Freie Universität Berlin

Optimizing the glycosylation of recombinant proteins

INAUGURAL-DISSERTATION

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy

of Freie Universität Berlin

by

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from Pirna

2015

The present work was conducted from 02/2011 to 12/2015 at the Department of Life Sciences and Technology of the Beuth University of Applied Sciences Berlin under supervision of Prof. Dr. Stephan Hinderlich.

1st Reviewer: Prof. Dr. Stephan Hinderlich

2nd Reviewer: Prof. Dr. Nora Kulak

Date of defense: April 21, 2016

LIST OF ABBREVIATIONS

A1AT	alpha-1-antitrypsin
ADCC	antibody-dependent cellular cytotoxicity
CDG	congenital disorders of glycosylation
CHO	Chinese hamster ovary
EPO	erythropoietin
ER	endoplasmic reticulum
Fc	fragment crystallizable
FcγRIIIa	Fcγ receptor IIIa
FCS	fetal calf serum
FDA	Food and Drug Administration
GalNAc	*N*-acetylgalactosamine
GDP	guanosine diphosphate
GlcNAc	*N*-acetylglucosamine
GM-CSF	granulocyte-macrophage colony-stimulating factor
HEK-293	human embryonic kidney-293
HER-2	human epidermal growth factor receptor-2
HPAEC-PAD	high-pH anion-exchange chromatography with pulsed amperometric detection
IgG	immunoglobulin G
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
ManNAc	*N*-acetylmannosamine
MS	mass spectrometry
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
OST	oligosaccharyltransferase
PNGase F	peptide-*N*-glycosidase F
PTM	post-translational modification
UDP	uridine diphosphate

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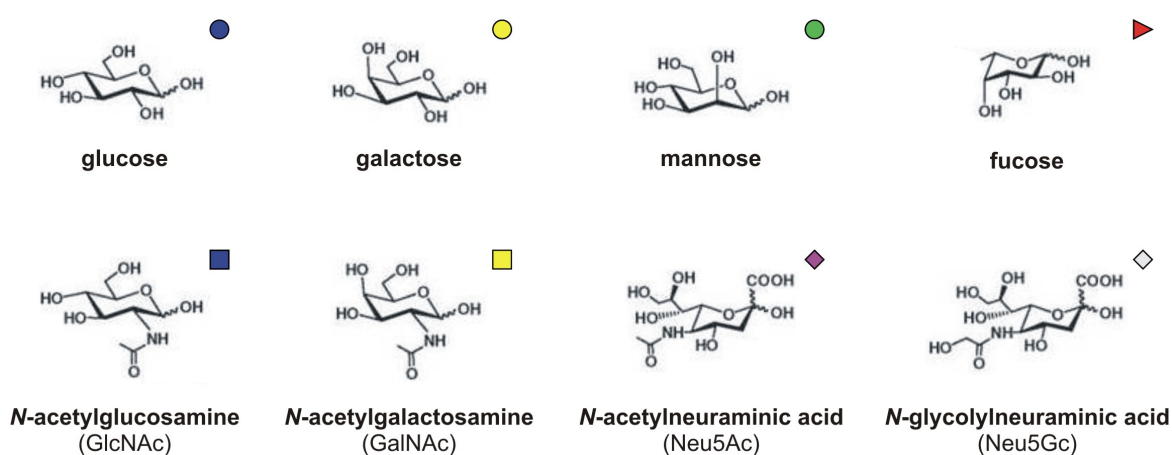
1 Introduction

1.1 Glycobiology

Carbohydrates, also termed (mono-, di-, oligo- or poly-)saccharides, are the most abundant class of biomolecules in nature and fulfill numerous functions in living organism. Historically, carbohydrates were mainly considered as a source (glucose, lactose) and storage (starch, glycogen) of energy and as major structural cell components for organisms such as insects or plants (chitin, cellulose). Despite their ubiquitous presence in the living world, carbohydrates were primarily believed to lack other biological activities. Moreover, their vast structural complexity, the difficulty in determining their sequences, and the fact that their biosynthesis could not be directly predicted from a DNA template, fundamentally impeded their exploration compared to the study of proteins or nucleic acids [1].

In 1988, the specific term "glycobiology" was coined by Rademacher *et al.* [2] joining the traditional disciplines of carbohydrate chemistry and biochemistry with a modern understanding of the molecular biology of carbohydrate moieties – termed glycans. Glycobiology is the emerging field of research contributing to the study of structure, function and biology of glycans that can be covalently linked to another (non-glycan) moiety by the enzymatic process of glycosylation [3]. Products of glycosylation, so-called glycoconjugates, comprise a broad spectrum of molecules including glycoproteins, glycopeptides, peptidoglycans, proteoglycans, glycolipids, glycosides and lipopolysaccharides. Recent progress in the field of glycobiology is being facilitated by the development of new strategies and methodologies for the high-throughput analysis of glycans – a type of study known as glycomics [4].

1.2 Protein glycosylation

The attachment of glycans to the polypeptide backbone by covalent glycosidic linkage, so-called protein glycosylation, is the most complex and versatile co- or post-translational modification (PTM) that a protein can undergo [5]. According to large-scale approaches, around 2% of the human genome have been predicted to encode proteins that are involved in glycosylation [6]. It has been estimated – deduced from the analysis of the SWISS-PROT database [7] – that more than the half of all human proteins are glycoproteins.

A small set of monosaccharides (in terms of mammalian glycans see **Fig. 1**) is used for glycan structure assembling. Generally, up to 12 monomer units are linked for a typical glycan molecule – but reported values of polymerization ranging up to > 370 residues in terms of polysialylated structures [8].

glucose   galactose   mannose   fucose

N-acetylglucosamine (GlcNAc)   *N*-acetylgalactosamine (GalNAc)   *N*-acetylneuraminic acid (Neu5Ac)   *N*-glycolylneuraminic acid (Neu5Gc)

Fig. 1: Monosaccharides of mammalian glycans. The recently updated symbol nomenclature of Varki *et al.* [9], that is used for graphical representation of all glycan structures throughout this work, is shown: blue circle, glucose; yellow circle, galactose; green circle, mannose; red triangle, fucose; blue square, *N*-acetylglucosamine (GlcNAc); yellow square, *N*-acetylgalactosamine (GalNAc); purple diamond, *N*-acetyl-neuraminic acid (Neu5Ac) and white diamond, *N*-glycolylneuraminic acid (Neu5Gc).

1.2.1 Major classes of glycans

Glycans are generally categorized into different groups based on the nature of their linkage between the sugar and the polypeptide moiety. Although several types of protein glycosylation have been described that way, the two major classes are, by far, *N*- and *O*-glycans.

1.2.1.1 N-glycans

N-linked glycosylation is initiated as a co-translational event during protein biosynthesis. It occurs when the GlcNAc at the reducing end of the glycan is covalently attached to the amid nitrogen of an asparagine present in the consensus sequence Asn-X-Ser/Thr, whereby X is any amino acid except proline [10]. All *N*-glycans share a common pentasaccharide core structure consisting of two GlcNAc and three mannose residues. Based on the nature of additional monosaccharides linked to the core, *N*-linked glycans are further classified into

three subgroups (**Fig. 2**). In case of highmannose *N*-glycans, only mannose units are attached to the core. In contrast, complex *N*-glycans are characterized by various combinations of monosaccharides such as GlcNAc, GalNAc, galactose, fucose and Neu5Ac, the predominant sialic acid. Up to five branches, termed antennae, are found in complex-type *N*-glycans [11]. As shown in **Fig. 2** for exemplary structures, fucosylation may occur at GlcNAc residues of the glycan core and/or of the antennae. The structural diversity of this *N*-glycan subclass is further increased by partial or complete sialylation of terminal galactose units. Finally, hybrid-type molecules combine the structural features of both, the highmannose and the complex type. Complex and hybrid *N*-glycans may also carry a so-called bisecting GlcNAc residue that is attached by a β1,4-glycosidic linkage to the central mannose of the core [12].
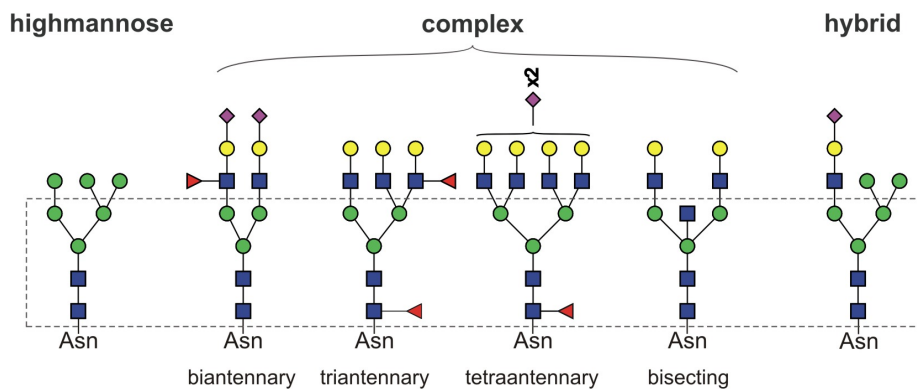
Fig. 2: Representative *N*-glycan structures of the highmannose, complex and hybrid type. The common core structure of all three *N*-glycan classes is highlighted by a dashed box. Asialo as well as partially and completely sialylated examples of the complex type are shown. Fucose units may be attached to GlcNAc residues of the core and/or the antennae.

The biosynthetic pathway of all eukaryotic *N*-linked glycans is initiated on the cytoplasmic side of the rough endoplasmic reticulum (ER) by assembling of an oligosaccharide precursor (**Fig. 3A**). Therefore, a dolichol pyrophosphate carrier serves as a membrane anchor and nucleotide sugar donors (uridine diphosphate (UDP)-GlcNAc, guanosine diphosphate (GDP)-mannose and UDP-glucose) provide activated monosaccharide units. Sequentially, two GlcNAc residues and five mannose units are added enzymatically by specific glycosyl-transferase reactions. Each individual glycosyltransferase step therefore shows strong substrate preference towards a single glycan structure resulting in a linear biosynthetic pathway of the branched oligosaccharide [13]. The sugar moiety is then translocated ("flipped") across the ER membrane bilayer to the luminal side of the ER – a mechanism known to be catalyzed by an enzyme termed as flippase, but that is not fully understood yet [14]. Since the ER membrane has no nucleotide-sugar transporters, further monosaccharide donors (made from activated nucleotide sugars in the cytosol) are also linked to dolichol phosphate and transferred into the ER lumen [15]. That way, four mannose residues and three glucose units are added forming the final oligosaccharide precursor composed of a total of 14 monosaccharide units.
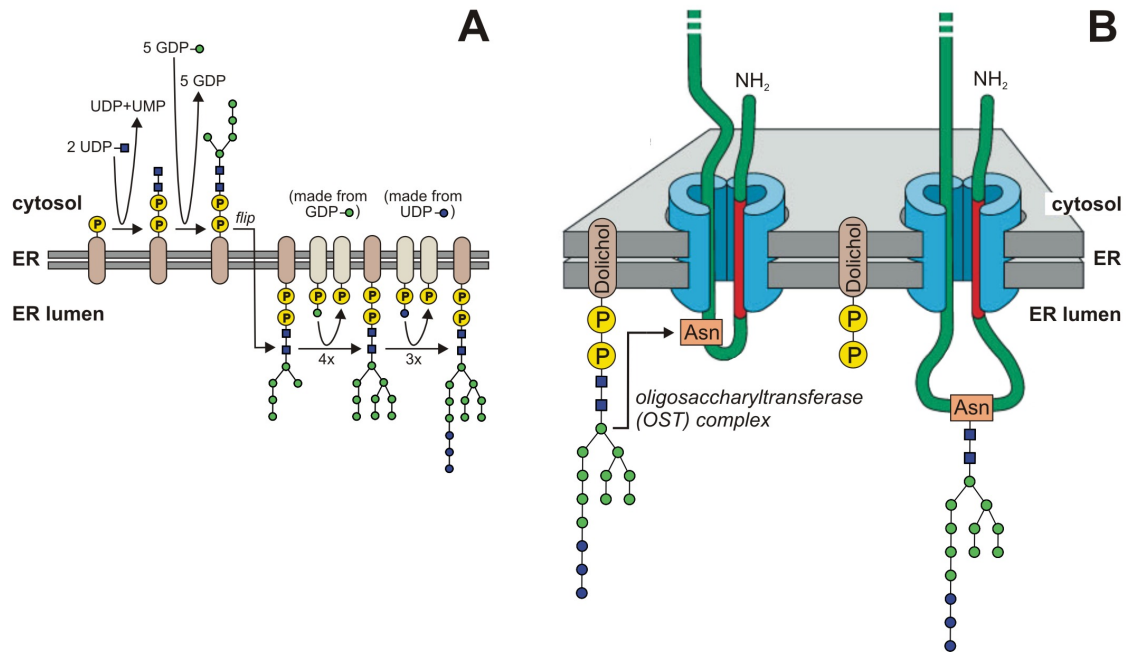
Fig. 3: Initial steps of the *N*-glycan biosynthesis (according to [15], with minor modifications). [A] Synthesis of a dolichol phosphate-linked precursor oligosaccharide from activated nucleotide sugars UDP-GlcNAc and GDP-mannose, which is transferred *en bloc* from the cytosol into the lumen of the endoplasmic reticulum (ER). Subsequent reactions involve transfers from dolichol phosphate-mannose and –glucose (synthesized on the cytosolic side of the ER and then thought to be flipped across the ER membrane). [B] Co-translational linkage of the glycan precursor to an Asn-X-Ser/Thr sequon in a protein that is being synthesized and translocated through the ER membrane.

As a co-translational event (**Fig. 3B**), a multimeric protein complex in the ER membrane, called oligosaccharyltransferase (OST), scans the nascent polypeptide chain for the occurrence of the *N*-glycosylation motif (Asn-X-Ser/Thr). OST catalyzes the *en bloc* transfer of the glycan precursor to the recipient protein when the consensus sequon has been translocated 12-14 residues into the ER lumen [16]. Subsequent to the covalent attachment to an Asn residue, the oligosaccharide is sequentially modified by a multitude of enzyme reactions including the hydrolytic removal (trimming) and the re-glycosylation with additional monosaccharide units (processing). Initial trimming by the removal of the two terminal glucose residues occurs in the ER and has critical roles in proper glycoprotein folding and quality control via interaction with lectin chaperons [17]. In a process known as the calnexin/calreticulin cycle, glycans serve as sorting signals reflecting the folding status of the protein. Misfolded variants are recognized and referred to refolding until the proper conformation is accomplished or the protein is degraded by the ER-assisted degradation (ERAD) system [17]. The removal of the remaining glucose residue displays that the glycoprotein is ready for transit from the ER to the *cis*-face (entry) of the Golgi apparatus undergoing further trimming and terminal glycosylation [18]. In the Golgi complex, *N*-glycan biosynthesis makes use of soluble nucleotide precursors that are imported from the cytosol by specific transporters [19] – lipid-linked intermediates, such as those in the ER, are not required. While high-mannose-type structures are assembled in the *cis*-Golgi, the synthesis of basic hybrid and

complex *N*-glycans mainly occurs in the *medial*-Golgi. Of the original glycan precursor built in the ER, only five monosaccharides remain forming the common core structure of all *N*-glycans. The addition of further monosaccharides (such as galactose or terminal sialic acid), mostly taking place in the *trans*-Golgi, convert the limited repertoire of *N*-linked glycans into an extensive set of highly diverse structural variants [20].

1.2.1.2 *O-glycans*

O-glycans, however, are a more heterogeneous and less understood group of glycans by far. *O*-linked glycosylation is typically initiated in the Golgi apparatus as a post-translational event. Several types of *O*-glycans exist, including those *O*-linked by fucose, mannose or GlcNAc. In terms of the most common mucin-type *O*-glycans (also termed *O*-GalNAc glycans), the covalent linkage by an *O*-glycosidic bond occurs via a first GalNAc monomer to the hydroxyl group of Ser or Thr residues within a fully folded polypeptide chain. This first step of mucin-type *O*-glycan biosynthesis is catalyzed by a polypeptide-*N*-acetylgalactosaminyl-transferase, demonstrated to be localized in the *cis*-Golgi. At least 21 different variants of this membrane-bound enzyme are known so far, partly showing overlapping acceptor substrate specificities [21]. Further linear and branched elongations by stepwise addition of galactose, GlcNAc, GalNAc or polylactosamine (repeating units of the disaccharide galactose-GlcNAc) as well as terminal modifications by sialylation and fucosylation are described [22]. Extensions of the initial GalNAc residue can generate eight different core structures, whereby four are common in human glycoproteins while the others show extremely restricted occurrence [21]. In contrast to the initial steps of *N*-glycosylation, no lipid-linked intermediates are involved in mucin-type *O*-glycan biosynthesis, and glycosidases do not appear to process *O*-GalNAc glycans within the Golgi [21]. Since also no amino acid consensus sequence for the prediction of glycosylation sites exists for *O*-glycans, any Ser and Thr residue is a potential target for *O*-glycosylation. Structural studies have to face the challenge, that *O*-linked carbohydrate moieties are usually located in clusters hindering the analysis of glycopeptides containing a single glycosylation site. Moreover, *O*-glycan analysis remains challenging due to the lack of enzymatic tools for universal *O*-glycan release from the protein backbone (comparable to the endoglycosidases for *N*-glycan release, see paragraph **1.3.1**) [23].

1.2.2 *Structural diversity and complexity of glycans*

Although there is just a limited set of monosaccharides used for glycan assembling in mammalians (see **Fig. 1**), a huge diversity of glycans can be observed in nature. The large quantity of structural variants is possible due to a varying number, sequence and confor-mation of the particular sugar units and their glycosidic linkage – in a linear or branched way – that can be formed in two stereoisomers at the anomeric carbon (α or β) via different

atoms of the ring structure [24]. Moreover, a multitude of modifications (including sulfation, acylation, methylation or phosphorylation) can occur at various positions within a carbohydrate to modulate biodiversity [25].

Trimming and processing of glycans arise in a protein-, cell-type, tissue- and species-specific manner. In contrast to other cellular processes such as transcription or translation, biosynthesis of glycans is non-template driven and is, however, realized by a complex and sequential interaction of enzymes, in particular glycosyltransferases and glycosidases. Since each of those enzymes shows a high substrate preference [13], a glycoprotein that fails to be processed by one step can usually not undergo further processing. Different glycan structures that emerge from the *trans*-Golgi can therefore be considered as the end products of a series of incomplete enzyme reactions [26]. Accordingly, the outcome of glycosylation is mainly determined by the concentration and cellular distribution of glycosylation enzymes, the enzyme repertoire, substrate availability of activated nucleotide sugars and the transit time of the glycoprotein through the Golgi network [26]. Regarding the latter, an increase in polylactosamine elongations of glycans attached to lysosomal membrane glycoproteins has been shown for prolonged transit times through the glycosylation pathway – achieved by lowering the temperature of cell cultures from 37 °C to 21 °C [27].

Although *N*-glycosylation is restricted to Asn in the consensus sequence Asn-X-Ser/Thr of a polypeptide chain, not all of these motifs are mandatorily occupied by a *N*-glycan. This phenomenon is referred to as macroheterogeneity (or variable site occupancy) and is mainly dependent on the kinetics of the individual biosynthetic steps as well as the conformation of the polypeptide chain, thus, the accessibility of the respective glycosylation site [28]. It has been reported, that the tripeptide sequon of the form Asn-X-Thr is glycosylated two to three times more efficiently than Asn-X-Ser [29]. Moreover, statistical studies done by Gavel and Heijne [30] revealed that non-glycosylated motifs tend to occur more frequently towards the C-terminus of a protein.

A major contribution to the huge diversity of glycans is associated with a phenomenon termed as microheterogeneity – the structural diversity of oligosaccharides at a particular glycosylation site within a glycoprotein. Thus, a glycoprotein encoded by a single gene usually exists as a population of different glycosylated variants, wherefore the definition of "glycoforms" was introduced by Rademacher *et al.* [2]. The extent of this microheterogeneity can vary substantially from one glycosylation motif to another, from glycoprotein to glycoprotein or cell type to cell type and may also reflect the physiological status of the cell [1]. The formation of glycoforms is also true for glycoproteins containing just a single glycosylation site, as for chicken ovalbumin that shows the presence of highmannose, hybrid and also bi- up to pentaantennary complex-type *N*-glycan structures [31]. Soybean agglutinin, however, is

one of the rare examples in which no microheterogeneity can be observed. This plant lectin solely carries a highmannose *N*-glycan consisting of nine mannose residues and is therefore the best source for the preparative isolation of this particular oligosaccharide [32].

Despite its ubiquitous nature, the mechanism underlying glycan microheterogeneity and its biological relevance remains poorly understood and largely unexplored. It has been shown, that the set of glycoforms is highly regulated under constant physiological conditions and associated with cell growth, differentiation or disease – suggesting that the presence of particular glycoforms is indeed required for proper functions of an organism [33]. However, the huge diversity of glycosylation is a challenging issue of numerous glycoanalytic strategies and also a fundamental task in recombinant glycoprotein production (see chapter **1.3** and **1.5**, respectively).

1.2.3 Biological importance of glycosylation

1.2.3.1   Properties of glycosylated proteins

The attachment of carbohydrate moieties may have fundamental and versatile impact on basic properties of glycoproteins. Besides the importance for proper protein folding in the ER (see section **1.2.1.1**), glycosylation plays a crucial role in maintaining molecular stability, e.g. against thermal, pH or chemical denaturation [34]. Carbohydrates have been also found to protect proteins against proteolytic degradation. For example, the latter was reported for human interferon-γ, that showed sensitivity to protease attack for a non-glycosylated variant while full or partial proteolytic resistance was observed in terms of the glycosylated protein [35]. Shielding immunogenic epitopes of the protein sequence from recognition by the immune system and the improvement of protein solubility are further outcomes of protein glycosylation [36].

Particularly, terminal monosaccharides of complex-type *N*-glycans often play key roles in the biological function and activity of glycoproteins. Residues of fucose, for instance, may be attached in an α1,6-linkage to the initial GlcNAc of the core unit. The presence of such a core fucose in *N*-linked glycans of immunoglobulin G (IgG) significantly reduces the antibody-dependent cellular cytotoxicity (ADCC), wherefore afucosylated recombinant antibodies show an enhanced ADCC and efficiency of anti-tumor activity [37] (see also section **1.4.3**). Hence, the engineering of cell lines lacking core-fucose expression is a frequent approach for the biotechnological production of highly efficient therapeutic IgGs [38, 39].

Another target of glycoengineering strategies are sialic acids, a family of Neu5Ac derivatives, characterized by a nine-carbon backbone and its acidic nature. Sialylation level is known to crucially affect the serum half-life, and consequently, the pharmacologic efficiency of glycoproteins. Recombinant sialylated human erythropoietin (EPO) revealed a much

slower clearance from the circulation in contrast to its non-sialylated protein variant [40]. Sialylated glycoproteins are protected against the recognition by the asialoglycoprotein receptor (ASGPR) in hepatocytes while exposed galactose residues are captured mediating the clearance from the blood serum [41]. Various approaches have been used to increase the content of sialic acids for recombinant forms of EPO. The genetically engineered darbepoetin alfa (trade name Aranesp®), for instance, carries two additional *N*-glycans and shows a threefold prolongation of serum half-life in contrast to the recombinant EPO analogue [42].

1.2.3.2   Impact of glycosylation on cellular level

The glycocalyx, a fuzzy layer of glycoproteins and sugar moieties, is located on the external side of the plasma membrane of most cell types [43]. These carbohydrates serve as preferential points of attachment for other cells, infectious bacteria or viruses, hormones or many other molecules. Thus, glycosylation plays key roles in myriad events of development, recognition and signal transduction within and between cells (**Fig. 4**). In this regard, cellular mechanisms are often mediated by lectins (from the Latin *legere,* "to select"), a class of naturally occurring and highly specific carbohydrate-binding proteins primarily found on the cell surface [44].
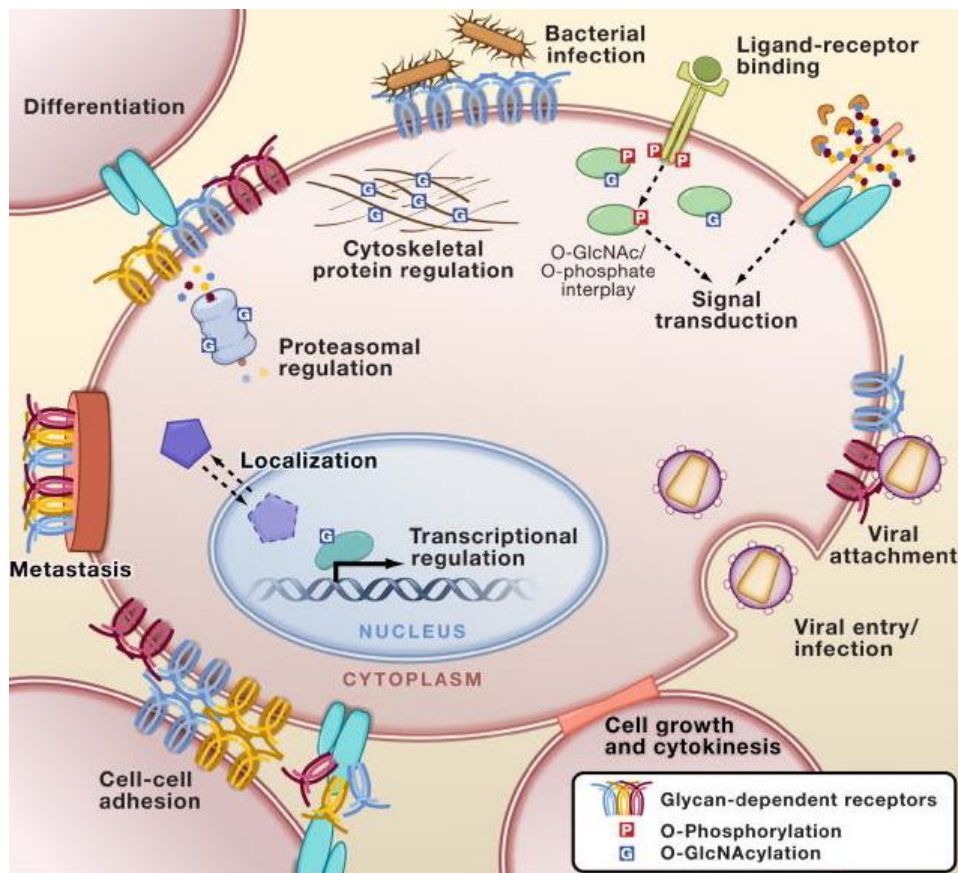
Fig. 4: Cellular mechanism mediated by glycosylation. Cell surface glycans are targets of microbes and viruses, regulate cell development, cell-cell adhesion, ligand-receptor interactions and influence metastasis of cancer cells [45].

Moreover, specific pattern of glycosylation have been shown to be characteristic for the metabolic and developmental stage of a cell. Thus, structural variations in the glycan repertoire at the cell surface produce numerous biomarkers – some of which also correlate with cell differentiation or disease [46]. Regarding the latter, glycosylation is also known to be involved in the metastatic spread of cancer. An increased degree of glycan branching mediated by the action of different GlcNAc transferases, for instance, has been reported for initial and advanced stages of cancer [47]. Since those changes in glycosylation are important cancer biomarkers, there is a high ongoing interest to use them in diagnostic and therapeutic approaches and to increase the scope of their clinical application [48].

1.2.3.3   *Glycosylation-related disorders*

Hereditary defects concerning the biosynthesis and metabolism of glycans lead to multi-systemic disorders in humans and come along with a broad spectrum of severe clinical symptoms. These defects are summarized as congenital disorders of glycosylation (CDG) and are primarily associated with neurological diseases with variable involvement of nearly all other organs. All CDGs identified so far are of autosomal recessive inheritance and can be classified into two groups: CDG-I defects are defined as those altering synthesis and transfer of the dolichol phosphate-linked precursor oligosaccharide to recipient proteins while CDG-II defects affect subsequent processing steps, mostly on *N*-linked sugar chains [49]. Since the first clinical description in 1980 by Jaeken *et al.* [50], physicians are becoming increasingly aware of CDGs and nearly 50 different defects have been elucidated so far [51].

Other glycosylation-related defects may also occur from defective *O*-glycan biosynthesis. A reduction in α-dystroglycan's ligand-binding capacity, for instance, results from its aberrant glycosylation [52]. In this regard, deficiency in *O*-mannosylation is a phenomenon that characterizes a growing subset of muscular dystrophies – genetic diseases associated with degeneration and disruption of muscle fibers, resulting in the progressive wasting of skeletal muscle. Moreover, lysosomal storage diseases – that comprise more than 40 heterogeneous disorders – are known to be caused by metabolic defects in glycoprotein catabolism resulting in the excessive accumulation of undegraded oligosaccharides derived from glycoproteins or other glycoconjugates [53].

1.3 Strategies of glycoanalysis

Caused by structural diversity, complexity and dynamic variation far beyond those in proteomics and genomics (see section **1.2.2**), glycoanalysis remains a challenging task. Since low-abundant glycoconjugates put additional pressure on the sensitivity of the analytical tools, the development of robust and precise methodologies is required [54]. No universal technique for the rapid and reliable identification of glycan structures is currently available; hence, research goals (e.g. including the exploration of structure-function relations or the monitoring of glycosylation in disease diagnosis and prognosis) dictate the most suitable method or, usually, a combination of methods for the experimental approach.

1.3.1 Glycan release

Generally, the first step in glycan analysis is to separate the carbohydrate moiety from the polypeptide backbone using enzymatic or chemical approaches. Isolation of *N*-linked glycans has been facilitated by the use of peptide-*N*-glycosidase F (PNGase F), an endoglycosidase purified from *Flavobacterium meningosepticum* capable of cleaving the glycosidic bond between the Asn residue of a glycosylated protein and the initial GlcNAc of the glycan portion. Besides other existing endoglycosidases often showing higher substrate specificities, PNGase F has emerged as the gold standard for detaching *N*-glycans [4]. Due to the common conserved core structure of the *N*-glycan family, PNGase F reliably liberates highmannose, hybrid as well as complex-type structures. In this regard, deglycosylation studies revealed that many native proteins were susceptible to this enzyme but that prior denaturation using sodium dodecyl sulfate, for instance, may improve enzyme accessibility and, thus, decrease the amount of required PNGase F for complete carbohydrate removal [55].

Considering the existence of eight different core structures only for mucin-type *O*-glycans, the enzymatic release of *O*-linked carbohydrate moieties is restricted to specific structures. Since no tools for universal cleavage of *O*-glycans have been developed so far, chemical methods including hydrazinolysis and alkaline (reductive and non-reductive) β-elimination are currently the preferred approaches for their release. Chemical toxicity (in terms of hydrazinolysis), the conversion of the reducing end impeding subsequent labeling reactions for glycan detection (in terms of reductive β-elimination) or the degradation (so-called "peeling reaction") of glycans as a consequence of strong basic conditions (in terms of non-reductive β-elimination) are major drawbacks causing that studies of *O*-linked glycosylation lag far behind those of *N*-glycans [54, 56].

1.3.2 Monosaccharide composition and structural analyses

Mass spectrometry (MS) is a widely used technique to precisely analyze glycans and their composition [57]. Since the ionization efficiency (in particular for sialylated molecules) is generally low, prior sample derivatization (e.g. by permethylation) is a common approach that also stabilizes terminal monosaccharides during MS analyses [58]. In combination with sequential enzymatic digestions of exoglycosidases or studies of fragment ions in tandem MS (MS/MS) approaches, glycans can be elucidated on a structural level. Compositional information of the carbohydrate moiety is also provided by quantitation of monosaccharides. In this regard, separation by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is useful since it does not require chemical derivatization prior detection [59]. Moreover, colorimetric assays or the use of lectins enable to assist glycan profiling with less technical effort, but often involve limited sensitivity. Facilitating the detection subsequent to chromatographic or electrophoretic separation, monosaccharides or glycans require a derivatization step to introduce a chromophore or fluorophore [60]. In this regard, capillary electrophoresis with laser-induced fluorescence (CE-LIF) even allows distinguishing between positional isomers and glycosidic linkages of glycans. However, the lack of a specific database for glycans and the intrinsic variability of this method essentially necessitate the link to other methodologies (such as MS) for data interpretation [54]. As already mentioned by Rudd and Dwek in 1997 [26], it is obvious that information is lost as long as the carbohydrate moiety and protein portion of a glycoprotein are perceived as separate entities. For this purpose, studies of the intact glycoprotein and/or glycopeptides (obtained by protease digestion) in terms of site-specific glycoanalysis represent an own research subject – the emerging field of glycoproteomics [61].

1.4 Therapeutic glycoproteins

Since the first monoclonal antibody (Orthoclone®) entered the market in 1986 for use in preventing kidney transplant rejections [62], this has opened the gateway for the approval of glycosylated biopharmaceuticals. Recently, the market of therapeutic glycoproteins is one of the fast-growing, profitable and also challenging fields of the pharmaceutical industry. These molecules play a crucial role in the treatment of a wide spectrum of human diseases, and include several protein classes (e.g. hormones, antibodies, enzymes, cytokines and coagulation factors) [63]. Among numerous clinical important glycoproteins, this work exemplarily focuses on a human serine protease inhibitor, an immunomodulatory cytokine as well as on a humanized IgG$_1$-type antibody – all are introduced in detail within the following sections.

1.4.1 Alpha-1-antitrypsin (A1AT)

A1AT, the most abundant plasma-circulating human serine protease inhibitor, belongs to the serpin superfamily [64]. The synthesis and secretion of A1AT into the blood stream mainly occur in hepatocytes and, to a much lesser extent, in monocytes and macrophages [65]. More than 2 g of A1AT is produced per day, leading to a plasma concentration of 1.5 to 3.5 g/L with a circulating half-life of four to five days in healthy individuals [66, 67].

The first part of the protein name originates from its migration in serum protein electrophoresis where it can be allocated to the α_1-globulin fraction [68]. In 1962, a protein with ability to inhibit trypsin activity was isolated from the α_1-globulin region of human serum and therefore named A1AT [69]. Since A1AT inhibits also a wide spectrum of other proteases (including chymotrypsin, thrombin or plasmin [70]), the synonym term alpha-1-proteinase inhibitor is occasionally preferred to A1AT in research publications. In clinical contexts, the original term A1AT is used more often in order to pay tribute to the investigators of the protein. However, the main physiological target of A1AT is neutrophil elastase, a key effector of the innate immune system with antimicrobial activity against harmful bacteria and other infectious agents [71]. Besides, neutrophil elastase is a destructive enzyme capable of degrading elastin, collagen and major connective tissue components [72]. The maintenance of its protease–antiprotease homeostasis in the lower respiratory tract protects the alveolar walls of the lungs against enzymatic destruction and is the primary site of action of A1AT [73]. With serum levels considerably rising in response to infection, injury or inflammatory processes and counterbalancing increased proteolytic activity, A1AT is a classical acute phase reactant [68].

1.4.1.1 Biochemical characteristics of the A1AT glycoprotein

Nascent A1AT consists of 418 amino acids including a hydrophobic pre- or signal peptide of 24 amino acids. The mature polypeptide chain is comprised of 394 amino acids equipped with three carbohydrate moieties giving an overall molecular weight of about 52 kDa [74, 75]. The complex-type oligosaccharide chains are *N*-linked to Asn residues at positions 46, 83 and 247 [76] representing about 15% of A1AT by molecular weight. According to Kolarich and coworkers [77], very little modification beyond biantennary structures takes place on the sites Asn[46] and especially Asn[247], while *N*-glycans at position Asn[83] exhibit considerable diversity ranging from biantennary up to traces of tetraantennary structures.

A1AT is a globular, highly ordered molecule possessing a tertiary structure of nine α-helices and three β-pleated sheets [75]. The metastable structure of A1AT undergoes massive conformational changes to a stable complex when inhibiting the target protease (**Fig. 5**). The reactive part of A1AT, whose peptide sequence acts as a pseudosubstrate for the protease, is

an exposed loop which is inserted as an extra strand into the central β-sheet during this inhibitory mechanism [78]. Translocating the target protease to the opposite end of the serpin molecule, the active site of the protease is distorted and, thus, its catalytic triad is inactivated. Finally, irreversible covalent complexes of A1AT and the inhibited protease are recognized by hepatic receptors and cleared from the circulation [79].
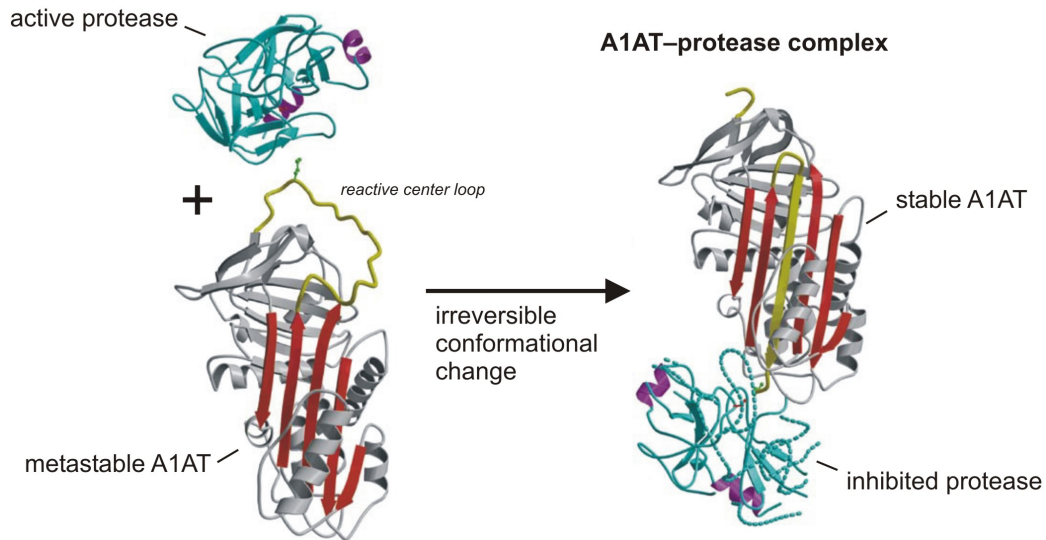

Fig. 5: Mechanism of inhibition of an active protease by A1AT. The reactive center loop of A1AT interacts with a target protease triggering a conformational change of both molecules and the formation of an A1AT– protease complex. The reactive center loop is inserted into the central β-sheet while the catalytic site of the protease is inactivated by distortion (according to [80], with minor modifications).

The lack of carbohydrate moieties on A1AT has been reported to increase the tendency of protein aggregation [81]. Furthermore, effects on stability and flexibility of the metastable serine protease inhibitor could be also ascribed to A1AT glycosylation [82]. Unglycosylated recombinant variants of A1AT showed a shorter serum half-life in rabbits compared to the glycosylated equivalent [83]. Regarding the clearance from the blood circulation, the level of terminal sialic acids and the number of exposed terminal galactose residues have been demonstrated to play a crucial role (see also section **1.2.3.1**) – while not being essential for the enzymatic inhibitory activity [84].

1.4.1.2   A1AT deficiency

A link between low serum levels of A1AT and symptoms of pulmonary emphysema was first discovered in 1963 by Laurell and Eriksson [85]. Only a few years later, a specific type of liver cirrhosis could also be associated with decreased activity of serum A1AT [86]. The A1AT deficiency disease is a genetic disorder inherited in an autosomal recessive manner. It is primary caused by point mutations of the *SERPINA1* gene encoding for the A1AT protein. A multiplicity of heterozygote and homozygote genetic variants has been reported so far, coming along with several forms and degrees of clinical symptoms.

The most prevalent type of severe A1AT deficiency (**Fig. 6**) arises from a genetic defect – defined as so-called Z mutation of the *SERPINA1* gene – that results in a single amino acid substitution (glutamic acid^{342} → lysine^{342}) within the A1AT polypeptide chain [87]. Its homozygous phenotype causes misfolded hepatic A1AT, leading to intracellular accumulation and liver cell damage or dysfunction. In consequence, most (~85%) of the protein is degraded prior to reach the circulation. Low serum levels of A1AT therefore expose lung tissue to uncontrolled proteolytic attack from neutrophil elastase, culminating in alveolar destruction and emphysema [88]. The general prevalence of the Z mutation in the population of Western Europe is approximately 1 to 2,500 with large geographic variations [87]. Early symptoms comprise shortness of breath, reduced ability to exercise, cough and wheezing, wherefore A1AT deficiency is commonly misdiagnosed initially as asthma or chronic obstructive pulmonary disease. Furthermore, the diagnostic delay typically exceeds five years, resulting in an average age at diagnosis of about 45 years [89].
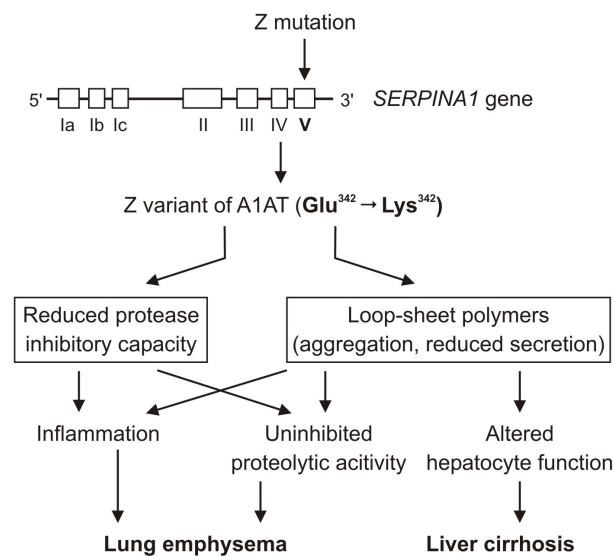
Fig. 6: Clinical consequences of the Z mutation in exon 5 of the *SERPINA1* gene encoding for the A1AT protein. (according to [87], with minor modifications)

A major goal in the treatment of patients suffering from A1AT deficiency is the prevention of lung disease and respiratory infections or the reduction of the progression rate that is already present. Augmentation therapy using concentrated preparations of human A1AT is available and US Food and Drug Administration (FDA)-approved to increase levels of circulating functional A1AT above the protective threshold of 0.5 g/L (11 μM) [90]. Weekly intravenous infusions of 60 mg purified plasma-derived A1AT per kg body weight are recommended despite high costs and therapeutic effort for the patients and medical staff [91]. Inhalation therapy offers the opportunity for a more safe and efficient delivery of aerosolized A1AT directly to the lungs [92], but the only long-term study is yet unpublished.

1.4.1.3   Recombinant sources of A1AT

Since the A1AT augmentation therapy is expensive (approximately $260,000 five-year total costs to treat a 70 kg-patient with the recommended weekly dose [93]) and concerns about safety and supply of the plasma-derived product come along, recombinant alternatives of A1AT are highly requested. Recombinant A1AT protein variants have been produced in numerous non-human hosts such as *Escherichia coli* [94], yeast cells of *Saccharomyces diastaticus* [95], the filamentous fungus *Aspergillus niger* [96], plant cultures of *Nicotiana benthamiana* [97], insect cell lines [98] or in milk of transgenic mice and sheep [99, 100]. In this regard, major drawbacks were attributed to the lack of human PTMs, especially glycosylation. For instance, *Escherichia coli*-derived A1AT is non-glycosylated and the absence of carbohydrate moieties affects the protein's folding and stability, resulting in altered pharmacokinetic properties [101]. Accelerated A1AT clearance from the blood circulation is problematic for yeast cell systems that produce variants of the protease inhibitor bearing exclusively highmannose-type *N*-glycans [102]. Detection of terminal non-reducing galactofuranose in terms of an A1AT expression in *Aspergillus niger* impeded its therapeutic use in humans [103]. Mammalian products, however, contain the non-human sialic acid Neu5Gc which may also raise immunogenicity and safety concerns.

In order to produce recombinant A1AT comparable to the plasma-derived inhibitor and avoid immunogenicity, human expression systems are focused to a greater extent. In 2011, Blanchard, Liu and coworkers published the first report on biological active A1AT produced in the human neuronal cell line AG1.HN® [104]. Despite strong similarities to the native equivalent, structural analysis of the A1AT *N*-glycan pattern revealed a lower degree of sialylation and higher level of core- and Lewis X-fucosylation most likely referable to features of neuronal *N*-glycosylation. The human primary embryonic retinal cell line PER.C6 offers a reliable source of functionally active A1AT – but requires the co-expression of an α2,3-sialyltransferase gene to overcome poor A1AT sialylation in these cells [105]. According to Wang *et al.* [67], similar pharmacokinetic profiles were obtained between a PER.C6 protein variant and plasma-derived A1AT, but also differences in fucosylation or *N*-glycan branching could be observed.

Although numerous efforts have been made to humanize A1AT glycosylation of well-established non-human hosts (e.g. with co-expression of a series of key glycosyltransferases, as shown for A1AT expressed in insect cells [98]) or to investigate novel human expression systems, no recombinant A1AT is available as a licensed therapeutic to date. The recombinant production still suffers from short circulating half-life due to increased serum clearance, low protein yields, immunogenicity or limited adaption to large-scale manufacturing.

1.4.2 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Colony-stimulating factors (CSF) mediate survival, proliferation, differentiation commitment, maturation and functional activation of hematopoietic cells and are crucial regulators of the immune system [106]. As part of this protein family, GM-CSF (which is also termed CSF-2) is an important immunomodulatory cytokine targeting at the macrophage and granulocyte lineage. GM-CSF signaling initiates the production of white blood cells through stem-cell stimulation in the bone marrow [107]. GM-CSF itself can be synthesized by multiple tissues and cell types including T cells, macrophages, mast cells, endothelial cells and fibroblasts. Besides constitute production by a number of tumor cell lines, GM-CSF secretion often requires inducing stimuli such as cytokines, antigens, microbial products, inflammatory agents or allergens [108]. Under physiological conditions, GM-CSF can be detected in serum concentrations ranging from 20 to 100 pg/mL [108].
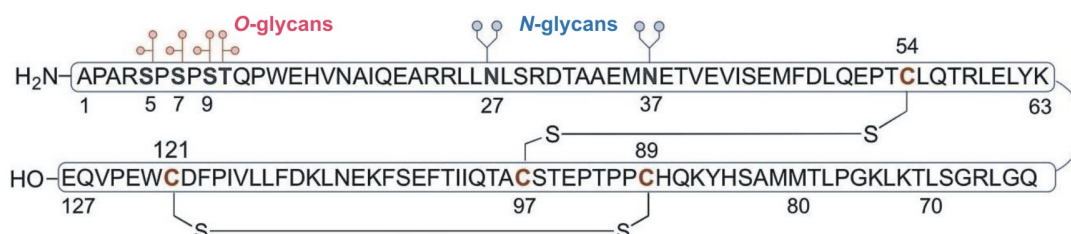
Fig. 7: Amino acid sequence of fully glycosylated human GM-CSF. ([107], with minor modifications)

Human GM-CSF is a monomeric and glycosylated protein. It is synthesized as a precursor of 144 amino acids including a secretory leader sequence at the amino-terminal end. The mature polypeptide chain (**Fig. 7**) consists of two intra-chain disulfide bonds and 127 amino acids accounting for a molecular mass of around 14 kDa. Due to varying degrees of glycosylation, the apparent molecular mass of GM-CSF varies from 18 to 32 kDa [109]. The glycoprotein contains two *N*-linked carbohydrate side chains of the complex type and four potential *O*-glycans clustered on Ser and Thr residues near the molecule's N-terminus [110]. So far, the functional role of the attached carbohydrates is not fully understood. There is a lot of evidence supporting the key role of carbohydrate chains in GM-CSF functions, such as pharmacokinetics, toxicity and immunogenicity [111]. Findings of Okamoto *et al.* [112] suggest that *N*-glycans play conflicting physiological roles (in terms of clearance from the blood circulation versus bioactivity) while *O*-linked carbohydrate moieties of GM-CSF do not have such effects.

1.4.2.1 Recombinant GM-CSF variants

Following the purification of human GM-CSF in 1984 [113], its molecular sequence was first elucidated in 1985. Within the next years, three recombinant GM-CSF variants were produced using yeast, bacterial and mammalian expression hosts: sargramostim (in

Saccharomyces cerevisiae), malgramostim (in *Escherichia coli*) and regramostim (in CHO cells) [114]. All synthetic forms of GM-CSF display the activity of the native molecule, but differ in their specific amino acid sequence, protein size and degree of glycosylation. The yeast-derived variant sargramostim contains a single amino acid substitution (proline[23] → leucine[23]) compared to endogenous human GM-CSF and may have a different carbohydrate moiety. Sargramostim is glycosylated to a lesser extent than regramostim, whereas malgramostim is not glycosylated at all [114]. Although the product from mammalian CHO cells is more similar to native GM-CSF and shows improved pharmacokinetic parameters [109], only the commercial preparations of aglycosylated malgramostim (approved in Europe) and yeast cell-derived sargramostim (approved in the USA) are presently available for clinical application [107].

Recombinant expression of human GM-CSF is also under investigation in various other hosts, such as human lymphoblastoid Namalwa cells [115], mammalian COS cells derived from monkey kidney tissue [116], in seeds of transgenic rice plants [117] or sugarcane [118]. All of these products showed biological GM-CSF activity, but none is approved for the treatment of patients.

1.4.2.2 Clinical applications of GM-CSF

Recombinant human GM-CSF has been available for therapeutic use since the early 1990s. Its main clinical application is to restore the hematopoietic dysfunction of patients after chemo- and/or radiotherapy [114]. In particular, for neutropenia (disorder characterized by an abnormally low number of neutrophils) and aplastic anemia (bone marrow failure that causes the inability of stem cells to generate mature cells of all three blood cell types) GM-CSF is employed for the physiological reconstitution of hematopoiesis. Recombinant GM-CSF is also widely used as immunostimulant to raise recovery from bone marrow transplantation and, thus, reduces the attendant morbidity of this procedure [109]. GM-CSF enhances the yield of peripheral blood stem cell transplantation und is usually administered at a dose of 5 mg per kg body weight a day either by intravenous infusion or subcutaneous injection [119]. Higher doses are rarely used because of associated side effects.

Besides its established role as hematopoietic growth factor in clinical practice, GM-CSF might be also important for driving inflammatory responses due to its proinflammatory activity. Since numerous reports describe the exacerbating influence of GM-CSF in established autoimmune-mediated disorders (such as rheumatoid arthritis), an antagonism of GM-CSF is postulated as a novel therapeutic target and might be an effective approach of treatment [120]. The therapeutical relevance of GM-CSF also becomes apparent in reports suggesting that the GM-CSF glycoprotein may hold promise as an immunoadjuvant for anticancer vaccines [107]. Since recombinant human GM-CSF has been shown to stimulate migration

and proliferation of endothelial cells and to promote faster wound healing, also applications for patients susceptible to mucosal damage or ulcers are under investigation [114]. According to the *clinicaltrials.gov* database, 968 studies were found to be related to sargramostim or GM-CSF in October 2013 [121] – most of them analyzing the usefulness of GM-CSF in hematological disorders, as adjuvant therapy in cancer treatment or adjuvant for prophylactic vaccines against HIV, sepsis or Crohn's disease. Due to the variety of biologic effects being attributed to endogenous GM-CSF, additional clinical applications are an emerging and promising subject of ongoing research.

1.4.3 Trastuzumab

Following the identification of the *human epidermal growth factor receptor (HER)-2/neu* oncogene from cancerous mouse cells in 1974, a milestone in the trastuzumab history was set by Slamon and coworkers establishing the linkage between *HER-2/neu* expression and breast cancer [122]. Overexpression of *HER-2/neu* occurs in 20 to 30% of invasive breast carcinomas indicating this transmembrane receptor as a major player in cancer pathogenesis.
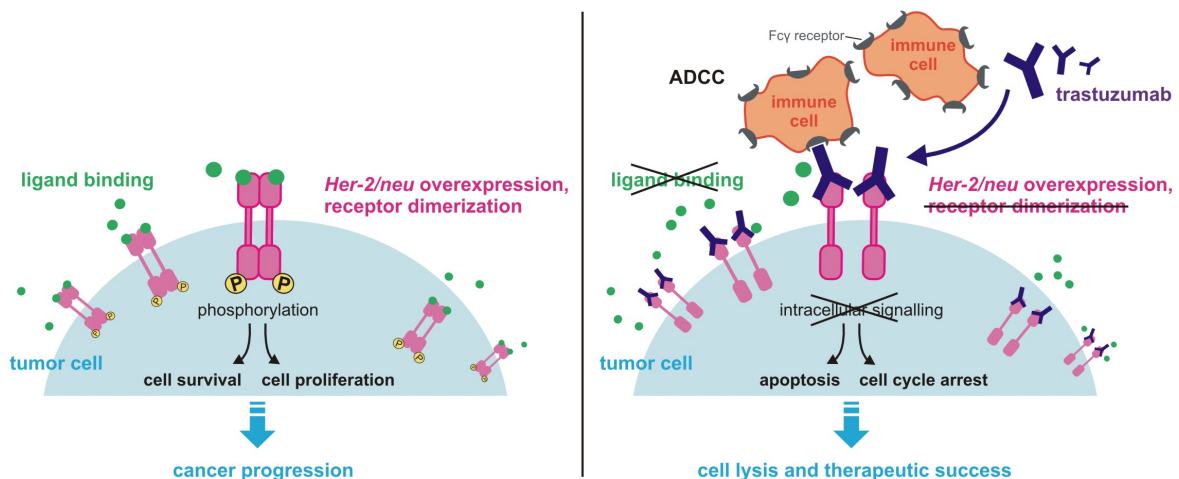
Fig. 8: Potential mechanisms of action of trastuzumab (according to [123])**.** Breast cancer cells overexpress the HER-2/neu transmembrane protein correlating with the severity of cancer progression (left). Trastuzumab treatment of these cells (right) prevents ligand binding, HER-2/neu dimerization, intracellular phosphorylation and, thus, tumor cell proliferation and survival signaling. Additionally, trastuzumab may recruit Fc-competent immune effector cells initiating ADCC and leading to tumor-cell death.

Trastuzumab (trade name Herceptin®, Gentech Inc., South San Francisco, CA, USA) is a medication strategy to selectively target HER-2/neu. It is a recombinant humanized mono-clonal antibody with specific binding affinity to the extracellular domain of the HER-2/neu protein. Its mechanism of action (**Fig. 8**) inhibits ligand binding, receptor dimerization and activation of intracellular tyrosine kinase activity and, thus, it prevents intracellular signaling in terms of tumor cell survival and proliferation. In addition to the direct effects on cancer cells, trastuzumab also acts indirectly via the immune system. It is suggested that the ADCC

mediated by the fragment crystallizable (Fc) portion of the IgG antibody plays an important role in the antitumor activity by recruiting immune effector cells [124].

In 1990, Gentech scientists characterized murine monoclonal antibodies active against HER-2/neu whereby 4D5 was the most effective growth inhibitor of a breast adenocarcinoma cell line [125]. Antibody humanization was later achieved by insertion of the complementary-determining regions of the parental murine 4D5 antibody into the consensus framework of a human IgG_1 [123]. In September 1998, the CHO cell-derived recombinant trastuzumab product received FDA approval for use in patients with invasive breast cancer overexpressing *HER-2/neu*. It was the first therapeutic antibody targeting at a specific cancer-related molecular marker that was approved for clinical use. More recently, the potential benefit of trastuzumab therapy has been investigated in several other HER-2/neu-amplified cancers including gastric cancer [126]. Approval for trastuzumab, in combination with chemotherapy, was granted in the USA in October 2010 for patients with HER-2/neu-positive adeno-carcinoma of the stomach or gastro-esophagael junction [127].

In terms of patient's resistance to trastuzumab treatment and significant adverse effects coming along with the need of chemotherapy, a novel antibody-drug conjugate was recently developed. Trastuzumab emtansine (trade name Kadcycla®, Gentech Inc., South San Francisco, CA, USA) consists of the monoclonal antibody trastuzumab stably linked to the cytotoxic agent DM1 and targets high-dose chemotherapy directly to the cancer cell [128]. Data clearly suggest an improved tolerability of trastuzumab emtansine compared to standard chemotherapy approaches. In 2013, the FDA approved this antibody-drug conjugate for treatment of HER-2/neu-positive and trastuzumab-pretreated advanced breast cancer [129].

1.4.3.1   *Impact of glycosylation on IgG effector functions*

Trastuzumab contains a single and highly conserved *N*-glycosylation site in each of both constant heavy chain 2 (C_H2) domains of the Fc region, as typical for IgG-type antibodies. The *N*-linked glycosylation occurs at Asn^{297} and studies indicate that the two Asn^{297} sites within a single IgG molecule may be differently glycosylated [130]. The magnitude of IgG glycosylation has been entirely described and is restricted to predominant biantennary complex-type *N*-glycans with varying degrees of terminal galactosylation, sialylation, bisecting GlcNAc and fucosylation. Although IgG glycans represent on average only 3% of the total molecule mass [131], their importance for pharmacokinetics, immunogenicity, protein conformation and thermodynamic antibody stability is throughout accepted. The functional relevance of particular glycoforms on immune effector functions is of great pharmaceutical interest since variations in the carbohydrate moiety have been shown to affect IgG affinity as well as its activity. In 2002, Shields and coworkers reported that antibodies lacking the α1,6-linked core

fucose at the *N*-glycan core showed an improved binding affinity to human Fcγ receptor IIIa (FcγRIIIa) of up to 50-fold and, thus, a significant enhancement of ADCC in the presence of natural killer cells compared to fucosylated IgG [37]. In this regard, fucosylation has emerged as a major target in drug development in order to optimize the properties of therapeutic antibodies. Reported methods achieving fucosylation control and the production of non-fucosylated IgG can be grouped into three methodologies [132]: (i) conversion of the non-mammalian *N*-glycosylation pathway into the humanized non-fucosylation pathway; (ii) inactivation of the fucosylation pathway of mammalian hosts and (iii) *in vitro* chemical synthesis of non-fucosylated glycans or enzymatic modification of *N*-glycans to non-fucosylated forms. This new generation of therapeutic antibodies most likely shows an improved clinical efficacy, in particular in cancer patients where cytotoxic antibodies are required [133].

1.5   Recombinant glycoprotein production

Therapeutic proteins were initially obtained from mammalian tissue or blood (e.g. insulin from pancreas or human serum albumin from plasma). The risk of contaminations by pathogens and, above all, the availability of biological material in the quantity required for an industrial production, primary impeded that way of protein extraction [134]. Today, approaches using recombinant DNA technology have mainly replaced the original animal-derived products for medical use. Bacteria, yeast, mammalian cell lines, plants, fungi, transgenic animals or insect cells are common systems for recombinant protein expression. From 2006 to June 2010, 32 of the 58 approved biopharmaceutical products were manufactured in mammalian systems, 17 in *Escherichia coli* and, at least, four in yeast *Saccharomyces cerevisiae* [135]. There is a steady increase in the prominence of mammalian over non-mammalian-based expression systems during the past two decades. This trend mainly comes along with the ongoing increase in the proportion of molecules that require PTMs, particularly human-like glycosylation [136]. Mammalian cells have significantly slower growth rates and product yields compared to microbes, and are much more complex concerning their nutritional and handling requirements. Although these systems are not at all competitive with well-established prokaryotic hosts in terms of scalability or operation costs [63], mammalian cell lines are the preferred platform for the production of glycosylated proteins. Among various existing systems, this work exemplarily focuses on the most common mammalian production cell line for industrial manufacturing, a famous human host for academic and pharmaceutical research purposes as well as on a novel human muscle-derived expression platform – all are introduced in detail within the following sections.

1.5.1 The Chinese hamster ovary (CHO) cell line

In 1957, the CHO cell line was established from an ovary biopsy of an adult Chinese hamster by Puck *et al.* in order to study genetics of mammalian cells [137]. In 1987, human tissue plasminogen activator produced in CHO cells became the first FDA-approved therapeutic protein from recombinant mammalian cell culture revolutionizing the market of biopharmaceuticals and the face of modern medicine [138]. Almost 30 years later, CHO-derived cells have emerged as preferred hosts for industrial manufacture of recombinant protein therapeutics. The ease of genetic manipulation, the multitude of available methods (in terms of cell transfection, gene amplification or clone selection) and the adaptability to large-scale suspension growth in serum-free and chemically defined media mainly promote the development of the CHO cell line as a workhorse for the pharmaceutic industry [139]. CHO cells have a proven track record as safe hosts for synthesis of medical products made from natural sources (termed as biologics). Moreover, the established history for regulatory approval of new recombinant products supports and facilitates upcoming CHO projects [139].

CHO cells have the capacity for efficient PTMs and possess the requisite cellular machinery to synthesize complex glycoforms that are usually compatible and bioactive in humans. Though, CHO cell-derived glycoproteins may contain traces of Neu5Gc [140], a non-human sialic acid and known immunogenic epitope. The cell line is also known to lack a functional copy of the gene coding for α2,6-sialyltransferase and, thus, exclusively α2,3-linked terminal sialic acids can be found in glycoproteins from CHO cells [141]. Moreover, the cell-type specific glycosylation profile lacks the so-called bisecting GlcNAc branch due to the absence of GlcNAc transferase III activity transferring GlcNAc residues to core mannose with a β1,4-linkage [142]. Umaña *et al.* [143] related the optimized ADCC of a recombinant therapeutic IgG antibody produced in an engineered CHO cell line to the occurrence of bisecting GlcNAc epitopes. Shinkawa, Nakamura and coworkers, however, observed that the lack of fucosylation – and not the presence of bisecting GlcNAc – has the most prominent effect in ADCC enhancement [144]. In fact, these observations are explicable by the reason that the presence of bisecting GlcNAc is always associated with low fucose content [133].

In order to optimize the properties of recombinant proteins, a wide panel of engineered variants and CHO glycosylation mutants has been developed and characterized so far (e.g. [145, 146]). In terms of IgG production, the heterologous expression of the prokaryotic enzyme GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD) is a reliable method to deflect the *de novo* fucose pathway by continuous removal of the key metabolic intermediate GDP-4-keto-6-deoxymannose and its conversion into a dead-end product (GlymaxX ® technology, [147]).

1.5.2 Human embryonic kidney (HEK)-293 cells

In 1973, the original HEK-293 cells were obtained in van der Eb's laboratory from kidney tissue of an apparently healthy and legally (under Dutch law) aborted human embryo of unknown parenthood [148]. The cell line derived from transformation of these cultivated cells with mechanically sheared fragments of human adenovirus type 5 DNA [149]. The results were published in 1977 by Graham *et al.* and the cell line was named HEK-293 due to Graham's habit of numbering his laboratory procedures: the original cell clone simply was the product of his 293rd experiment. Later, cloning and sequencing analyses of the transformation product revealed an incorporation of approximately 4.5 kilobases from the viral genome into the human chromosome 19 [150]. Since adenovirus 5 could have significantly disrupted cell expression and morphology, and embryonic kidneys are a heterologous mix of different cell types (particularly fibroblasts, endothelial or epithelial cells), the definite cellular origin of the HEK-293 cell line is unknown and still controversially discussed. Independent reports [148, 151] suggest a neuronal lineage and an embryonic adrenal precursor structure (the adrenal medulla, closely associated with the kidney during development) as the most likely origin of these cells. Based on this hypothesis, HEK-293 cells should not be used as an *in vitro* model for renal function [152].

However, HEK-293 is the second most commonly used cell line in cell biology studies (behind HeLa cells) and the second in frequency of their use in mammalian biotechnology approaches (behind CHO cells) [148]. In this regard, these cells are a popular expression host and a reliable platform for pharmaceutical and medical research purposes, in particular in small-scale productions. Benefits of using these cells for recombinant protein expression include the ease of cultivation, quick reproduction and maintenance, a highly efficient transfection of plasmid DNA using a wide variety of methods and a human protein processing [153]. Xigris®, an activated protein C, was the first biopharmaceutical generated in HEK-293 cells receiving FDA approval [154]. Recently, a number of reports have examined the feasibility of changing the expression host from classical CHO or baby hamster kidney (BHK) cell productions to HEK-293 [155, 156] – particularly due to improper protein glycosylation in non-human systems. A cell-type specific glycosylation pattern of HEK-293 cells is the occurrence of glycan antennae with terminal GalNAc instead of galactose, that is suggested to be a phenomenon of kidney(-derived) cells since it was also observed for the BHK cell line [157].

Among multiple derivatives of the HEK-293 cell line, the HEK-293T variant is a very popular one. It merely differs to the parental line in an additional expression of the large T antigen that is important for the replication of plasmids containing the simian virus 40 (SV40) origin of replication. Besides the generation of high-titer retroviruses [158], the high

transfectability of these cells is primarily exploited for recombinant protein production [159, 160]. The glycosylation potential of the HEK-293T cell line has been already investigated by Reinke *et al.* in terms of its cell surface *N*-glycan profile [161].

1.5.3 The rhabdomyosarcoma cell line TE671

The establishment of the TE671 cell line was first reported in 1977 by McAllister *et al.* [162], aiming at the generation of a permanent line of malignant human brain tumor cells for biological and biochemical studies. These cells were obtained from a brain tumor biopsy of a six-year-old Caucasian girl diagnosed with a cerebellar medulloblastoma. Morphologic, cytochemical and neurochemical characterizations suggest the medulloblastoma-derived TE671 cell line as an experimental model to measure *in vitro* drug sensitivity in order to develop innovative therapy approaches for this common type of brain tumor in children [163]. Surprisingly, findings of Schoepfer and coworkers [164] revealed the expression of a functional muscle-like acetylcholine receptor in the TE671 cell line despite its neuronal origin. Subsequent investigations including DNA fingerprinting, cytogenetic analysis and immunohistochemistry confirmed the identity of TE671 cells and the skeletal muscle cancer cells RD and suggested that TE671 is a subline of this human rhabdomyosarcoma cell line RD [165]. Consequently, the TE671 cell line was reclassified in 1989 refuting its neuronal origin. To date, these muscle-derived cells have served as a model for investigation of acetylcholine receptors on TE671 cell surface [166, 167], endogenous ATP-sensitive potassium channels [168], antiproliferative properties of anticancer drugs [169] and polysialylation in tumor diagnosis and progression [170, 171]. The TE671 cell line has not been considered as human expression host for therapeutic proteins or glycoproteins so far.

1.6 Modification of glycan composition and structure

Therapeutic proteins have revolutionized the treatment of many diseases. In this regard, the modification of protein-associated carbohydrate moieties to alter pharmacokinetic, biochemical or functional properties of the respective glycoproteins is an emerging strategy – an approach referred to as glycoengineering [36]. As already stated by Stanley in 1992 [172], major targets of these engineering techniques are the increase of biological protein activity and serum half-life, the improvement of molecule stability or solubility as well as the prevention of glycan antigenicity and reduction of structural heterogeneity to facilitate pharmaceutical production and quality monitoring.

1.6.1 Genetic glycoengineering

Genetic glycoengineering, for instance, primarily focuses the introduction of additional glycosylation motifs into the existing amino acid sequence. In this way, the hyper-glycosylated darbepoetin alfa variant of human EPO obtained an increased degree of sialylation and, thus, revealed a significant prolongation of its serum half-life compared to the unmodified recombinant equivalent [42]. Also A1AT, the therapeutic model glycoprotein used in this work, has been studied in the course of genetic glycoengineering. Resulting A1AT neoglycoproteins, in particular a variant with an additional *N*-glycan at Asn^{123}, exhibited an up to 60% increase in the circulating half-life [173]. Since this approach requires the site-directed mutagenesis of the underlying parental DNA template, it may come along with crucial adverse effects regarding protein folding, molecule stability or biological activity. Accordingly, the transfer of well-known glycosylation domains from one protein to the N- or C-terminus of a target protein seems to cause less structural and functional interference. Grabenhorst and coworkers transferred a *N*-glycosylation motif of human interferon-β to interleukin-2 and yielded proper *N*-glycosylation, as this is the case for the wild-type variant of interferon-β [174]. Kaup, Saul *et al.* designed novel peptide tags with unnatural *N*-glycosylation sites (termed GlycoTags) and successfully attached them e.g. to the C-termini of human EPO or A1AT [175]. Hence, genetic glycoengineering cannot be considered as a universal strategy for therapeutic glycoproteins, but as a promising tool that always has to be applied and optimized anew for each specific target. Since heterogeneity in the glycosylation profile of therapeutic glycoproteins causes difficulties for process reproducibility and can lead to variable therapeutic efficiencies, genetic glycoengineering can also be applied to eliminate distinct glycosylation sites in order to gain a more homogenous protein glycosylation [172].

1.6.2 Cell-based glycoengineering

There are several ways to engineer a given host cell line in order to achieve a modified and customized glycosylation pattern. The conventional technique is to generate a genetically modified organism by introducing or deleting DNA sequences relevant for a desired glycosylation – mainly genes coding for glycosyltransferases and/or glycosidases. In terms of non-mammalian expression systems such as yeast, bacteria or plants, one of the major objectives is to humanize the respective glycosylation pathway. This was recently achieved by glycoengineering of *Nicotiana benthamiana* finally lacking plant-specific glycosylation pattern (i.e. xylose residues and α1,3-core fucosylation) but showing bisected and branched complex *N*-glycans [176]. The strategy was also applied for recombinant plant-derived A1AT

[97] that could be decorated with α2,6-linked terminal sialic acids (corresponding to the native therapeutic counterpart) by coexpression of the respective sialyltransferases gene.

Another cell-based method for recombinant production of therapeutics with consistent and unique glycan structures comprises the screening of cell populations for genetic defects in their glycosylation machinery. For instance, CHO glycosylation mutants isolated on the basis of their resistance to cytotoxic plant lectins are considered as potential host cells to produce therapeutic proteins with enhanced efficacies [177, 178].

1.6.3 Metabolic glycoengineering

Metabolic glycoengineering, also referred to as metabolic oligosaccharide engineering, is a straightforward strategy to create modified glycans by incorporation of exogenously supplied monosaccharides via salvage pathway. Gu and Wang, for instance, successfully improved the sialylation of recombinant interferon-γ by feeding of the metabolic precursor *N*-acetyl-mannosamine (ManNAc) in CHO cell culture [179]. A milestone of methodology development was published in 1992 by Reutter and coworkers [180] reporting about the substrate permissivity of the sialic acid biosynthesis pathway for ManNAc derivatives with unnatural elongations at the *N*-acyl side chain. Subsequently, multiple functional groups (such as ketones, thiols, azides or alkynes) have been introduced and served as targets for chemo-selective ligation reactions enabling glycan detection for quantitative and functional studies or visualization and localization of glycoconjugates [181]. Since the sialic acid metabolism pioneered this technique and is considered as the methodical workhorse, the scope of metabolic glycoengineering has been broadened to further glycosylation pathways, including GlcNAc, GalNAc and fucose [182]. Low membrane permeability of the monosaccharide analogues due to the lack of specialized membrane transporters so far limited the large-scale adaption of this technology in biotechnology approaches. In this regard, masking hydroxyl groups of sugar residues by acetylation (that is removed intracellularly by unspecific esterases) was introduced by Sarkar *et al.* [183] as a strategy to increase substance hydrophobicity and enhance cellular uptake across the plasma membrane barrier. Although metabolic glycoengineering has its major current application in regard to the study of glycan structure-function relations, it also holds immense potential to manipulate the glycan decoration of selected proteins and, thus, to increase glycoprotein quality.

1.6.4 Impact of cell culture and bioprocess conditions

The optimization of protein glycosylation within a bioprocess primarily focuses the maintenance of a consistent glycan pattern. Curling *et al.* revealed an alteration of recombinant interferon-γ glycosylation with increasing time in CHO cell batch culture [184]. It was concluded, that this was due to a change of specific cell growth and metabolic rate rather than due to instability of the glycoprotein carbohydrate residues over time. Based on the same model system, Wong and coworkers studied the impact of cell culture conditions on *N*-glycosylation quality in CHO cell approaches [185]. In this regard, low glutamine or glucose concentrations (affecting the amount of intracellularly available UDP-GlcNAc) led to decreased glycan chain extensions caused by nutrient limitations. Moreover, altered interferon-γ *N*-glycan structures have been shown to be determined by low culture viability which can be attributed to extensive degradation by intracellular glycosidases released via cell lysis [185].

The necessity to perform serum-free cultivations for therapeutic approaches generally requires an adaption procedure of the cell line from fetal calf serum (FCS)-supplemented medium to new environmental conditions. The sialylation level of recombinant murine EPO produced in CHO cells, for instance, has been reported to be crucially affected by the presence of FCS in the culture medium which can be considered as a rich source of free and covalently bound monosaccharides and glycoconjugates [186]. So far, numerous other environmental factors have been described to profoundly influence glycosylation within a bioprocess. These include the respective production method [187], number of cell passage [188] or basic cultivation parameters such as pH of the medium [189], cultivation temperature [190] or ammonium concentration [191]. A deeper understanding of those correlations and detailed monitoring are required and become increasingly aware to the glycobiology community.

2 Scientific goals

Control and modification of glycosylation is turning into an emerging aspect in the development of biopharmaceuticals, as it has a key impact on protein properties such as stability, bioactivity, bioavailability and antigenicity. Applying different methodical approaches, the superordinate goal of this thesis aims at the optimization of glycosylation of recombinant proteins in terms of their therapeutic applicability. Illustrating the pivotal relevance of glycosylation for various protein classes, the work focuses on the recombinant expression of three different therapeutic glycoproteins: the serine protease inhibitor A1AT, the hematopoietic cytokine GM-CSF and the humanized IgG_1-type antibody trastuzumab.

In order to minimize the risk of antigenic side effects and to achieve high product efficacy, mammalian expression platforms are used to assure a complex-type glycan pattern similar to those in humans and therefore compatible to the recipient. Besides protein-specific characteristics, glycan decoration of recombinant proteins dependents on the underlying cell type and tissue origin. Therefore, the usability and glycosylation potential of the human muscle-derived cell line TE671 have to be investigated – aiming at the establishment of a novel human production platform for recombinant glycoproteins. The glycosylation specificity of closely related human cell lines, e.g. the A1AT *N*-glycan profiles derived from two HEK-293 cell variants, have to be opposed. Since literature records showed the crucial impact of serum supplementation on cell growth and behavior, a detailed comparison of A1AT glycosylation under both cell culture conditions is necessary, which may contribute to a more in-depth understanding of this complex protein modification. In order to modify the glycan decoration in a target-oriented manner, the metabolic glycoengineering strategy is exploited. Using the

example of the fucose biosynthetic pathway, acylated fucose analogues could be investigated to facilitate the transport across the plasma membrane barrier, to reduce the extracellular required monosaccharide concentration and to allow, prospectively, a large-scale application of this methodology. While the *O*-hydroxyl-modification via acetyl and propanoyl residues targets at the lowering of substance hydrophilicity, the introduction of novel polyethylene glycol groups shall avoid potential difficulties in terms of analogue solubility.

Throughout all methodical approaches, this work faces the challenge of structural glycan heterogeneity, variability and complexity. As no universal strategy for a reliable and rapid identification of glycan compositions and structures is currently available, the particular research goals have to dictate the set-up of analytical technologies. This work focuses solely on *N*-glycosylation, for which an enzymatic *N*-glycan release by PNGase F is the initial step common to the majority of all experimental approaches. While sensitive studies on mono-saccharide compositions are provided by HPAEC-PAD, structural glycan analyses are primarily obtained by MS, in particular by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. The use of monosaccharide-specific lectins further assists glycan profiling and data validation.

3 Publications

3.1 The human rhabdomyosarcoma cell line TE671 – Towards an innovative production platform for glycosylated biopharmaceuticals
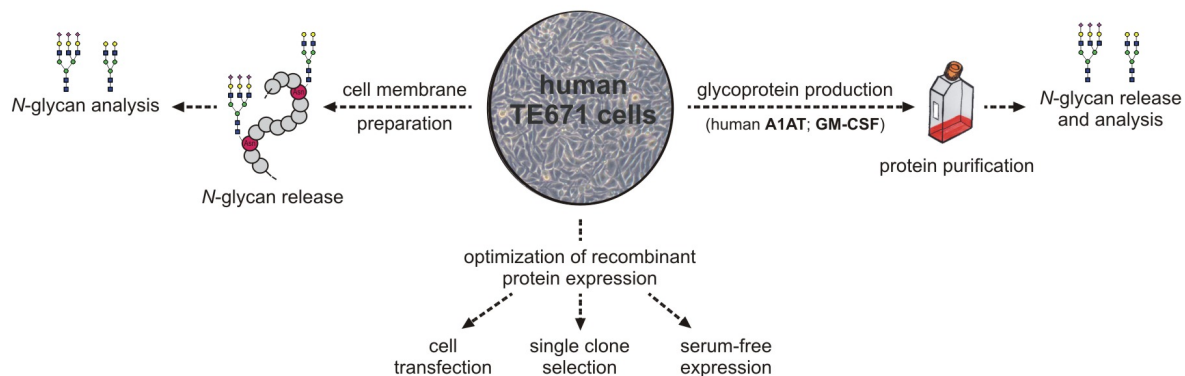
This chapter was published in the following journal:

Initial analyses of its *N*-glycan pattern on cell membrane glycoprotein level provided promising insights into the glycosylation potential of the muscle-derived cell line TE671. In order to establish a novel human system for glycoprotein production, this work focused the recombinant expression of A1AT and GM-CSF.

Conception and execution of all experiments as well as the preparation of the manuscript were performed in the scope of this dissertation under supervision of Prof. Dr. Stephan Hinderlich. Coauthors provided preliminary results, technical and/or practical assistance.

The original research article as well as the Supplementary data is available online at:

http://dx.doi.org/10.1016/j.pep.2015.08.008

3.2 Recombinant glycoproteins: The impact of cell lines and culture conditions on the generation of protein species
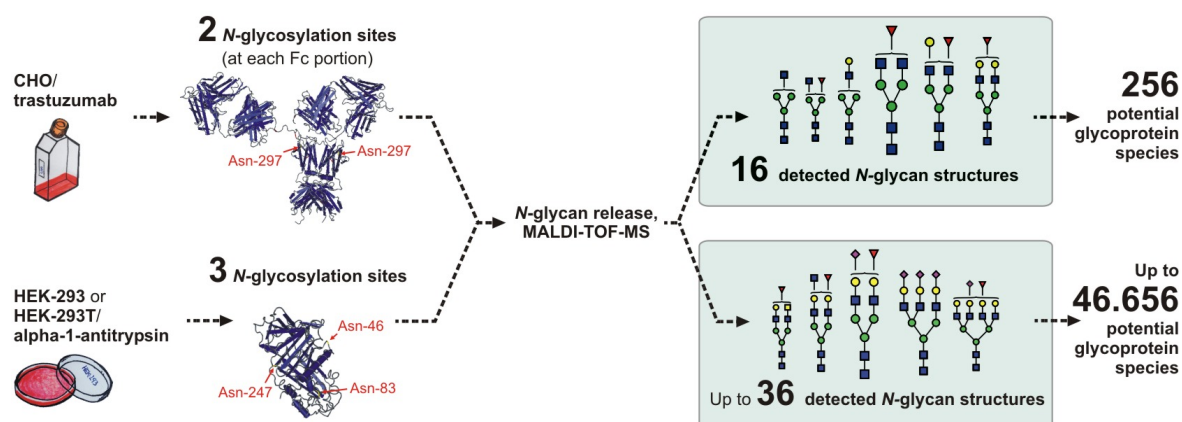
This chapter is published in the following journal:

The complexity and heterogeneity of glycosylation was demonstrated by means of exemplary model glycoproteins expressed in mammalian and human cell lines. Altering glycosylation profiles due to the presence or absence of FCS in the respective cell culture media (as already observed for TE671, see chapter **3.1**) were reported. A new term, called "glycoprotein species", was defined in order to introduce the definition of glycoforms from glycobiology into the field of protein species.

Conception and execution of all experiments as well as the preparation of the manuscript were performed in the scope of this dissertation under supervision of Prof. Dr. Stephan Hinderlich. Coauthors provided preliminary findings, practical assistance or content-related support.

The original research article is available online at:

http://dx.doi.org/10.1016/j.jprot.2015.08.011

3.3 Applying acylated fucose analogues to metabolic glyco-engineering
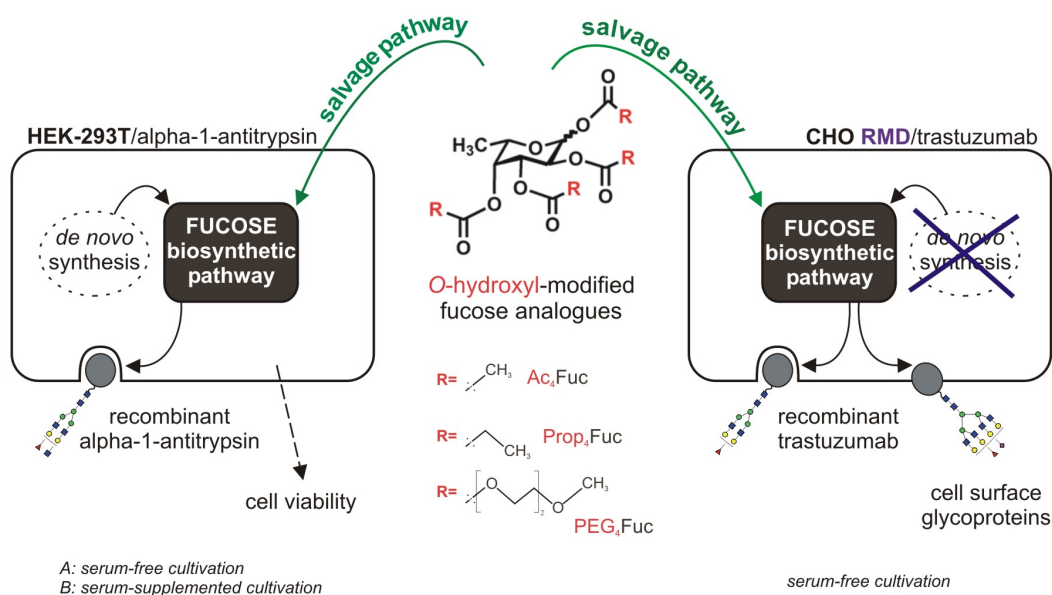
This chapter is published in the following journal:

The modulation of fucosylation by exploiting the salvage pathway of fucose biosynthesis via metabolic glycoengineering was the key concern of this work. Applying acylated fucose derivatives in cell culture approaches, the glycosylation profiles on the level of cell membranes and recombinantly expressed glycoproteins were analyzed for a human and a mammalian cell line.

Conception of this work and the preparation of the manuscript were performed in the scope of this dissertation under supervision of Prof. Dr. Stephan Hinderlich. Chemical fucose analogue syntheses were conducted under guidance of Dr. Verena Böhrsch who also evaluated the corresponding analytical data. This study was complemented by the results of the FcγRIIIa binding assay from coauthors while all other results were obtained in experiments of this thesis. Moreover, coauthors provided practical assistance or content-related support.

The original research article as well as the Supplementary data is available online at:

http://dx.doi.org/10.3390/bioengineering2040213

4 Summary

Recombinant therapeutic glycoproteins, in particular the notably increasing demand and commercial relevance of antibodies, cytokines, hormones or coagulation factors, have emerged as a profitable but highly challenging sector of the pharmaceutical industry. Thereby, glycans, their monosaccharide composition and structural heterogeneity have fundamental and versatile impact on basic glycoprotein properties, such as protein folding, molecular stability, biological function, serum half-life or immunogenicity.

Mammalian cell production platforms are currently the preferred systems for therapeutic glycoprotein manufacturing due to their natural ability to perform human-like glycosylation. Moreover, human expression hosts come to the fore throughout evading the synthesis of non-human glycan pattern and, in consequence, product antigenicity. Since the number of available human systems is presently limited and cell-type specific glycosylation characteristics attach further importance, the TE671 cell line was established as a novel tool for glycoprotein production within this work. Analyses of their membrane proteins and two recombinant model glycoproteins with therapeutical relevance, alpha-1-antitrypsin (A1AT) and granulocyte-macrophage colony-stimulating factor (GM-CSF), provided first insights into the glycosylation potential of this rhabdomyosarcoma cell line. An optimized method of cell transfection, the feasibility of cultivation under serum-containing and –free media conditions and, above all, the synthesis of complex and highly sialylated *N*-glycans characterized TE671 as a serious alternative to other existing human platforms.

Highlighting the crucial importance of environmental impact factors on the final glycosylation profile, the novel term "glycoprotein species" was introduced within this work

to join the definition of glycoforms (coined by Rademacher *et al.,* 1988) with the concept of protein species (coined by Jungblut *et al.*, 1996). Based on the analysis of their *N*-glycans, the theoretical numbers of glycoprotein species were determined for the therapeutic antibody trastuzumab and the serine protease inhibitor A1AT expressed in different cell lines in the presence and absence of serum. Regarding these low-complex examples, the resulting magnitude of structural heterogeneity was already much higher compared to other post-translational modifications and was primarily characterized by varying levels of terminal monosaccharides, such as fucoses and sialic acids. Thus, considering the aspect of glyco-protein species, in particular in process development and monitoring, is strongly suggested for future approaches in glycobiotechnology.

Metabolic glycoengineering – the exogenous supply of unnatural metabolic precursor and their incorporation into glycoconjugates via salvage pathway – allows modulating the glyco-sylation of selected glycoproteins to improve protein quality or functional properties. Within this study, acylated fucose derivatives applied to a genetically engineered Chinese hamster ovary (CHO) cell line with blocked *de novo* fucose synthesis revealed an increase in fuco-sylation on cell membrane level and for the recombinant model antibody. A thus regulated gradual fucosylation of expressed trastuzumab could be shown to correlate with a reduced antibody's binding affinity in a Fcγ receptor IIIa binding assay. Addressing the fucose bio-synthesis pathway by metabolic glycoengineering therefore can be considered as an appropriate strategy to directly influence glycosylation and, in consequence, functional aspects of recombinant glycoproteins.

In summary, the goal of optimizing the glycosylation of recombinant model proteins was successfully realized by three independent approaches: the establishment of a novel human expression system on the basis of the rhabdomyosarcoma cell line TE671, the in-depth investigation of the impact of cell culture conditions on the number and occurrence of glycoprotein species, and the application of acylated fucose analogues in the course of metabolic glycoengineering. The respective publications of the applied promising techniques may prospectively contribute to improve the manufacturing of protein therapeutics with desired and defined glycan decoration.

5 Zusammenfassung

Rekombinante, therapeutische Glykoproteine, insbesondere die beträchtlich wachsende Nachfrage und wirtschaftliche Bedeutung von Antikörpern, Zytokinen, Hormonen oder Gerinnungsfaktoren, haben sich als ein gewinnbringender aber in hohem Maße herausfordernder Zweig der pharmazeutischen Industrie entwickelt. Dabei haben Glykane, ihre Monosaccharid-Zusammensetzung und strukturelle Heterogenität wesentliche und vielseitige Bedeutung für grundlegende Eigenschaften von Glykoproteinen – wie Proteinfaltung, molekulare Stabilität, biologische Funktion, Serumhalblebenszeit oder Immunogenität.

Säugerzell-Produktionssysteme sind derzeit bei der Herstellung therapeutischer Glykoproteine wegen ihres naturgegebenen Vermögens human-ähnliche Glykosylierung zu erzeugen, bevorzugt. Überdies rücken humane Expressionswirte in den Fokus, die die Synthese nicht-humaner Glykanstrukturen und folglich die Antigenität des Produktes gänzlich vermeiden. Da die Zahl verfügbarer humaner Systeme derzeit begrenzt ist und zudem Zelltyp-spezifischen Glykosylierungsmerkmalen Bedeutung zuzumessen ist, wurde im Rahmen dieser Arbeit die TE671-Zelllinie als neuartiges Werkzeug zur Glykoprotein-Herstellung etabliert. Die Analysen ihrer Membranproteine und zweier rekombinanter und therapeutisch relevanter Modellglykoproteine, Alpha-1-Antitrypsin (A1AT) und Granulozyten-Makrophagen-Kolonie-stimulierender Faktor (GM-CSF), lieferten erste Erkenntnisse bezüglich des Glykosylierungspotentials dieser Rhabdomyosarkom-Zelllinie. Eine optimierte Zelltransfektion, die Kultivierbarkeit unter serumhaltigen und –freien Medienbedingungen und vor allem die Synthese von vornehmlich komplexen, hoch-sialylierten *N*-Glykanen zeichneten TE671 als ernstzunehmende Alternative zu anderen existierenden humanen Plattformen aus.

Um die entscheidende Bedeutung der umgebungsbedingten Einflussfaktoren auf das finale Glykosylierungsprofil hervorzuheben, wurde der neuartige Begriff „Glykoprotein-Spezies" im Rahmen dieser Arbeit eingeführt, um die Definition von Glykoformen (geprägt durch Rademacher *et al.,* 1988) mit dem Konzept der Protein-Spezies (geprägt durch Jungblut *et al.,* 1996) zu verbinden. Basierend auf der Analyse ihrer *N*-Glykane wurde die theoretische Anzahl an Glykoprotein-Spezies für den therapeutischen Antikörper Trastuzumab und den Serinprotease-Inhibitor A1AT, die in verschiedenen Zelllinien in An- und Abwesenheit von Serum exprimiert wurden, bestimmt. Bereits hinsichtlich dieser wenig komplexen Beispiele war das resultierende Ausmaß der strukturellen Heterogenität viel größer im Vergleich zu anderen posttranslationalen Modifikationen und zeichnete sich vorrangig durch verschiedene Anteile terminaler Monosaccharide, wie Fucosen und Sialinsäuren, aus. Demnach wird dringend empfohlen, den Aspekt der Glykoprotein-Spezies, insbesondere in der Prozessentwicklung und –überwachung, in zukünftigen Ansätzen in der Glykobiotechnologie zu berücksichtigen.

Metabolisches *Glycoengineering* – die exogene Zuführung von unnatürlichen metabolischen Vorläufern und ihr Einbau in Glykokonjugate über den *Salvage*-Weg – erlaubt die Glykosylierung ausgewählter Glykoproteine zu modulieren, um ihre Proteinqualität oder funktionelle Eigenschaften zu verbessern. Im Rahmen dieser Studie zeigten acylierte Fucose-Derivate am Beispiel einer gentechnisch veränderten *Chinese hamster ovary* (CHO)-Zelllinie eine Steigerung der Fucosylierung auf Zellmembran-Ebene und für den rekombinanten Modellantikörper. Für eine auf diese Weise gesteuerte, graduelle Fucosylierung von exprimiertem Trastuzumab konnte eine Korrelation mit gesenkter Bindungsaffinität des Antikörpers in einer Fcγ-Rezeptor IIIa-Bindungsstudie gezeigt werden. Die Adressierung des Fucose-Biosyntheseweges durch metabolisches *Glycoengineering* kann daher als eine zweckdienliche Strategie betrachtet werden, um die Glykosylierung, und folglich funktionale Aspekte von rekombinanten Glykoproteinen, zielgerichtet zu beeinflussen.

Das Ziel der Optimierung der Glykosylierung rekombinanter Modellproteine wurde somit durch drei unabhängige Ansätze erfolgreich umgesetzt: die Etablierung eines neuartigen humanen Expressionssystems auf Grundlage der Rhabdomyosarkom-Zelllinie TE671, die detaillierte Untersuchung des Einflusses von Zellkulturbedingungen auf die Zahl und Ausprägung von Glykoprotein-Spezies und die Anwendung von acylierten Fucose-Analoga im Zuge des metabolischen *Glycoengineerings*. Die entsprechenden Publikationen der angewendeten, aussichtsreichen Techniken können zukünftig einen Beitrag leisten, die Herstellung von Proteintherapeutika mit erwünschter und definierter Glykanausstattung zu verbessern.

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