aberrant *N*-glucosylation is possibly triggering autoantibody response in Multiple Sclerosis (MS) [1]. In fact, by the synthetic β-hairpin peptide structure CSF114(Glc), optimally exposing on the tip of the β-turn the minimal epitope Asn(Glc), we detected for the first time autoantibodies in MS patients' sera by ELISA. In this scenario, we decided to extend our "chemical reverse approach" to other autoimmune conditions, proposing CSF114 as an "Universal Peptide Scaffold" to be modified for specific biomarkers recognition. We demonstrated that modification of the CSF114 β-turn structure with different glycosyl moieties linked to different amino-acid side chains, each one specific for different autoimmune diseases, can lead to a family of Synthetic Antigenic Probes to be used to develop specific diagnostic/prognostic tests for guiding personalised therapeutic treatments.

[1] (a) Lolli, F. et al. *P.N.A.S. U.S.A.* **2005**, *102*, 10273-10278; (b) Lolli, F. et al. *J. Neuroimmunology* **2005**, *167*, 131-137; (c) Papini, A.M. *Nat. Med.* **2005**, *11*, 13; (d) Carotenuto, A. *J. Med. Chem*. **2006**, *49*, 5072-5079; (e) Papini, A.M.; Rovero, P.; Chelli, M.; Lolli, F. Granted U.S.A. Patent & PCT Application WO 03/000733 A2.

Poster No. III-14

AN N-GLUCOSYLATED PEPTIDE AS MULTIPLE SCLEROSIS ANTIGENIC PROBE TO FISHING OUT AUTOANTIBODIES IN SERUM AND MONITORING DISEASE ACTIVITY

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Multiple Sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) in which an autoimmune mechanism against myelin antigens is thought to contribute to the immunopathological mechanisms of the disease. The components of normal CNS myelin or post-translational modified (PTM) forms of these myelin proteins are the most extensively studied putative self-antigens. It is now clear that some PTMs can create new self-antigens or even mask antigens normally recognized by the immune system. In our laboratory, we developed CSF114(Glc), a structure-based designed glucosylated peptide, as the first Multiple Sclerosis Antigenic Probe (MSAP) accurately measuring autoantibodies, as specific biomarkers of disease activity, by ELISA experiments on sera of a statistically significant MS patients' population [1]. In order to characterise the epitope(s) mimicked by CSF114(Glc), by a bioinformatic approach we selected myelin proteins with sequence and/or conformation homologies. Moreover, we evaluated the role in antibody recognition of the amino acid sequence surrounding the minimal epitope Asn(Glc) and we investigated the size of the peptide-binding groove interacting with the wild-type mimicked epitope(s). The synthetic glucosylated peptides of the selected sequences were tested in ELISA on sera of MS patients' positive and negative to CSF114(Glc).

[1] (a) Lolli, F. et al. *P.N.A.S. U.S.A.* **2005**, *102*, 10273-10278; (b) Lolli, F. et al. *J. Neuroimmunology* **2005**, *167*, 131-137; (c) Papini, A.M. *Nat. Med.* **2005**, *11*, 13; (d) Carotenuto, A. *J. Med. Chem*. **2006**, *49*, 5072-5079; (e) Papini, A.M.; Rovero, P.; Chelli, M.; Lolli, F. Granted U.S.A. Patent & PCT Application WO 03/000733 A2.

Poster No. III-15

MUTATION IN THE NUCLEAR LOCALIZATION SIGNAL OF THE CMP-SIALIC ACID SYNTHETASE CAUSES A LETHAL PHENOTYPE IN MICE

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The CMP-sialic acid synthetase (CMP-sia syn) catalyses the activation of sialic acid (Sia) which is a prerequisite for the incorporation of Sia into glycoconjugates.

A unique feature of CMP-Sia syn is its nuclear localization, as all other sugar activating enzymes are cytoplasmic proteins. The nuclear targeting of CMP-Sia syn is mediated by a canonical nuclear signal (NLS). Using site directed mutagenesis, we previously showed that the NLS shares primary structure elements with the active site of the enzyme. However, despite of their cytoplasmic localization some NLS-mutants could fully complement a CMP-Sia syn deficient Chinese Hamster Ovary cell line. This led us to conclude that enzymatic activity and sub cellular localization are independent functions. To study the relevance of nuclear localization at a systemic level, we generated a mouse model in which the endogenous CMP-Sia syn gene was replaced by a functionally active NLS-mutant. Homozygote mutant mice showed normal embryonic development, but died soon after birth. Biochemical analyses carried out to evaluate the sialylation patterns in mutants did not display differences to wild type and strongly argued for the integrity of the sialylation pathway. Similarly, histological studies in a number of organs did not reveal any significant morphological changes. Unexpectedly however, and in marked contrast to the cellular system, we found the mutant enzyme to localize in the nucleus of transgenic animals. Moreover, Western blot analysis of brain samples revealed a marked decrease in the amount of mutant protein.

Together, our data argue for a second yet unknown function of CMP-Sia syn in the cell nucleus. Based on the observation that the sialylation machinery seems to be intact, this may be a function exerted by the C-terminal domain. Using a yeast two hybrid screen we are currently searching for potential interaction partners.

Poster No. III-16

IDENTIFICATION OF PROTEINS, MUCINS AND THEIR *O*-GLYCOSYLATION IN THE ENDOCERVICAL MUCUS DURING THE MENSTRUAL CYCLE

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The mucus filling the human cervical opening blocks the entry to the uterus, but at the same time must undergo alterations during the menstrual cycle to allow for penetration of the sperms at ovulation. We have studied this mucus, its content of proteins, mucins, and the mucin *O*-glycosylation in cervical secretions before, during and after ovulation. Cervical mucosal secretions from twelve subjects were collected, reduced-alkylated, separated with polyacrylamide or agarose/polyacrylamide gel electrophoresis, stained with silver, Alcian blue or Coomassie blue stain. Protein and mucin bands from before and during ovulation were digested and subsequently analyzed by nanoLC-FT-ICR MS and MS/MS. We identified 194 proteins after searches against the NCBI non-redundant protein database and an in-house mucin database. Three gel-forming (MUC5B, MUC5AC, and MUC6) and two transmembrane mucins (MUC16 and MUC1) were identified. For the analysis of mucin *O*-glycosylation, separated mucins from six individuals were blotted to PVDF-membranes, the *O*-glycans released by reductive β-elimination and analyzed with capillaryHPLC-MS and MS/MS. At least 50 neutral, sialic acid- and sulfate-containing oligosaccharides were found. An increase of GlcNAc-6GalNAcol core 2 structures and terminal Fuc residues are typical for ovulation, and NeuAc-6GalNAcol and NeuAc-3Gal- epitopes for the non-ovulation phases. The cervical mucus at ovulation is thus characterized by neutral and fucosylated oligosaccharides. This comprehensive characterization of the mucus during the menstrual cycle suggest mucin glycosylation as the major alteration at ovulation, but the relation to the altered physicochemical properties and sperm penetrability is still not understood.

Poster No. III-17

TRANSFECTION OF PANCREATIC ADENOCARCINOMA CELL LINES WITH α2,3-SIALYLTRANSFERASES TO DETERMINE THEIR ROLE IN THE BIOSYNTHESIS OF SIALYLATED ANTIGENS INVOLVED IN INVASIVE AND METASTATIC PROCESSES

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One of the features of tumor cells is usually an increase in sialic acid content at the cell surface and in secreted glycoproteins. In particular, the expression of the carbohydrate antigens sialyl-Lewis^a (SLe^a) and sialyl-Lewis^x (SLe^x) can be related to the metastatic potential of tumor cells. This aberrant expression of sialylated antigens could be related to abnormal expression of sialyltranferases (STs). Our studies have focused on the expression pattern of the

sialylated antigens, SLe^a and SLe^x, in pancreatic adenocarcinoma cells, before and after transfection with specific α2,3-STs: ST3Gal III and ST3Gal IV and their influence in the adhesion properties of these cell lines to E-selectin.
The pancreatic cell lines studied (MDAPanc-3, Capan-1, MDAPanc-28 and Panc-1), which are of different degree of differentiation and metastatic potential, presented different levels of the α2,3-ST transcripts and had also different pattern of sialylated antigens on their cell surface. Capan-1 and MDAPanc-28 were chosen for transfection as they showed the lowest levels of ST3Gal III or ST3Gal IV transcripts. ST3Gal III transfection of Capan-1 produced an increase of the antigen SLe^a, which was poorly expressed in these cells, an increase up to 100% of SLe^x, which was already expressed at high levels, and a remarkable decrease of Le^y, Le^x and H2 antigens, which were highly expressed in the parental cells. ST3Gal IV transfectants had also an increase in SLe^a, and showed a moderate and a slight decrease of the SLe^x and Le^x antigens, respectively.
Adhesion experiments with these transfected cell lines using E-selectin and hyaluronic acid demonstrated changes in the pattern of adhesion that could be correlated to the Lewis antigens expression and ST3Gal III and ST3Gal IV expression levels. Invasion experiments are in progress and will also allow determining the implication of these enzymes in the metastatic and invasion processes of pancreatic cancer cells.

Poster No. III-18

HELICOBACTER PYLORI ADHERENCE AND SIGNALING THROUGH FUCOSYLATED BLOOD GROUP ANTIGENS EXPRESSED BY RECOMBINANT CHO CELLS

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The prime receptor for *Helicobacter pylori* in the gastric mucosa is the ABH/Leb blood group antigens that define blood groups A, B and O. The cognate attachment protein (adhesin) for these histo-blood group antigens in the gastric mucosa is the blood group antigen binding adhesin BabA. *H. pylori* can also bind to sialylated oligosaccharides (glycans) through the sialic acid binding adhesin SabA. For detailed investigation on the adherence of *H. pylori* to its (glycans) receptors, we have developed a representative cell culture model that expresses these antigens. Here, we have transfected Chinese Hamster Ovary (CHO) cells with plasmids carrying glycosyltransferase cDNAs encoding β3GlcNAc-T6, β3Gal-T5, and FUT3 with or without FUT2 in order to make CHO cells expressing Leb or Lea antigens, respectively. Bacterial-cell adhesion experiments show that *H. pylori* efficiently adhere to Leb- but not Lea-expressing CHO cells, which is an important verification that adherence of *H. pylori* to CHO cells require Fucα1.2-substituted blood group antigens. To further characterize the cellular response to *H. pylori* binding to blood group antigens we studied calcium signaling in response to *H. pylori* adherence in these transfected cells using FURA-2. The results show that bacterial adhesion to cellular Leb antigens induces a calcium flux. This response is not seen in cells that *H. pylori* cannot adhere to, here the CHO Lea. These results suggest that signaling cascades are induced by *H. pylori* attaching to its host cell receptor and, that such attachment contributes to the complexity of biological responses seen in *H. pylori* infections.

Poster No. III-19

VIRUS-INDUCED TRANSCRIPTIONAL ACTIVATION OF HOST FUT GENES ASSOCIATED WITH NEO-EXPRESSION OF LE(Y) IN CYTOMEGALOVIRUS- AND SIALYL-LE(X) IN VARICELLA-ZOSTER VIRUS-INFECTED DIPLOID HUMAN CELLS

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Cell surface glycoepitopes including sLe(x) and Le(y) are engaged in important recognition phenomena in normal and malignant tissue. We explored the possible influence on the expression of such antigens induced by two viruses, varicella-zoster virus (VZV) and cytomegalovirus (CMV) that are involved in persistent infections of humans. We found that infection of human diploid fibroblasts with both viruses resulted in transcriptional activation of several fucosyltransferase genes that were either dormant or expressed at low levels in uninfected cells. Both viruses induced *FUT3*, *FUT5*, and *FUT6*, encoding α1,3 and or 1,4-specific fucosyltransferases. CMV, but not VZV induced transcription of *FUT1* (encoding an α1,2-specific fucosyltransferase). The changes in transcription of fucosyltransferase genes were expectedly, due to the *FUT1* induction in CMV-infected cells, associated with expression of Le(y) in CMV-infected cells and sLe(x) in the VZV-infected fibroblasts although no expression of these antigens was observed in uninfected cells. The induced carbohydrate antigens in CMV- and VZV-infected cells could be of significance for virus spread or possible decoying of immune responses and is therefore a potential target for chemotherapeutic intervention.

Poster No. III-20

P^K BLOOD GROUP EXPRESSION PROVIDES PROTECTION AGAINST HIV INFECTION WHILST THE P^K-DEFICIENT p PHENOTYPE IS HYPER-SUSCEPTIBLE

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Many blood group systems including ABH, Lewis and P1/P/P^k antigens are glycosphingolipids (GSLs), a group of molecules implicated in HIV-host cell fusion. Several GSLs bind to HIV outer envelope gp120 *in vitro*. We recently showed that a soluble P^k blood group analogue inhibits HIV infection irrespective of the viral tropism. Our studies on patients with Fabry disease, a genetic defect causing P^k accumulation, indicated that P^k over-production may provide resistance to HIV-1 infection. However; the exact role of P^k is unknown. We investigated the effects of differentially expressed P^k levels as presented on blood cells from individuals with the naturally-occurring histo-blood group phenotypes P_1^k and p, on R5 or X4 HIV-1 infection. In addition, we determined the effects of exogenously introduced P^k by liposome fusion into Jurkat T-cells on HIV susceptibility. Peripheral blood mononuclear cells (PBMCs) over-expressing P^k (P_1^k) showed resistance to productive R5 or X4 HIV-1 infection. Conversely, PBMCs lacking P^k (p) were hypersensitive to R5 or X4 HIV-1 infection. Higher expression of total P^k levels in P_1^k-PBMCs was confirmed by FACS and thin layer chromatography. Differences in CD4 or CCR5/ CXCR4 expression measured by FACS on P_1^k-PBMCs could not account for the observed resistance. Jurkat cells expressed P^k following exogenous P^k fusion which reduced productive X4 HIV-1 infection by 50%, without affecting cell viability (measured as annexinV-FITC/PI staining) or HIV receptor/co-receptor expression.

Overall, HIV infection is inhibited when P^k is highly expressed on the cell surface, while its absence enhances infection. Furthermore, exogenously introduced P^k seems to mimic natural P^k over-expression. These findings support a protective role for P^k in providing natural resistance to HIV-1 infection.

Poster No. III-21

DIFFERENTIAL EXPRESSION OF GLYCOSYLATION RELATED GENES INDUCED BY *HELICOBACTER PYLORI* STRAINS IN HUMAN GASTRIC CELLS

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Gastric carcinoma is the second cause of cancer death worldwide. *Helicobacter pylori* (Hp) causes gastritis, ulcer, and carcinoma. The first step for Hp infection is the adhesion to epithelial cells. Hp BabA adhesin binds to Le^b, a terminal carbohydrate structure expressed in normal mucosa. Hp binding/interaction with host cells is known to alter the host's gene expression profile, including genes related with the inflammatory response. Persistent Hp infection is accompanied by expression of the sialyl-Le^x antigen in the gastric epithelium. Sialyl- Le^x is a ligand for the SabA Hp adhesin. These observations suggest that Hp is capable to induce the host tissue to retailor the gastric mucosal glycosylation patterns to a more favorable environment for its adhesion.

This study reports an analysis of gene expression induced by a set of Hp strains differing markedly in virulence using a gastric cell line model. Gene expression was evaluated using the GLYCOv2 array from the Consortium for Functional Glycomics, by semi-quantitative PCR, and by quantitative Real-Time PCR. We observed that Hp induces significant expression alterations in 168 of the 1031 human genes tested. The highly pathogenic *cag* PAI (+) *Hp* strain 26695, but not the *cag* PAI (-) Tx30a strain, induced an increased expression of glycosyltransferases participating in the biosynthesis of the lactoseries and neo-lactoseries on glycolipids, which may explain the synthesis of Sialyl-Le^x antigen. In addition, our results showed that genes involved in the regulation of the inflammatory response displayed the most remarkable increase and the pattern of these alterations suggests a preponderant role of NF-κB transcription factor in Hp infection. Finally, *Hp* strain 26695, but not the Tx30a strain, induced significant alterations in syndecan-4 expression, a proteoglycan implicated in inflammation. None of the other proteoglycans showed alterations. This study show that Hp is capable of altering several glycosylation-related genes and that the strains' factors of virulence are related with gene expression alterations induced in host cells.

Supported by FCT (POCI/SAU-OBS/56686/2004) and AICR (Grant 05-088). The Gene Microarray Core resources and collaborative efforts provided by The Consortium for Functional Glycomics were funded by NIGMS - GM62116.

Poster No. III-22

HUMAN NOROVIRUS RECOGNIZE SIALYL LEWIS X NEOGLYCOPROTEIN

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Virus like particles (VLP) were constructed from a human genogroup II.3 norovirus, isolated from an immunocompromised patient with a chronic norovirus infection. The receptor binding characteristics of the VLP were investigated using well defined carbohydrates conjugated to serum albumin in combination with saliva samples from individuals genotyped for *FUT2* (secretor) and *FUT3* (Lewis) and phenotyped for ABO and Lewis blood groups. The VLP had a secretor gene dependent binding to saliva samples and bound to samples from secretor positive individuals regardless of ABO blood group or Lewis status, but did not bind to samples from any secretor negative individuals. Surprisingly H type 1 serum albumin conjugate did not bind to the VLP whereas Sialyl Lewis x, Sialyl di Lewis x and Sialylated type 2 chain conjugates did. The VLP did not bind to Lewis x or Sialyl Lewis a conjugates, demonstrating that the terminal sialic acid is essential for binding and that the interaction is highly specific. The minimal requirement for this receptor structure is thus Neu5Acα3Galβ4(Fucα3)GlcNAcβ3Galβ- where Fuc is not absolutely necessary for binding.

Our study shows that certain human noroviruses have at least two binding specificities, one sialic acid dependent, which is defined by a specific carbohydrate, Sialyl Lewis x, and one secretor gene dependent, which is related to a gene product but not yet to a defined carbohydrate structure.

Poster No. III-23

NANOGLYCOBIOLOGY OF THE S-LAYER GLYLCOPROTEIN OF THE PERIODONTAL PATHOGEN *TANNERELLA FORSYTHIA*

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Tannerella forsythia (*Tf*), a filamentous, non-motile, Gram-negative oral anaerobe, has been identified as an important periodontal pathogen with implications for systemic disease, including cardiovascular disease. How-ever, potential virulence factors of *Tf* have not yet been adequately characterized and little information is avail-able on the mechanisms by which *Tf* mediates host-parasite interaction, leading to periodontal tissue breakdown.

Preliminary studies suggest that the surface layer (S-layer) of *Tf* is structurally unique in being possibly composed of two glycoprotein subunits and has pathogenic potential as a virulence determinant. The *Tf* S-layer is proposed to be involved in hemagglutination, adherence/ invasion, and coaggregation with *Porphyromonas gingivalis*, another crucial periodontopathic microorganism. *Tf* is the only Gram-negative microorganism that has been reported thus far to possess a glycosylated S-layer protein. Interestingly, important steps in pathogenesis of Gram-negative bacteria have been linked to the glycan substitution of surface proteins, indicating that glycosylation of bacterial proteins might serve specific functions in infectious processes.

Detailed studies of the *Tf* S-layer glycoproteins may reveal novel pathogenic mechanisms in Gram-negative organisms. A prerequisite for unraveling such a mechanism is the detailed investigation and characterization of the *Tf* S-layer glyco-nanolattice. Our investigations include (i) ultrastructural studies of the *Tf* glyco-nanolattice, (ii) determination of the glycan structure, glycosylation sites, glycan chain length, and mass of the constituting S-layer glycoproteins, and (iii) identification of a putative S-layer glycosylation gene cluster encoding the glycosylation event of the *Tf* S-layer protein(s) to unravel key modules of *Tf* S-layer protein glycosylation.

The long-term nanobiotechnological goal resides in the unique combination of a glycoprotein self-assembly system with carbohydrate engineering using an S-layer based molecular construction kit as a novel strategy for interfering with the pathogen's ability to establish infection in periodontal disease.

Poster No. III-24

HUMAN GASTRIC GLYCOSPHINGOLIPIDS RECOGNIZED BY *HELICOBACTER PYLORI* VACUOLATING CYTOTOXIN VACA

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Many bacterial toxins utilize cell surface glycoconjugate receptors for attachment to target cells. In the present study the potential carbohydrate binding of *Helicobacter pylori* vacuolating cytotoxin VacA was investigated by binding to human gastric glycosphingolipids on thin-layer chromatograms. Thereby a distinct binding of the toxin to two compounds in the non-acid glycosphingolipid fraction was detected. The VacA-binding glycosphingolipids were

isolated and characterized by mass spectrometry and proton NMR as galactosylceramide (Galβ1Cer) and galabiosylceramide (Galα4Galβ1Cer). Comparison of the binding preferences of the toxin to reference glycosphingolipids from other sources showed an additional recognition of glucosylceramide (Glcβ1Cer), lactosylceramide (Galβ4Glcβ1Cer) and globotriaosylceramide (Galα4Galβ4Glcβ1Cer). No binding to the glycosphingolipids recognized by the VacA holotoxin was obtained with a mutant toxin with deletion of the 37 kDa fragment of VacA (P58 molecule). Collectively our data show that the VacA cytotoxin is a glycosphingolipid binding protein, where the carbohydrate recognition is mediated by the 37 kDa moiety. The ability to bind to short chain glycosphingolipids will position the toxin close to the cell membrane, which may facilitate the internalization of the toxin.

Poster No. III-25

INTERVENTION OF VIRUS–GLYCOSAMINOGLYCAN INTERACTIONS: SCREENING FOR NOVEL ANTIVIRALS

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A substantial number of enveloped viruses utilize glycosaminoglycans for host cell adherence. Such interactions constitute a potential target for antiviral intervention. Two minilibraries, each of approximately 100 compounds, were screened for their activities against herpes simplex virus types 1 and 2 (HSV-1; HSV-2) and/or respiratory syncytial virus (RSV). These libraries comprised of sulfated oligosaccharides (di-heptasaccharide of various sugar backbone and linkage), different sulfated polysaccharides, and low molecular weight mono- and di-sulfated cyclitols. Of the sulfated oligosaccharides maltotetraose, maltotriitol, maltopentitol, isomaltotetraose, cellotetra- and –hexaose, sulfated beta- and gamma-cyclodextrins, and different sulfated lactose derivatives exhibited anti-HSV and anti-RSV effects in cultured cells. Of the sulfated polysaccharides different derivatives of heparin as well as chondroitin sulfate E, fucoidan, carrageenans, pentosan polysulfate, and dextran sulfate were active against HSV-1, HSV-2 and RSV.

Anti-HSV activity was also exhibited by two novel di-sulfated cyclitols. Their antiviral activities were mainly due to interference with the virus attachment to cells; however, these compounds also were able to directly inactivate the virus particles. A possible application as virucidals against sexually transmitted viruses is being pursued.

Poster No. III-26

INVESTIGATING THE POTENTIAL OF LPS-BASED VACCINES TO PROTECT AGAINST MENINGOCOCCAL DISEASE

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Despite the success of glycoconjugate vaccines based on the capsular polysaccharides of several serogroups of *Neisseria meningitidis*, the quest for an effective vaccine to combat meningococcal Group B (*Nm*B) disease continues. Similarity between the α-2,8 polysialic acid capsule of *Nm*B and the post-translational sialylated proteins of certain cell-surface expressed molecules of human cells has raised serious safety issues. Alternative immunogens have therefore been sought. Of these, outer membrane proteins of *Nm*B are leading candidates. However, fulfilling the several requirements of a protective antigen, including conservation, consistent expression, immunogenicity and induction of bactericidal antibodies has been difficult. In particular, the extensive antigenic variability of candidate cell surface expressed *Nm*B proteins has proved problematical. We are considering approaches based on the candidacy of lipopolysaccharide (LPS). The outer core of LPS contains several host-like structures and this potential cross-reactivity with human cell surface antigens coupled with the variability of the outer core structures precludes their consideration as vaccine candidates. In contrast to the outer core structures, the inner core is relatively conserved. We have demonstrated that a monoclonal antibody (designated mAb B5), directed to an inner core epitope on an immunotype L3 *galE* mutant, reacted with 76% of *Nm*B strains and could bind to the surface of intact *Nm*B bacteria. Additionally, using bactericidal assays and passive protection studies, this mAb was shown to kill *Nm*B *in vitro* and to reduce or eliminate bacteremia *in vivo* in an infant rat model. To investigate if active immunization with a glycoconjugate displaying this conserved inner core structure could induce specific antibodies with protective activity, we have explored several conjugation strategies to optimize appropriate presentation of LPS inner core epitopes. We have found that conjugation strategies involving coupling through the detoxified lipid A region could elicit a functional immune response.

Poster No. III-27

IDENTIFICATION OF A NOVEL CANCER-SPECIFIC IMMUNODOMINANT GLYCOPEPTIDE EPITOPE IN THE MUC1 TANDEM REPEAT

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The cell membrane mucin MUC1 is over-expressed and aberrantly glycosylated in many cancers and cancer-associated MUC1 glycoforms represent potential targets for immunodiagnostic and therapeutic measures. We have recently shown that MUC1 with Tn and STn O-glycosylation are cancer-specific glycoforms and that Tn/STn-MUC1 glycopeptide-based vaccines can override tolerance in human MUC1 transgenic mice and induce humoral immunity with high specificity for MUC1 cancer-specific glycoforms (Sorensen et al. 2006).

In order to further characterize the immune response to Tn/STn-MUC1 glycoforms we generated monoclonal antibodies with specificity similar to the polyclonal antibody response found in transgenic mice. In the present study we define the immunodominant epitope on Tn/STn-MUC1 glycopeptides to the region including the amino acids GSTA of the MUC1 20-amino acid tandem repeat (HGVTSAPDTRPAPGSTAPPA). Most other MUC1 antibodies are directed to the PDTR region, although patients with antibodies to the GSTA region have been identified. A panel of other MUC1 glycoform-specific monoclonal antibodies was included for comparison.

The study demonstrates that the GSTA region of the MUC1 tandem repeat contains a highly immunodominant epitope when presented with immature short O-glycans. The cancer-specific expression of this glycopeptide epitope makes it a prime candidate for immunodiagnostic and therapeutic measures. MUC1 glycopeptide cancer vaccines based on this glycopeptides epitope is currently being tested in preclinical and clinical studies.

Poster No. III-28

POSTTRANSLATIONAL MODIFICATION OF COLLAGEN TYPE II; EFFECTS ON ANTIGEN SPECIFIC T CELL TOLERANCE IN COLLAGEN-INDUCED ARTHRITIS

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Collagen type II (CII), the major component of hyaline cartilage, has been proposed as a possible autoantigen participating in the pathogenesis of rheumatoid arthritis (RA). The CII-peptide recognized by the Aq-restricted T-cells is located in between positions 259-273 of the CII molecule. The only difference between heterologous (rat, human) CII and mouse CII within this region is a single substitution at position 266. This epitope also contains 2 lysine residues that can become postranslationally modified by hydroxylation and subsequent glycosylation with a mono- or disaccharide. Both features have been shown to be of importance for development of arthritis in CIA.

In order to study the fate of CII-specific T cells in CIA we have established a murine system with a double-transgene. MMC transgenic mice, which express the heterologous CII-peptide in cartilage, were found to have a reduced susceptibility to CIA and a reduced T cell response to heterologous CII. Whereas an earlier established mouse expressing a transgenic T cell receptor (TCR)-beta chain, obtained from a CII-specific T cell clone, showed a strongly enhanced T cell response to heterologous CII and an increased susceptibility to severe CIA. Combining of the two transgenes on Aq background leads to an intermediate response and disease. However, the T cell repertoire of the transgenic mouse is still heterogeneous, as the beta chain can be combined with different endogenous alpha chains. To assess the frequency, distribution and phenotype of CII-specific T cells under the influence of the TCR-beta chain and the MMC transgene, we have generated a clonotypic antibody recognizing CII-specific TCRs. In order to address the establishment of CII-specific tolerance, non-tolerized CII-specific T cells have been isolated from mice, expressing the TCR beta chain and transferred into MMC transgenic mice. Following transfer, donor T cells were monitored for phenotypic changes.

Poster No. III-29

PECTIC POLYSACCHARIDES FOR COATING OF MEDICAL DEVICES

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The plant cell wall is an extracellular matrix, which is rather well understood with regard to composition and progress over the past decade has shed some light on the biochemistry and molecular biology of cell wall polysaccharide biosynthesis. Functional properties of the cell wall *in toto* or of the individual polymers are appreciated due to many practical applications.

We try to exploit the physical properties such as self assembly and water binding as well as biological properties of pectic polysaccharides for the coating of medical devices. Surfaces of medical materials and devices that come into direct contact with human tissues need to be fine-tuned with regard to both physical and biological properties. Pectic polysaccharides have biological properties also in a mammalian context and we propose to exploit these to impart material surfaces with appropriate biological properties. Additional, we attempt to make these more applicable to

human tissue by modification through further controlled glycosylation adding on sugar residues known in mammalians.

Poster No. III-30

MUCIN-TYPE FUSION PROTEINS CARRYING MANNOSYLATED OR α1,3-GAL TERMINATING STRUCTURES AS VACCINE ADJUVANTS – MAKING USE OF NATURES OWN DEFENCE SYSTEMS

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Many infectious diseases can be prevented by vaccines. Vaccines may also offer novel therapeutic possibilities when it comes to chronic diseases and cancer. One of the major problems facing vaccine developers is the fact that tumor-associated peptides, proteins and carbohydrates that may be used as vaccinogens are usually poorly immunogenic. In order to increase their immunogenicity and break self tolerance various strategies may be used, including the use of vaccine adjuvants. For this purpose, we propose to use mucin-type fusion proteins carrying carbohydrate determinants that may enhance immunogenicity by improving antigen uptake and presentation.

To this end, we have engineered host cells that secrete a mucin-type immunoglobulin fusion protein (PSGL-1/mIgG_{2b}) carrying terminal Galα1,3Gal (PSGL-1/mIgG_{2b}/Gal) or mannosyl (PSGL-1/mIgG_{2b}/Man) structures as verified by MS and Western blotting. PSGL-1/mIgG_{2b}/Gal was produced in glyco-engineered CHO cells coexpressing core 2 β1,6-*N*-acetylglucosaminyltransferase I and the porcine α1,3-galactosyltransferase, while PSGL-1/mIgG_{2b}/Man was produced in *Pichia pastoris*. The fusion proteins will be covalently conjugated to model antigens and well-studied antigens used as vaccines, and their ability to enhance immunogenicity will be evaluated in mouse models.

Poster No. III-31

GLYCOPROTEIN BASED BIOMARKERS OF THE RATE OF AGEING

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It has been established that serum protein glycosylation profiles change in relation to various diseases including rheumatoid arthritis, nephropathy, liver cirrhosis, diabetes and cancers.

There are clear indications that glycosylation of lipids and proteins is also affected by ageing, although research in this area is limited. One of the few well-documented findings is that the glycosylation of serum IgG changes with age. Such dynamic glycosylation can be seen as an accurate measure for changes in metabolic processes in cells producing particular glycoconjugates or glycan-modulatory proteins. The identification of biological mechanisms that are crucial for the determination of the human rate of ageing and their contribution to age-related disease is hampered by the absence of biomarkers, parameters that mark 'biological age'. Can we identify a biomarker for longevity in glycosylation patterns?

We have established a 96-wells plate method for high-throughput N-glycan release from total serum and affinity purified glycoproteins using PNGase F, and fluorescent labeling (Rudd et al., 1997). The samples are then analyzed using both LC with fluorescence detection and off-line ESI-MS. The LC method has the advantage of separating isobaric structures, while MS has a high resolution, but cannot discriminate isobaric structures. Profiling of released N-glycans is now a straightforward and feasible approach for large scale population studies on small serum samples.

Rudd PM, Guile GR, Küster B, Harvey DJ Opdenakker G and Dwek RA, Oligosaccharide sequencing technology, *Nature* **388**, 205-207.

Poster No. III-32

PLATELETS LACKING SIALIC ACID CLEAR RAPIDLY FROM THE CIRCULATION DUE TO INGESTION BY ASIALOGLYCOPROTEIN RECEPTOR-EXPRESSING LIVER MACROPHAGES AND HEPATOCYTES

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Although surface sialic acid is considered a key determinant for the survival of circulating blood cells and glycoproteins, its role in platelet survival is unclear. We investigated the importance of sialic acid for platelet clearance using mice deficient in the ST3GalIV sialyltransferase gene (ST3GalIV$^{-/-}$ mice) and identified a novel clearance mechanism previously unrecognized for platelets. ST3GalIV catalyzes the addition of sialic acid onto exposed galactose residues of cell surface glycoproteins. ST3GalIV$^{-/-}$ mice have increased platelet surface galactose exposure, a 70% reduction in platelet count, and prolonged bleeding times. We report that ST3GalIV$^{-/-}$ platelets

transfused into wild-type C57BL/6J mice exhibit markedly reduced recoveries and shortened survivals respectively compared to littermate wild-type platelets. Infusion of asialofetuin, an antagonist of the asialoglycoprotein-receptor (ASGPR) restored platelet recovery time and initial circulation of ST3GalIV$^{-/-}$ platelets to normal values. Immunohistochemical studies of organ specimens harvested shortly after transfusion of biotin-labeled platelets demonstrated the predominant clearance of ST3GalIV$^{-/-}$ platelets by the liver Kupffer cells and, unexpectedly, hepatocytes. Megakaryocytes cultured from ST3GalIV$^{-/-}$ mice produced proplatelets normally compared to megakaryocytes generated from wild-type littermates, indicating that the thrombocytopenia in the ST3GalIV$^{-/-}$ mice is not due to reduced platelet production. Comparison of ST3GalIV$^{+/+}$ and ST3GalIV$^{-/-}$ platelet surface receptor expression as evidenced by flow cytometry and preliminary in vitro activation studies did not reveal any significant differences in the two genotypes. We conclude that the absence of terminal sialic acid residues on platelet surfaces exposes galactose residues to the lectin domain of ASGPR on both hepatocytes and liver Kupffer cells, resulting in platelet clearance from the circulation.

Poster No. III-33

GLYCOPROFILING OF A BIOMARKER FOR COLORECTAL CANCER

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Tissue inhibitor of metalloproteinases-1 (TIMP-1), a doubly *N*-glycosylated protein, is a marker for early detection of colorectal cancer (CRC). As many glycoproteins alter their glycan composition as a consequence of malignancy, the glycoprofile of TIMP-1 may improve the biomarker specificity, if it undergoes malignancy-induced changes. To investigate this, we have initially developed a method for rapid and sensitive mass spectrometric glycoprofiling of TIMP-1 using recombinant sources. Subsequently, the established method was used to determine the individual glycoprofiles of plasma TIMP-1 from healthy donors and CRC patients.

Hydrophilic interaction liquid chromatography at nanoscale level was used to enrich for glycopeptides prior to the glycoprofiling, which was performed using matrix-assisted laser desorption/ionisation MS and MS/MS. The method proved to be fast and sensitive and yielded a comprehensive site-specific glycan analysis, allowing a differentiation of the glycoprofiles of the two sources of recombinant protein.

As shown by immuno-assays, the plasma TIMP-1 concentrations of CRC patients were significantly increased compared to healthy donors, indicating that the additional TIMP-1 was a result of the malignancy. TIMP-1 was purified from plasma using immuno-affinity chromatography and gel electrophoresis and identified using western analysis and MS. Unexpectedly; the glycoprofiling showed that the glycan structures of TIMP-1 from healthy donors and CRC patients were highly similar. Relative quantization of the observed glycopeptides revealed that the glycan composition of the healthy donors and the CRC patients varied minimally and only to the same extent as the internal variation within the two groups. The lack of malignancy-induced alterations excludes the use of TIMP-1 glycan fingerprints as a CRC marker and illustrates that malignancy-induced glycosylation alteration is not a universal phenomenon.

Poster No. III-34

UNIVERSAL RED CELLS – NOVEL FAMILIES OF BACTERIAL GLYCOSIDASES CAN MAKE IT HAPPEN

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Enzymatic removal of blood group A and B antigens to develop universal red blood cells was a pioneering vision originally proposed 25 years ago by Jack Goldstein (*Science* **215**, 168-170 (1982)). Goldstein envisioned the enzymatic digestion of the A and B antigens on RBCs to the H antigen by the use of exoglycosidases for selective removal of αGalNAc and αGal residues of the immunodominant A and B trisaccharide antigens. Feasibility of this approach was previously demonstrated in clinical trials for group B red blood cells. However, because these enzymes had poor kinetic properties and a low pH optimum the conversion process was not economically viable. Conversion of group A RBCs was only achieved with the weak A_2 subgroup.

Here we present two novel bacterial glycosidase gene families that provide enzymes capable of efficient removal of the A and B antigens at a neutral pH with minimal consumption of recombinantly generated enzymes. These enzymes have unique catalytic mechanisms and X-ray crystallography of the α-*N*-acetylgalactosaminidase reveals a new structural fold for glycosidases. Complete removal of A and B antigens from RBCs was achieved in a low ionic strength conversion buffer as evaluated by agglutination typing with routine licensed reagents and methods, as well as

by sensitive FACS analysis. Further biochemical analysis of ECO cells included mass spectrometry and thin-layer chromatography (TLC) of glycolipids as well as SDS-PAGE Western blot of total membrane (glyco)protein extracts. These current enzyme processes hold promise for finally achieving the goal of producing universal red blood cells, which would significantly improve the blood supply while enhancing the safety of clinical transfusions.

Poster No. III-35

THE N-GLYCOME OF HUMAN EMBRYONIC STEM CELLS

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Complex carbohydrate structures, glycans, are crucial components of glycoproteins, glycolipids, and proteoglycans. While individual glycan structures such as the SSEA and Tra antigens are already used to define undifferentiated human embryonic stem cells (hESC), the whole spectrum of stem cell glycans has remained unknown. We undertook a global study of the asparagine-linked glycoprotein glycans (N-glycans) of hESC and their differentiated progeny using MALDI-TOF mass spectrometric profiling and proton NMR spectroscopy of unmodified N-glycosidase F liberated glycans. The data demonstrated that stem cells have a unique N-glycome which consists of a constant part and a variable part that change during hESC differentiation (circa 75%/25%, respectively). Significantly, certain hESC-associated N-glycans were lost and novel glycans emerged in the differentiated cells. By use of novel quantitative data analysis methods for the mass spectrometric glycan profiles, we were able to deduce N-glycan structural features typical to each cell type and also evaluate the extent of the changes in the N-glycome. The applicability of the analysis methods were verified by use of nano-scale proton NMR N-glycan profiling as well as specific exoglycosidase digestions. We found that both N-glycan core structures and their decorations were changed during hESC differentiation, while complex fucosylation was the most characteristic glycosylation feature of undifferentiated hESC. These results provide an overview of the glycobiology of hESC and form the basis for strategies to target stem cell glycans.

BENZON SYMPOSIUM No. 54

GLYCOSYLATION:
OPPORTUNITIES IN DRIG DEVELOPMENT

JUNE 11-14, 2007, COPENHAGEN, DENMARK

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Abstracts - THURSDAY, June 14, 2007

PROTEIN GLYCOSYLATION IN CELLULAR MECHANISMS OF HEALTH AND DISEASE

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Glycosylation of cellular proteins represents a major form of enzymatic post-translational modification, producing secretory, cell surface, and extracellular glycans of enormous abundance and structural diversity. The enzymes responsible for glycosylation are the glycosyltransferases and their regulation produces a dynamic cellular glycan repertoire. The past decade of biomedical research has been associated with remarkable discoveries revealing that glycosyltransferases uniquely contribute to the development and function of physiologic systems in the context of living organisms. The biologic activities of mammalian glycans are derived from an endogenously regulated portfolio of glycosyltransferases and substrates that have been retained in an evolutionary investment encompassing millions of years and spanning 1-2% of the genome. Glycosyltransferases generate a significant amount of structural variation in biological systems and thereby modulate intermolecular interactions by steric influences and lectin binding. We have been investigating the in vivo functions of mammalian glycans and the mechanistic paradigms by which glycans participate in physiology as well as contribute to disease pathogenesis. Remarkably, recent findings indicate tissue-, cell type-, and glycoproteinspecific dysfunction in the etiology of glycosyltransferase-deficient phenotypes, as well as therapeutic benefits of specific glycan deficiency states in the pathogenesis of disease. Research encompassing glycobiology is increasingly successful in explaining the origin of extracellular signals and how cell-cell communication as well as intracellular signal transduction is established. Glycans participate in cell adhesion, self-non-self recognition, molecular trafficking and clearance, receptor activation, signal transduction, and endocytosis. These findings span multiple fields of biomedical research including immunology, neurobiology, hematology, metabolism, and the molecular origins of genetic disease. These and future discoveries emanating from glycobiology will be necessary to acquire the knowledge needed in deciphering the biologic systems that comprise living organisms.

TUMOR METABOLISM AND DRUG SENSITIVITY IS DEPENDENT ON N-GLYCAN PROCESSING

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Golgi β1,6N-acetylglucosaminyltransferase V (Mgat5) produces tetra-antennary β1,6GlcNAc-branched N-glycans on cell surface glycoproteins that bind to galectins with higher affinity than the less branched structures. Disruption of galectin binding in $Mgat5^{-/-}$ cells or by lactose competition in $Mgat5^{+/+}$ cells increases EGF receptor mobility in the plane of the membrane. Thus, galectin binding restricts receptor mobility and inhibits partitioning into both caveoli and internalization via coated-pit endocytosis. As such, the galectin/glycoprotein cross-linking limits receptor internalization and maintains downstream signaling sensitivity. Mammary tumor formation is delayed and metastasis suppressed in $Mgat5^{-/-}$ polyomavirus middle T (PyMT) transgenic mice. $Mgat5^{-/-}$ carcinoma cells are less sensitive to growth factors and TGF-β *in vitro*, but are nonetheless tumorigenic when injected into mice. Here we report that PyMT $Mgat5^{-/-}$ cells are checkpoint impaired, and following serum withdrawal, fail to down regulate glucose transport, protein synthesis, reactive oxygen species (ROS), and activation of Akt and Erk. A screen of pharmacologically active compounds revealed synthetic $Mgat5^{-/-}$ phenotypes including hypersensitivity of tumor to the ROS inducer 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), and hyposensitivity to tyrosine kinase inhibitors, to Golgi disruption by brefeldin A, and to topoisomerase inhibition by camptothecin. Regulation of ROS, glucose uptake and sensitivities to EGF and TGF-β were rescued by Mgat5 expression or by hexosamine supplementation to N-glycan processing in $Mgat5^{-/-}$ cells. Thus, the Mgat5 deficiency reveals PyMT as primarily a stimulator of glucose

metabolism and cell-autonomous growth signaling, while Mgat5/N-glycan processing promotes responsiveness to extracellular cues that control tumor cell invasion.

SIGLECS AS MODULATORS OF INNATE AND ADAPTIVE IMMUNE RESPONSES

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The siglecs are a family of up to 12 sialic acid binding Ig-like lectins, all but one of which are expressed as membrane receptors in the immune system. They are involved in both cell-cell adhesive interactions and in signaling functions. Each protein displays a unique glycan binding preference and exhibits complex and overlapping expression profiles amongst the many cell types of the immune system. Sialoadhesin (siglec-1) is an unusual siglec with 17 Ig domains expressed on macrophages within tissues and is induced on inflammatory macrophages. Mice deficient in sialoadhesin exhibit a mild phenotype, but following immunisation and induction of autoimmune diseases by either genetic or peptide-induced mechanisms, there are striking alterations in immune and inflammatory responses, suggesting an important role for this sialic acid binding receptor in cell-cell interactions with immune cells. The CD33-related siglecs are a separate supgroup of receptors sharing high sequence similarity with evidence for rapid evolution between mammalian species. Rather than mediating cell-cell interactions, CD33-related siglecs mediate primarily cis ineractions with neighbouring glycans on the same cells. In addition, they have typical features of 'inhibitory receptors' and contain two tyrosine-based signalling motifs that recruit a subset of SH2 domain containing signalling molecules including the tyrosine phosphatases SHP-1 and SHP-2 and the SOCS3 suppressor of cytokine signalling protein. Recent studies on mice lacking individual CD33-related siglecs suggest that these molecules can negatively regulate cellular activation and expansion that lead to a dampening of inflammatory and immune responses. Based on structural studies of the sialic acid binding V-set Ig domain of selected siglecs, it may be feasible to design carbohydrate-based drugs that could either promote or antagonise siglec function and modulate inflammatory responses to the benefit of the patient.

ATTENUATION OF A SPECIFIC GLYCOSYLATION REACTION IN BREAST CARCINOMA CELLS INHIBITS CELL MIGRATION AND INVASIVENESS

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Altered cell surface glycosylation is a hallmark of oncogenic transformation; in some cases, specific glycan changes have been shown to be driven by oncogenic signaling and to regulate cell adhesion, migration, and invasiveness both *in vitro* and *in vivo*. Patients with human colorectal or breast carcinoma that over-express a particular glycan, the N-linked [Man beta1,6 GlcNAc] branch, exhibit a significant decrease in 5-year survival. The enzyme that synthesizes this branch, GnT-Va, therefore, is a potential therapeutic target. To provide evidence that inhibition of GnT-Va affects tumor cell invasiveness, siRNA specific for GnT-Va was expressed in a highly invasive human breast carcinoma cell line, MDA-MB231. The results demonstrated that the siRNA-expressing cells showed a marked decrease in GnT-Va activity and glycan products, assayed by L-PHA binding. The GnT-Va-attenuated cells showed significantly higher cell-matrix adhesion and decreased rates of migration in several assay systems, suggesting that inhibiting GnT-Va expression resulted in these phenotypic effects associated with suppressed invasiveness. *In vivo* tumorigenicity studies are in progress. Attenuation of GnT-Va caused a marked inhibition of epidermal growth factor (EGF)-induced dephosphorylation of focal adhesion kinase (FAK) and activation of the tyrosine phosphatase SHP-2, consistent with the inhibition of invasiveness-related phenotypes in the cells expressing GnT-Va. No changes in EGF binding levels were observed; however, ligand (EGF-) induced internalization of the EGF receptor (EGFR) in the GnT-Va knockdown cells was inhibited, causing increased receptor residence on the cell surface. Cell surface levels of EGFR in the absence of EGF were not affected. These and other data suggest that the effects of decreased invasiveness after attenuation of GnT-Va result at least in part from the inhibition of EGFR internalization and subsequent endosomal signaling, and they point to GnT-Va as a target for inhibition to suppress breast carcinoma invasiveness.

RECOMBINANT MUCIN-TYPE FUSION PROTEINS AS VERSATILE INHIBITORS OF PROTEIN-CARBOHYDRATE INTERACTIONS

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Cell surface carbohydrates are essential for a multitude of biomedically important interactions taking place at the cell surface. Carbohydrate-binding proteins are therefore significant targets for the development of carbohydrate-based inhibitors. Because of their multivalent character, monovalent low molecular-weight sugar homologues or analogues are usually poor inhibitors of these interactions. Recent advances in organic and chemoenzymatic synthesis of carbohydrates will undoubtedly increase the pace by which new multivalent carbohydrate-based drugs are developed. Knowledge gained on the glycosyltransferases involved in glycan biosynthesis can be used to engineer host cells for

recombinant production of proteins with tailored glycan substitution. In particular, recombinant mucin-type proteins can serve as natural scaffolds for multivalent presentation of therapeutic carbohydrate determinants. We have thus engineered a number of CHO-K1 cell lines secreting mucin-immunoglobulin fusion proteins carrying different carbohydrate determinants on defined O-glycan core structures. These include sialyl-Le^x, sialyl-Le^a, Le^a, Le^b, Le^y, α-Gal and blood group A and B determinants. The fusion proteins have been tested *in vitro* with regard to their anti-blood group A/B and anti-αGal antibody binding activity as well as their capacity to inhibit *H. pylori* adhesion to its neoglycoprotein receptor. The picomolar concentrations of fusion protein needed in the latter example implicates its superior efficacy as compared to free oligosaccharides or low valency glycoconjugates. Clinical applications of the mucin-type fusion proteins include extracorporeal adsorption columns for anti-blood group A/B and α-Gal antibody removal prior to transplantation as well as inhibitors of microbial adhesion.

NEURITOGENIC ACTIVITY AND MECHANISM OF BRAIN CHONDROITIN/DERMATAN SULFATE HYBRID CHAINS

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Chondroitin sulfate (CS) and dermatan sulfate (DS) hybrid chains are involved in the brain development and critical roles for oversulfated disaccharides and IdoUA residues have been implicated in the growth factor-binding and neuritogenic activities of these chains. In the pursuit of sources of CS/DS with neuritogenic activity and therapeutic potential, two novel CS/DS preparations were isolated from shark liver. The major (80%) low-sulfated and minor (20%) highly sulfated fractions had an average molecular mass of 3.8-38.9 and 75.7 kDa, respectively. Digestion with various chondroitinases (CSases) revealed a large panel of disaccharides with either GlcUA or IdoUA scattered along the polysaccharide chains in both the fractions. The higher Mr fraction, richer in IdoUA(2S)alpha1–3GalNAc(4S) (iB) and GlcUAbeta/IdoUAalpha1–3GalNAc(4S,6S) (E/iE) units, exerted greater neurite outgrowth-promoting (NOP) activity and better promoted the binding of various heparin-binding growth factors including pleiotrophin (PTN), midkine, HB-EGF, VEGF, FGF-2, FGF-7, and hepatocyte growth factor (HGF). These activities were largely abolished by digestion with CSase ABC or B, but only moderately affected by a mixture of CSases AC-I and AC-II. In addition, the NOP activity of the larger fraction was markedly reduced by desulfation with alkali, suggesting a role for the 2-O-sulfate of iB. The NOP activity of the larger fraction and that of the embryonic pig brain-derived CS/DS fraction were also suppressed to a large extent by antibodies against HGF, PTN, and their individual receptors cMet and anaplastic lymphoma kinase, revealing the involvement of the HGF and PTN signaling pathways in the activity.
(Li, F., Shetty, A.K., & Sugahara, K. J. Biol. Chem., in press.)

ENZYME REPLACEMENT AND ENHANCEMENT THERAPIES FOR LYSOSOMAL DISEASES

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In 1964, Christian de Duve first suggested that lysosomal storage diseases (LSDs) could be treated by replacing the defective enzyme with its normal counterpart. Early tissue culture, animal model, and pilot clinical studies demonstrated “proof of concept” for enzyme replacement therapy (ERT). In the 1970’s, the discovery of the mannose-6-phosphate receptor-mediated pathway for the cell uptake and delivery of glycoproteins to the lysosome provided the rationale for the treatment of LSDs by ERT. However, it was not until the early 1990’s that ERT became a reality with the demonstration of its safety and effectiveness in Type 1 Gaucher disease. Encouraged by the success in Gaucher disease, investigators were stimulated to develop ERT for other LSDs. Today, ERT is approved for six LSDs (Gaucher, Fabry, Mucopolysaccharidoses I, II and VI, and Pompe diseases) and clinical trials are underway for Niemann-Pick B disease. The lessons learned from ERT in Gaucher and Fabry diseases will be presented, with emphasis on the general principles for effective ERT.

Most LSDs have later-onset subtypes due to missense mutations encoding enzymes with residual activities. Pharmacologic chaperone therapy (PCT) is a novel strategy to rescue unstable/misfolded proteins/enzymes. Small molecules, including substrate analogues, are used to bind to the active site, stablize the mutant enzymes, and facilitate their lysosomal targeting/delivery, thereby increasing their residual activity. In the lysosome the chaperone dissipates, is replaced by the high concentration of substrate which is then degraded. Advantages include oral administration, delivery to most/all tissues/cells, including CNS delivery. Preclinical studies demonstrated the feasibility of PCT in Gaucher, Fabry and other LSDs. A successful Phase 1 trial of PCT for Fabry disease has been completed, and a multi-center Phase 2 clinical trial is underway. Future studies will evaluate the safety/effectiveness of PCT for LSDs and other protein-misfolding diseases.

MOST CARBOHYDRATE CANCER ANTIGENS CAN BE MADE IMMUNOGENIC

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Melanomas, sarcomas, and neuroblastomas express the gangliosides GM2, GD2 and GD3 at the cell surface; small cell lung cancers express GM2, fucosyl GM1, the hexasaccharide globo H, and polysialic acid; while epithelial cancers express GM2, globo H, sialyl Lewis^{a} (sLe^{a}), Le^{y}, the mono or disaccharides Tn, sTn, and TF, and the glycoprotein antigens MUC1 and KSA (EpCAM). All of these are available by extraction or synthesis for vaccine construction. Since most are carbohydrates and not known to be recognized by T-lymphocytes, we have focused on antibody mediated treatments. Antibodies, whether administered or vaccine induced, are ideally suited for eradication or free tumor cells and micrometastases after surgery and/or chemotherapy have eliminated known disease, and have done so in a variety of preclinical models. If antibodies of comparable titer can be induced against human tumor antigens in patients, it should also result in elimination of free tumor cells from the circulation and micrometastases making establishment of new metastases no longer possible. This would dramatically change our approach to treatment of even the patient with known metastatic cancer.

Vaccine purpose determines vaccine design. For vaccines that induce antibodies we have found conjugation of defined antigens to KLH plus the saponin immunological adjuvant QS-21 to be optimal. Using this approach GM2, Fucosyl GM1, globo H and MUC1 consistently induce IgM and IgG antibodies in cancer patients which react both with the synthetic antigens by ELISA and with antigen positive cancer cells by flow cytometry. The remaining antigens require modifications to be comparably immunogenic. GD2 and GD3 require acid treatment resulting in lactones with one or more internal rings; Tn, TF and sTn require presentation as trimers for induction of antibodies better able to react with the cancer cell surface; and polysialic acid requires N-propionylation resulting in antibodies which cross react with the unmodified (N-acetyl) polysialic acid. Only the glycolipid Lewis^{y} and the protein KSA have defied our best efforts.

The antibodies induced by these monovalent conjugate vaccines have been almost exclusively IgM, IgG1 and IgG3. They have all activated complement at the cell surface, but only antibodies against antigens intimately associated with the cell surface lipid bilayer have induced complement dependent cytotoxicity. We are currently preparing GMP batches of these vaccines for testing as polyvalent vaccines in randomized trials.

CHANGES IN MUCIN-TYPE O-GLYCOSYLATION IN CANCER

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The differences in glycosylation patterns seen in malignancy, strongly influence the final structure of membrane and secreted glycoproteins, and these novel tumour-associated glycoforms can modify the behaviour of the malignant cell and its interactions with immune effector cells.

Changes in mucin type O-glycosylation occur in most carcinomas, which develop from epithelial cells, and these are strongly manifest in the production of aberrantly glycosylated mucins carrying multiple O-glycans. The MUC1 membrane mucin is the most widely expressed and in the change to malignancy in breast cancer truncated O-glycans are added to the core protein of MUC1 instead of, or in addition to, the core 2 structures which are added to the normally mucin.

In vitro synthesis of MUC1 based glycoproteins and glycopeptides carrying a specific O-glycan (either Tn,T, SialylTn, SialylT), has allowed an investigation of how individual glycoforms affect the immune response and interact with immune effector cells. Moreover, cloning of glycosyltransferases has allowed modification of glycosylation patterns in tumour cell lines, and evaluation of how these changes affect tumour growth.

It is becoming clear that some cancer associated MUC1 glycoforms can induce humoral responses, even when MUC1 is expressed as a self antigen, while others are immuno-suppressive.

The induction of antibodies to some cancer associated glycoforms of MUC1 – and indeed of other mucins - suggests that such antibodies may be useful as biomarkers. On the other hand, understanding how the different cancer associated glycoforms induce or inhibit the immune response is important for the design of clinical studies using MUC1-based antigens.

CARBOHYDRATES AND T-CELL IMMUNITY

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The critical recognition event in the antigen specific immune response is the interaction between the T cell receptor (TCR) and an antigenic peptide bound to a peptide receptor on antigen presenting cells. This peptide receptor is a major histocompatibility complex (MHC) class II molecule. The MHC class II molecule determine the immune response and is expressed on antigen presenting cells (APC), like dendritic cells, B cell or macrophages. Exogenous derived proteins are digested into peptides and these peptides are bound to MHC class II molecules. The peptide-MHCII complex is subsequently transported to the cell surface and exposed for T cells. The interaction between the TCR and the MHC class II molecule requires the formation of an immunological synapse between the APC and the T

cell with participation of several membrane proteins and soluble factors. Importantly, the MHC class II molecules are specialized to bind peptides and normally the T cells only recognize peptides on the MHC class II molecules. Exceptionally the peptides are modified by various posttranslational modifications. One such exception occurs in recognition of cartilage derived type II collagen (CII) which in fact is a critical event for development of autoimmune arthritis.
Immunization of mice with CII induces arthritis, collagen induced arthritis (CIA). This disease is dependent on recognition of glycosylated peptide derived from positions 260-270 on CII and the critical TCR recognition structure is a lysine sidechain on position 264. This side chain can be hydroxylated, galactosylated and glucogalactosylated. The T cells can in fact recognize minor modification on the galactose. Many T cells recognize only the carbohydrate and not the peptide although all T cells needs to recognize the gal-peptide in its proper orientation on the MHC class II Aq molecule. Recognition of this gal-peptide Aq complex is the critical recognition event for development of collagen induced arthritis in mice. This observation can also be used for treatment as injection of recombinant Aq-galpeptide complex vaccinate against arthritis.
Interestingly, these observations is possible to transfer to human rheumatoid arthritis (RA) as the same carbohydrate structure is recognized in humans and mice humanized to express the human MHC class II molecule DR4 behave the same way as mice with the murine MHC class II Aq molecule.
In conclusion, recognition of carbohydrates are critical for development of arthritis in mice and play an important role in humans. This gives new possibilities for vaccination approaches to prevent development of RA.

GLYCANS IN NERVOUS SYSTEM STABILITY AND AXON REGENERATION

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Glycosphingolipids are the major glycans in the mammalian brain, constituting >80% of cell surface saccharides. Among these, gangliosides, sialylated glycosphingolipids are major determinants on nerve cells and axons. Mammals share the same four major ganglioside structures, two of which (GD1a and GT1b), carry the non-reducing terminal glycan "NeuAcα2-3Galβ1-3GalNAc", which is the preferred binding determinant for a brain lectin, myelin-associated glycoprotein (MAG, Siglec-4). Binding studies reveal that GD1a and GT1b are high-avidity binding glycans for MAG.
MAG is expressed on the inner-most wrap of myelin, the multilamellar membrane that enwraps axons throughout the nervous system. Myelination of axons is required for their rapid nerve conduction, but also stabilizes axons, enhances axon cytoarchitecture, and down-regulates axon sprouting. MAG binding to axonal gangliosides is required each of these myelin functions. Mice engineered to lack the N-acetylgalactosaminyltransferase required for complex ganglioside biosynthesis, and that lack the "NeuAcα2-3Galβ1-3GalNAc" MAG-binding terminus on gangliosides, display the same phenotypic deficits as MAG-null mice, including axon degeneration, disrupted axonal cytoarchitecture, progressive motor behavioural deficits, and seizure susceptibility.
MAG-ganglioside binding also regulates axon regeneration. The central nervous system is an inhibitory environment for axon regeneration, limiting recovery from traumatic (e.g. spinal cord) injuries. In part, this is because of endogenous axon regeneration inhibitors, including MAG on residual myelin at the injury site, inhibit axon outgrowth. Experimental disruption of ganglioside expression or structure on neurons *in vitro* overcomes MAG-mediated inhibition, enhancing axon outgrowth. In traumatic spinal cord injury models in the rat *in vivo*, infusing the enzyme sialidase, which removes the terminal sialic acid from MAG-binding gangliosides GD1a and GT1b, enhances spinal axon outgrowth and functional recovery.
This work was supported by the US NIH.