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Production of Recombinant A1AT with Human Glycosylation Profile In CHO Cells and Its Interaction with Asialoglycoprotein Receptors

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Résumé

L'alpha-1 antitrypsine (A1AT) est un inhibiteur de sérine protéase sécrété principalement par le foie et libéré dans la circulation où sa concentration physiologique est de 1,5 à 3,5 g/L. La principale fonction de l'A1AT est d'inhiber l'activité de l'élastase des neutrophiles (NE) afin de maintenir l'équilibre protéase/anti-protéase dans les poumons. Son déficit (A1ATD) touche plus de 3,4 millions d'individus dans le monde chez qui l'élastase des neutrophiles décompose l'élastine, provoquant ainsi une diminution de l'élasticité du poumon ainsi qu'une dégradation de son tissu conjonctif. En conséquence, l'A1ATD entraîne des troubles respiratoires tels que l'emphysème ou la maladie pulmonaire obstructive chronique et ceux qui en sont atteints nécessitent des injections fréquentes d'A1AT purifiée à partir du sang d'un donneur. Cependant, l'A1AT plasmatique est hétérogène dans son état de glycosylation et sa qualité varie d'un lot à l'autre. De plus, il y a un risque, même très faible, de transmission d'agents pathogènes avec l'administration d'A1AT purifiée par plasma. Par conséquent, il existe un besoin pour une version recombinante.

La glycoprotéine mature possède trois sites de *N*-glycosylation comprenant principalement des structures de type complexe bi-antennaires afucosylées et α-2,6-di-sialylées, A2G2S2 (6,6). Bien que la glycosylation ne soit pas essentielle à l'activité inhibitrice de l'A1AT, il a été démontré qu'elle a un impact significatif sur sa demi-vie *in vivo*. Notamment, l'acide sialique, un monosaccharide terminal chargé négativement présent sur les *N*-glycanes, aide à prolonger la demi-vie de l'A1AT dans le sérum en empêchant l'interaction entre l'avant-dernier galactose (Gal) du *N*-glycane et les récepteurs hépatiques des asialoglycoprotéines (ASGPRs), composés de deux sous-unités appelées lectines hépatiques (HL) 1 et 2, qui se lient aux glycoprotéines asialylées contenant un Gal terminal et conduisent à leur dégradation. Par conséquent, il est important de produire A1AT dans un système d'expression qui peut effectuer les modifications post-traductionnelles (PTM) appropriées à des fins thérapeutiques.

Jusqu'à présent, la production d'A1AT recombinante (rA1AT) a été tentée dans différents systèmes d'expression cellulaire avec un succès limité. Malgré la disponibilité de diverses lignées cellulaires, les cellules ovariennes de hamster chinois (CHO) ont été largement utilisées pour la

production de glycoprotéines thérapeutiques car ces cellules sont compatibles avec des stratégies de glyco-ingénierie pour produire des glycoprotéines recombinantes composées de glycanes de type humain. Cependant, ces cellules synthétisent des *N*-glycanes de type complexe comprenant de la fucosylation centrale et de l'acide sialique lié en α-2,3. Par conséquent, dans ce projet, l'objectif était de développer une version recombinante d'A1AT avec un profil de glycosylation humaine exprimée en cellules CHO modifiées et qui se prête à des utilisations thérapeutiques.

À cette fin, dans notre étude, nous avons d'abord empêché l'α-2,3 sialylation ainsi que la fucosylation centrale en éliminant les gènes responsables via la technologie CRISPR/Cas9, suivie de la surexpression de l'α-2,6-sialyltransférase humaine à l'aide d'un système d'expression inductible au cumate. Nous avons ensuite montré la supériorité du promoteur inductible CR5 pour l'expression de A1AT par rapport à cinq promoteurs constitutifs forts couramment utilisés dans l'industrie. En utilisant le promoteur CR5, nous avons généré des populations de CHO stables modifiées par glyco-ingénierie produisant plus de 2,1 g/L pour la forme native et 2,8 g/L pour la version mutée d'A1AT avec des *N*-glycanes analogues au produit clinique dérivé du plasma, la Prolastin-C. L'effet bénéfique de la supplémentation en N-acétylmannosamine du milieu de culture cellulaire sur la glycosylation de l'A1AT a également été démontré. Enfin, nous avons montré que l'activité anti-élastase des rA1ATs est comparable à celle de la Prolastin-C, et que la substitution des résidus méthionines critiques par des valines rendait A1AT significativement plus résistante à l'oxydation.

Nous avons ensuite étudié l'impact de la glycosylation d'A1AT sur son interaction avec les orthologues d'ASGPR. Pour cela, nous avons initialement utilisé un test d'internalisation cellulaire basé sur la lignée cellulaire hépatique humaine HepG2 connue pour exprimer les ASGPRs à sa surface et avons examiné leur interaction avec les rA1ATs possédant divers profils de glycosylation. Comme le test d'internalisation basé sur les cellules HepG2 a démontré un faible rapport signal sur bruit (SNR) ainsi qu'un niveau élevé de signal de fond d'internalisation, nous avons cherché à développer un nouveau test basé sur des cellules CHO surexprimant des orthologues ASGPR recombinants. Alors que la sous-unité HL-1 humaine seule était suffisante pour lier et internaliser l'A1AT asialylée, les sous-unités HL-1 et HL-2 étaient nécessaires pour former des récepteurs fonctionnels et ayant une forte affinité pour les ASGPR de rat et de souris.

Afin d'améliorer le SNR de notre test cellulaire d'internalisation, le tri cellulaire activé par fluorescence (FACS) a été utilisé pour enrichir les populations de cellules CHO pour celles exprimant des niveaux élevés d'orthologues ASGPR. Enfin, en utilisant des structures de glycanes remodelés par voie enzymatique de Prolastin-C, nous n'avons observé aucune internalisation lorsque les glycanes sont terminés avec α-2,6-Neu5Ac ni α-2,8-Neu5Ac-α-2,6-Neu5Ac par l'ASGPR de l'humain, du rat et de la souris. D'autre part, l'absorption de Prolastin-C portant des glycanes bi-antennaires avec une branche terminée par de l'acide sialique α-2,3 et l'autre par du galactose terminal, par l'ASGPR de souris a été statistiquement plus élevée que celle de l'humain et du rat.

En somme, l'A1AT recombinante résistante à l'oxydation décrite dans ce projet pourrait représenter un meilleur médicament biothérapeutique tout en offrant une alternative sûre et plus stable pour la thérapie d'augmentation. Nous avons également contribué à une meilleure compréhension de l'impact de la sialylation de l'A1AT sur son internalisation cellulaire par les orthologues ASGPR.

Mots-clés : A1AT, cellule CHO, CRISPR/Cas9, glyco-ingénierie, *N*-glycosylation, récepteur de l'asialoglycoprotéine, sialylation, internalisation

Abstract

Alpha-1 antitrypsin (A1AT) is a serine protease inhibitor secreted primarily by the liver, and released in the circulation where its physiological concentration is 1.5-3.5 g/L. The main physiological function of A1AT is to inhibit the activity of neutrophil elastase (NE) to maintain the protease/anti-protease balance in the lung. The A1AT deficiency (A1ATD) is affecting more than 3.4 million individuals worldwide where neutrophil elastase breaks down elastin, thereby causing a decrease in the elasticity of the lung as well as a degradation of its connective tissue. As a result, A1ATD leads to respiratory disorders such as emphysema or chronic obstructive pulmonary disease. Treatment of this health condition requires frequent injections of A1AT purified from donor blood. However, plasma A1AT is heterogeneous in its glycosylation state and its quality varies from batch to batch. Moreover, there is a risk, however very low, of pathogen transmittance with plasma-purified A1AT administration. Therefore, there is a need for recombinant version.

The mature glycoprotein has three *N*-glycosylation sites possessing mostly afucosylated, α-2,6-di-sialylated bi-antennary complex-type structures, A2G2S2 (6,6). Though glycosylation is not essential for A1AT's inhibitory activity, it has been shown to have a significant impact on its *in vivo* half-life. Notably, sialic acid, a terminal negatively charged monosaccharide present on *N*-glycans, helps to prolong the half-life of A1AT in serum by preventing the interaction between the penultimate galactose (Gal) of the *N*-glycan and the hepatic asialoglycoprotein receptors (ASGPRs), composed of two subunits termed hepatic lectin (HL) 1 and 2, which bind to asialylated glycoproteins containing terminal Gal and lead to their degradation. To this extend, it is important to produce A1AT in an expression system that can carry out the appropriate post-translational modifications (PTMs) for therapeutic purposes.

Thus far, the production of recombinant A1AT (rA1AT) has been attempted in different cell expression systems with limited success. Despite the availability of various cell lines, Chinese hamster ovary (CHO) cells have been widely used to produce therapeutic glycoproteins as these cells can tolerate glycoengineering strategies to produce recombinant glycoproteins with human-like glycans. However, these cells synthesize complex-type *N*-glycans with core-fucosylation along

with α-2,3-linked sialic acid. Therefore, in this research project, the aim was to develop a recombinant version of A1AT with human glycosylation pattern expressed in genetically engineered CHO cells that would be amenable to therapeutic uses.

To this end, in our study, we first prevented α-2,3 sialylation as well as core-fucosylation by eliminating the corresponding genes via CRISPR/Cas9 technology, followed by overexpressed human α-2,6-sialyltransferase using a cumate-inducible CHO expression system. We then showed superiority of the CR5 inducible promoter compared to five strong constitutive promoters commonly used in the industry. Using the CR5 promoter, we generated glycoengineered stable CHO pools producing over 2.1 g/L of the wild-type and 2.8 g/L of the mutein forms of A1AT, with *N*-glycans analogous to the plasma-derived clinical product, Prolastin-C. The effect of *N*-acetylmannosamine supplementation to the cell culture media on the A1AT glycosylation was also demonstrated. Finally, we showed that the anti-elastase activity of rA1ATs is comparable to that of Prolastin-C, and that substitution of critical methionine residues with valines rendered A1AT significantly more resistant to oxidation.

We then studied the impact of A1AT glycosylation on its interaction with ASGPR orthologs. For this, we initially used a cell-based internalization assay based on the human HepG2 hepatic cell line known to express ASGPRs at its surface and examined their interaction with rA1ATs possessing various glycosylation profiles. As HepG2 cell-based internalization assay demonstrated poor signal-to-noise ratio (SNR) as well as high level of background internalization signal, we then aimed at developing a new assay based on CHO cells overexpressing recombinant ASGPRs orthologs. While human HL-1 subunit alone was sufficient to bind and internalize asialylated A1AT, both HL-1 and HL-2 subunits were required to form functional and high affinity receptors for the rat and mouse ASGPRs. To enhance SNR of our cell-based uptake assay, fluorescence-activated cell sorting (FACS) was used to enrich the CHO pools for cells expressing high levels of ASGPR orthologs. Finally, using enzymatically remodelled glycan structures of Prolastin-C, we observed no uptake when glycans are terminated with α-2,6-Neu5Ac nor α-2,8-Neu5Ac-α-2,6-Neu5Ac by human, rat, and mouse ASGPR orthologs. On the other hand, the uptake of Prolastin-C bearing bi-antennary glycans with one branch terminated with α-2,3 sialic acid and the other

with terminal galactose, by mouse ASGPR was observed to be statistically higher than that by human and rat ASGPR orthologs.

Collectively, the oxidation-resistant recombinant A1AT described in this project could represent a viable biobetter drug while offering a safe and more stable alternative for augmentation therapy. We also contributed a better understanding of the impact of A1AT sialylation on its cellular uptake by ASGPR orthologs.

Keywords : A1AT, CHO pool, CRISPR/Cas9, glycoengineering, *N*-glycosylation, asialoglycoprotein receptor, sialylation, cellular uptake

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List of Acronyms and Abbreviations

A1AT : alpha-1-antitrypsin

A1ATD : alpha-1-antitrypsin deficiency

AAL : *Aleuria aurantia* lectin

AAV : adeno-associated virus

ADCC : antibody-dependent cell-mediated cytotoxicity

ASF : asiaofetuin

ASGP : asialylated glycoproteins

ASGPR : asialoglycoprotein receptor

Asn : asparagine

ASOR : asialoorosmuccoid

ATS : American Thoracic Society

BChE : butyrylcholinesterase

BHK : baby hamster kidney cells

CAG : cytomegalovirus enhancer fused to the chicken beta-actin promoter

Cas9 : CRISPR-associated protein 9

CHEF1 : Chinese hamster elongation factor-1 alpha

CHIP-seq : chromatin immunoprecipitation sequencing

CHO : Chinese hamster ovary

CMAS : CMP-Sia synthetase

CMP : cytidine 5'-monophosphate

CMV : cytomegalovirus

COPD : chronic obstructive pulmonary disease

CRD : carbohydrate recognition domain

CRISPR : clustered regularly interspaced short palindromic repeats

CST : CMP-sialic acid transporter

Cys : cysteine

DANA : dehydro-2-deoxy-*N*-acetylneuraminic acid

dCO_2 : dissolved carbon dioxide

DHFR : dihydrofolate reductase

DMSO : dimethyl sulfoxide

DNA : deoxyribonucleic acid

DNMT : DNA methyltransferase

DO : dissolved oxygen

dpi : days post-induction

DSB : double-stranded break

ECL : *Erythrina cristagalli agglutinin* lectin

E.coli: Escherichia coli

EF1α : human elongation factor-1 alpha

EMA : European Medicines Agency

EPO : erythropoietin

ER : endoplasmic reticulum

ERS : European Respiratory Society

EU : European Union

FACS : fluorescence-activated cell sorting

FBS : fetal bovine serum

Fc : fragment crystallizable

FcR : Fc receptor

FDA : US Food and Drug Administration

FI : fluorescence Intensity

FITC : fluorescein

FSC : forward scatter

Fuc : fucose

Fut8: α-1,6-fucosyltransferase

Gal : galactose

GalNAc: *N*-acetylgalactosamine

GAPDH : glyceraldehyde 3-phosphate dehydrogenase

GDP : guanosine diphosphate

GFPP : GDP-fucose pyrophosphorylase

Glc : glucose

GlcNAc : *N*-acetylglucosamine

GS : glutamine synthetase

GT : galactosyltransferase

HEK293 : human embryonic kidney cells 293

hHL : hepatic lectin

HILIC : hydrophilic interaction liquid chromatography

HPAEC-PAD : high-performance anion-exchange chromatography with pulsed amperometric detection

IFN-γ : interferon-gamma

Ig : immunoglobulin

KO : knockout

LC-MS/MS : liquid chromatography with tandem mass spectrometry

mAb : monoclonal antibody

MAL II : *Maackia amurensis* lectin II

MALDI-TOF : matrix assisted laser desorption/ionization- time-of-flight

Man : mannose

ManNAc: *N*-acetylmannosamine

Met : methionine

mHL : mouse hepatic lectin

miRNA : microRNA

MS : mass spectrometry

MSX : methionine sulfoximine

MTX : methotrexate

NaBu : Sodium butyrate

NANP : Neu5Ac-9-phosphate phosphatase

NANS : Neu5Ac 9-phosphate synthase

NE : neutrophil elastase

Neu5Ac : *N*-acetylneuraminic acid

Neu5Gc : *N*-glycolylneuraminic acid

NHEJ : non-homologous end joining

NO : nitric oxide

OST : oligosaccharyltransferase

PAM : protospacer adjacent motif

PD : pharmacodynamics

PEI : polyethylenimine

PK : pharmacokinetics

PNGase: Peptide-*N*-Glycosidase F

PSA : polysialic acid

PTMs : post-translational modifications

rA1AT : recombinant A1AT

RCL : reactive center loop

rcTA : reverse transactivator

rHL : rat hepatic lectin

RME : receptor mediated endocytosis

RNA : ribonucleic acid

SARS-CoV2 : severe acute respiratory syndrome- coronavirus 2

SD : standard deviation

SEAP : secreted alkaline phosphatase

SEC : serpin enzyme complex

Ser : serine

sgRNA : single guide RNA

shRNA : short hairpin RNA

Sia : sialic acid

siRNA : small interfering RNA

SNA : *Sambucus nigra agglutinin*

SNP : single nucleotide polymorphism

SNR : signal-to-noise ratio

SSC : side scatter

ST : sialylatransferase

ST3Gal : β-galactoside α-2,3-sialyltransferases

ST6Gal : β-galactoside α-2,6-sialyltransferases

TALEN : transcription activator like nuclease

Thr : threonine

TMPRSS2 : transmembrane serine protease 2

TNFα : tumor necrosis factor α

tPA : tissue plasminogen activator

UDP : uridine diphosphate

UPLC : ultra performance liquid chromatography

Val : valine

WT : wild-type

ZFN : zinc finger nuclease

To my dad …

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Chapter 1: Introduction

Context

Over the past decade, there has been a significant rise in demand and commercial importance of recombinant protein expression for the treatment of numerous diseases (1). Majority of these recombinant biopharmaceuticals require post-translational modifications (PTMs) that can significantly impact their biological functions and activities. Particularly, proper *N*-glycosylation can improve the properties of recombinant proteins, such as enhancing their stability and prolonging their half-life in the bloodstream, while modulating their immunogenicity (2-4). Concerning human proteins for therapeutical applications, the choice of the expression system is of particular interest since it has a direct effect on *N*-glycosylation and varies greatly across various prokaryotic and eukaryotic expression hosts. Among them, mammalian cells are the preferred host for the production of such therapeutics as they are capable of expressing glycoproteins with human-like PTMs that are comparable to their native counterparts (5). Notably, Chinese hamster ovary (CHO) cells has emerged as the predominant mammalian production platform, and are involved in the production of over 70% of recombinant biopharmaceutical proteins (5). Benefits of using CHO cells include their ease of scale-up, adaptation to grow in suspension culture under serum-free conditions and producing safe as well as effective glycoprotein therapeutics with human-like *N*-glycosylation pattern at high yields (5). However, despite many advantages, one of the biggest challenge is that CHO cells are unable to produce glycoproteins with human-like α-2,6-sialylation (6), a common terminal modification found on human *N*-glycans, which was addressed in this thesis.

Among numerous clinically important glycoproteins, alpha-1-antitrypsin (A1AT) has gained a huge interest over several years. The first description of A1AT was made by Laurell and Eriksson in 1963 when they found that the serum protein electrophoresis of several individuals with early-onset severe obstructive lung disease did not display a band for alpha-1 globulin (7). It was later discovered that the absence of this electrophoretic band indicated an inherited deficiency of a particular protein that can inactivate serine proteases, such as trypsin and

therefore named A1AT (8, 9). Since A1AT can inhibit a broad range of proteases, including chymotrypsin, thrombin, and plasmin (10), the term "alpha-1-proteinase inhibitor" is occasionally favored over A1AT in research papers. However, the main physiological function of A1AT is shown to inhibit the activity of neutrophil elastase (NE) in the lung to maintain protease/anti-protease balance (11). In the event of A1AT deficiency (A1ATD), the unrestrained NE, which is released from the neutrophil as part of the defense mechanism against infectious agents during inflammation, starts to break down elastin, collagen and major connective tissue components, thereby causing a decrease in the elasticity of the lung while leading to emphysema or chronic obstructive pulmonary disease (COPD) (12). Currently, the only treatment for patients with A1ATD consists of a life-time intravenous augmentation therapy where A1AT is purified from human blood donors and administrated to the patients weekly (13). However, there exist significant concerns related to this therapy, including batch-to-batch heterogeneity of plasma-purified product in terms of glycosylation and quality (13). Another issue is that commercial plasma purified A1AT is often in short supply, expensive and might harbor a risk, however very low, of pathogen transmission (14). Although many groups have successfully produced recombinant A1AT (rA1AT) in various prokaryotic and eukaryotic systems, there is currently no approved source of rA1AT for clinical use. This may be due in part to the difficulty in producing it with natural human glycosylation pattern, and/or in achieving productivity levels necessary for the commercial viability of the recombinant product. Thus, it would be highly beneficial to develop alternative A1AT production methods that can provide humanized glycosylation patterns crucial for the therapeutic success in order to alleviate plasma-derived A1AT shortages while reducing the risk of disease transmission and ensuring a batch-to-batch A1AT glycosylation homogeneity.

Hypothesis and Objectives of the Thesis

We hypothesized that genetically engineered CHO cell lines represent a reliable source for biologically active recombinant A1AT production due to their ability to provide humanized glycosylation pattern and high expression levels. As there is currently no recombinant source of A1AT, first objective of this thesis is to develop an oxidation-resistant recombinant version of A1AT with human glycosylation pattern expressed in glycoengineered CHO cells for therapeutic use. Second objective of this thesis is then to assess the effect of different A1AT sialylation on its cellular uptake by asialoglycoprotein receptors.

Author Contributions

Article 1 : *High-level Production of Wild-type and Oxidation-resistant Recombinant Alpha-1-antitrypsin in Glycoengineered CHO Cells*

Authors: Izel Koyuturk, Surbhi Kedia, Anna Robotham, Alexandra Star, Denis Brochu, Janelle Sauvageau, John Kelly, Michel Gilbert and Yves Durocher

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DOI: 10.1002/bit.28129.

My contribution involves the definition of the subject and its limits, the conceptualization and execution of the experiments, the analysis of the results and their presentation. I also produced the bibliography, wrote the manuscript and made the corrections requested by the reviewers.

Surbhi Kedia participated in the generation of stable CHO pools expressing A1AT under different promoters and contributed in the analysis of the results.

Anna Robotham and Alexandra Star participated in the analysis of glycans by LC-MS/MS.

Denis Brochu participated in the analysis of glycans by HILIC-UPLC under the supervision of Michel Gilbert.

Janelle Sauvageau supervised the analysis of the samples by HPAEC-PAD, proofread and corrected the manuscript.

John Kelly supervised the analysis of the samples by LC-MS/MS Analysis, proofread and corrected the manuscript.

Michel Gilbert supervised the analysis of the samples by HILIC-UPLC, proofread and corrected the manuscript.

Yves Durocher supervised the entire study, validated the subject, its limits, the results and their analysis. He proofread and corrected the manuscript before submitting it.

Article 2 : *Impact of A1AT sialylation on its cellular uptake by asialoglycoprotein receptor orthologs*

Authors: Izel Koyuturk, Melissa J. Schur, Michel Gilbert and Yves Durocher

Current Status of Article: Submitted to *Scientific Reports*

Same as before, my contribution involves the definition of the subject and its limits, the conceptualization and execution of the experiments, the analysis of the results and their presentation. I also produced the bibliography and wrote the manuscript.

Melissa J. Schur performed *in vitro* remodelling of Prolastin-C under the supervision of Michel Gilbert.

Michel Gilbert supervised the analysis of the samples by HILIC-UPLC, proofread and corrected the manuscript.

Yves Durocher supervised the entire study, validated the subject, its limits, the results and their analysis. He proofread and corrected the manuscript before submitting it.

Chapter 2: Literature Review

Biologics, Biosimilars, and Biobetters

Biologics are medications whose active ingredient is produced by or obtained from a living organism through techniques including recombinant DNA technology with regulated gene expression (15, 16). They have become conventional treatments for a number of malignancies, hematological, cardiovascular, autoimmune, retinal, and other rare genetic illnesses (16). Biologics are complex large molecule proteins having a somewhat narrow spectrum of indications for usage in relatively uncommon and frequently orphan illnesses. They also involve complex production procedures (17). These life-saving treatment techniques, which are generally patented and expensive, are frequently out of the financial reach of patients in many developing nations and dramatically raise the cost of healthcare in rich nations that use socialized medicine (18). In the US, for instance, biologics account for 38 % to 40 % of all pharmaceutical spending (19).

A biological product that is highly similar to the approved innovator product, without any clinically meaningful differences in safety, purity, and potency, is referred to as a "biosimilar" (15, 16, 19). Biosimilars, also called follow-on biologics, biogenerics, similar biologics, comparable biotherapeutic medicines, and generic biologics, provide generally cheaper, broadly accessible, and effective treatment choices while also enhancing access to healthcare (16, 18). Worldwide, the first biosimilar, somatropin (a recombinant human growth hormone), was approved in the European Union (EU) in 2006, whereas in the US the first biosimilar, filgrastim, a granulocyte colony-stimulating factor analogue, was approved in 2015 (20). The cost and production time of biosimilars are roughly one tenth of those of the innovator biologic (21). An animal toxicity assessment, a brief clinical study, and analytical verification of biosimilarity are vital determining factors whether a product is approved rather than independently proving the proposed product's efficacy and safety (22). The discovery stage, phase II study, and comprehensive clinical trials are skipped during the creation of a biosimilar because the therapeutic target is already known and the dosage, efficacy as well as side effects are similar to those of the originator medication (18).

As a result, the price of the biosimilar can be significantly reduced in a market that is already set up to use the innovator product, with a good chance of capturing market share and making a profit. However, the price difference between biosimilar and the innovator product is currently only 20-40% (18). There appears to be room to cut the cost of biosimilars from its present level. This issue should be fully covered by public policies. Since the introduction of biosimilars, patient access to biologic medicines has increased by as much as 100 % (20).

On the other hand, biobetters, also known as me-betters, are brand-new, highly potent biosuperiors developed from better scientific research and antibody technologies that improve upon existing, approved biologics in terms of potency, bioavailability, half-life, safety, immunogenicity, and efficacy across a wider range of indications (18). The main purpose of biobetters is to treat patients who are resistant to treatment, have relapsed, have difficult dosing regimens, or who have safety issues. As opposed to biosimilars, which must be price sensitive since they are not providing the patient with anything new or better, biobetters can demand a premium price as they are more effective than biosimilars or the reference molecule (23). However, reduced dosage, an improved half-life of the drug, and a longer shelf life should be able to offset this premium price, which is expected to result in a reduction in the overall cost of the treatment (23).

All biological therapies are subject to the inherent metabolic variability caused by post-translational modifications (PTMs), involving glycosylation, phosphorylation, deamidation, methylation, and acetylation (24). These PTMs can directly affect the biologic activity (potency), pharmacokinetics (PK), pharmacodynamics (PD), or immunogenicity of therapeutic proteins (25). Among them, glycosylation can be considered as the most complex PTM, which greatly influence the protein folding, stability, biological activity, immune responses as well as the serum half-life, and it is very difficult to manage due to its complexity and susceptibility to change dependent on process circumstances (26, 27). It is for this reason why protein glycosylation is typically considered as a critical quality attribute by the developer of biosimilars as well as biobetters, and thus will be our main focus for this work.

Protein Glycosylation

Protein glycosylation is the most complex and versatile co- or post-translational modification, which involves attaching one or more sugar chains called glycans to the polypeptide backbone through covalent glycosidic linkage to form glycoproteins (28). Glycans are generally classified into different groups based on the type of bond that attaches the sugar to the protein. For instance, while *O*-glycans are attached to the oxygen (O-) atom of the hydroxyl group of a serine (Ser) or threonine (Thr) residue, *N*-glycans are attached to the nitrogen (N-) of the amide group of an asparagine (Asn) residue of the protein. According to the SwissProt protein database, more than 50% of eukaryotic proteins are glycoproteins, with an estimated 90% of them likely to feature *N*-glycosylation (28-30). For glycoproteins with therapeutic value, *N*-glycosylation plays a vital role in regulating various aspects such as pharmacokinetics, immunogenicity, bioactivity, solubility, stability as well as protein folding, and therefore will be our main focus (25).

N-glycosylation

N-linked glycosylation is initiated as a co-translational event during protein biosynthesis. All eukaryotic *N*-glycosylation starts in the endoplasmic reticulum (ER) and continues throughout the Golgi apparatus (31). As shown in Figure 1, the synthesis starts with the formation of dolichol phosphate-linked precursor consisted of five mannoses (Man) and two N-acetylglucosamines (GlcNAc) residues on the cytoplasmic side of the ER. This lipid precursor is then flipped to face the luminal side of the ER, where four additional Man residues and three glucose (Glc) units are added to form a 14-sugar structure. The $Glc_3Man_9GlcNAc_2$ glycan precursor is then transferred en bloc onto a nascent protein at an Asn-X-Ser/Thr site (where X is any amino acid except proline) by oligosaccharyltransferase (OST) (32). Following the formation of the carbohydrate–protein conjugate, it undergoes an additional modification in the ER, where three glucoses are sequentially removed by α-glucosidases (α-Glc) and one mannose is cleaved by the ER α-mannosidase (ER α-Man). After a quality-control checkpoint, the glycoprotein bearing $Man_8GlcNAc_2$ moves to the *cis*-Golgi for further trimming of mannose residues by a series of α-mannosidases (α-Man) prior to being transferred to the *medial*-Golgi for maturation. It is then *medial*- and *trans*-Golgi compartments that are responsible for the production of hybrid and

complex type *N*-glycans through the addition of GlcNAc, galactose (Gal), sialic acid (Sia), and fucose (Fuc) sugars (32).

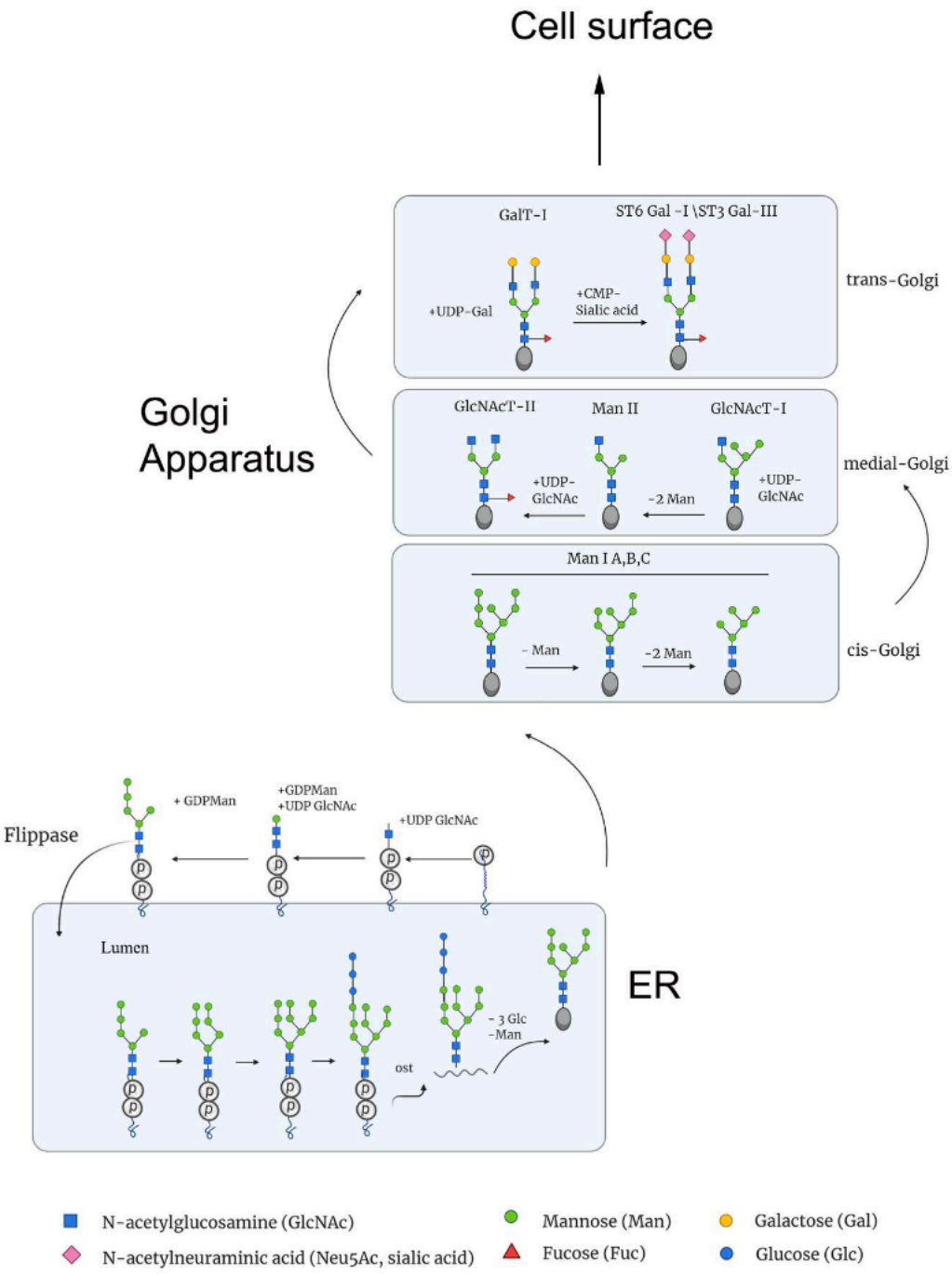

Figure 1. Overview of *N*-glycan biosynthesis in human (Figure adapted from Viinikangas *et al.*, 2021 (33)).

O-glycosylation

Compared to *N*-glycosylation pathway, *O*-glycans have a far less clear mechanism of association with proteins. Although *O*-glycosylation can occur on amino acids with functional hydroxyl groups, which are most often Ser and Thr residues, there is no shared glycan core structure (32, 34). As a result, various kinds of *O*-linked glycosylation can occur through the initial monosaccharide linkage of Man, Gal, Fuc, Glc, xylose, or GlcNAc, which are typically initiated in the Golgi apparatus as a post-translational event.(34). However, *N*-acetylgalactosamine (GalNAc)-linked *O*-glycans, also known as mucin-type *O*-glycans, are the most common *O*-glycans that are abundant on many extracellular and secreted glycoproteins, including mucins, which form a crucial interface between epithelial cells and the external mucosal surfaces of the body (32). The first step of this mucin-type *O*-glycan biosynthesis is catalyzed by a family of approximately 20 GalNAc transferases, which add GalNAc (from UDP-GalNAc) onto coil, turn, and linker regions of the folded protein (34). The specificity of these GalNAc transferases for amino acid motifs varies, although they are frequently promiscuous, which regulates the location and manner in which *O*-glycans are attached (32). Extensions of this initial GalNAc residue can then generate eight different core structures, where four of them typically extend further stepwise by a number of glycosyltransferases, leading to heterogeneity, including topology, from linear to extensively branched potential glycan structures (34).

Types of *N*-linked glycans in human

All eurkaryotic *N*-glycans share a common pentasaccharide core structure comprised of two GlcNAc and three Man residues. Based on the nature of additional monosaccharides linked to the core structure, *N*-linked glycans can be categorized into three subgroups: high-mannose, complex-type and hybrid (Figure 2) (35) While high-mannose *N*-glycans only contain mannose residues that are attached to the core structure, complex *N*-glycans are characterized by the attachment of two or more GlcNAc residues to each of the mannoses residues of the core structure. Depending on the number of the branch or antenna coming off the terminal mannose of the core structure, complex-type *N*-glycan can exist in bi-, tri- and tetra-antennary forms. Each of these antennae consist of, in order, GlcNAc, Gal and *N*-acetylneuraminic acid (Neu5Ac), which

is the most prevalent type of sialic acid in mammals (36). Finally, the hybrid type combines the structural characteristics of both high-mannose and complex type *N*-glycans, in which only mannose residues are attached to the Manα1–6 arm of the core structure, and Manα1–3 arm is extended by a complex-like GlcNAc residue. As shown in Figure 2, complex and hybrid type glycans can be further modified by adding fucose sugar to the asparagine linked GlcNAc residues of the glycan core and/or to the antennae, as well as by attaching a β1,4-GlcNAc residue to the trimannosyl core, resulting in bisecting GlcNAc.

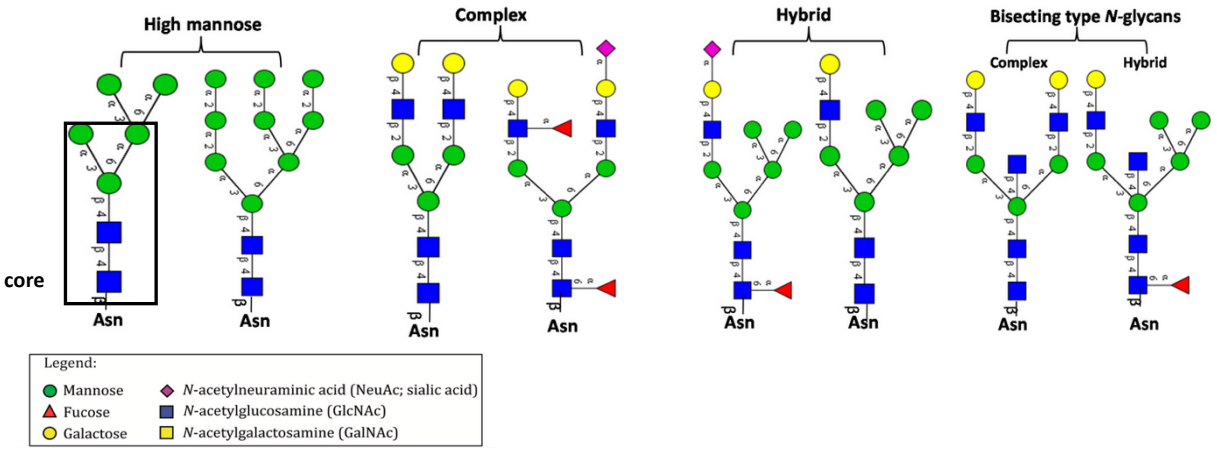

Figure 2. *N*-glycans structures of high-mannose, complex and hybrid type (Figure adapted from Sethi and Fanayan, 2015 (37), with minor modifications).

Fucosylation

Fucosylation, or the addition of fucose sugar to glycoforms, can be catalyzed by both de-novo and salvage pathways (Figure 3). In the first pathway, guanosine diphosphate (GDP)-mannose is produced by the absorption of D-glucose into the cytoplasm. GDP-mannose is then converted into GDP-fucose with the help of GDP-mannose4,6-dehydratase and GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase enzymes (38). The salvage pathway, on the other hand, uses L-fucose from extracellular and lysosomal sources. L-fucose is first phosphorylated to L-fucose-1-phosphate by fucokinase, and then GDP-fucose pyrophosphorylase (GFPP) converts L-fucose-1-phosphate into GDP-fucose. Following transportation of GDP-fucose to the Golgi apparatus by the GDP-fucose transporter, fucose is finally added to the glycan chains of

developing glycoproteins by fucosyltransferases, including α-1,6-fucosyltransferase (FUT8) for the *N*-glycan core fucosylation, which are crucial targets for cell engineering since fucosylation can strongly affect antibody-dependent cell-mediated cytotoxicity (ADCC) (39).

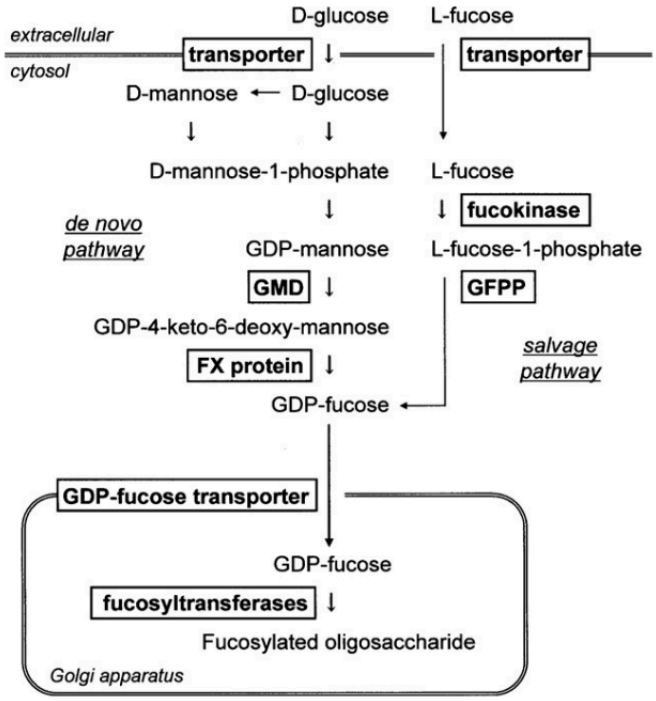

Figure 3. Fucosylation pathways in mammals (Figure adapted from Kanda *et al.*, 2007 (38))

Sialylation

Sialic acids (Sia), a large family of neuraminic acid derivatives, are negatively charged monosaccharides with a nine-carbon backbone. The numbering of the carbon atoms, C, starts at the carboxylate carbon and continues along the chain, as demonstrated in Figure 4. *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) are the two most common members of the Sia family in mammals, which differ by the presence of an extra hydroxyl group at C5 (40). Although Neu5Gc is a major Sia in most mammals, humans are genetically unable to produce Neu5Gc due to a deleterious mutation in the *CMAH* gene that codes the enzyme converting Neu5Ac to Neu5Gc (41). However, metabolic incorporation of small amount of Neu5Gc still occurs in humans as it can be obtained through dietary sources (42).

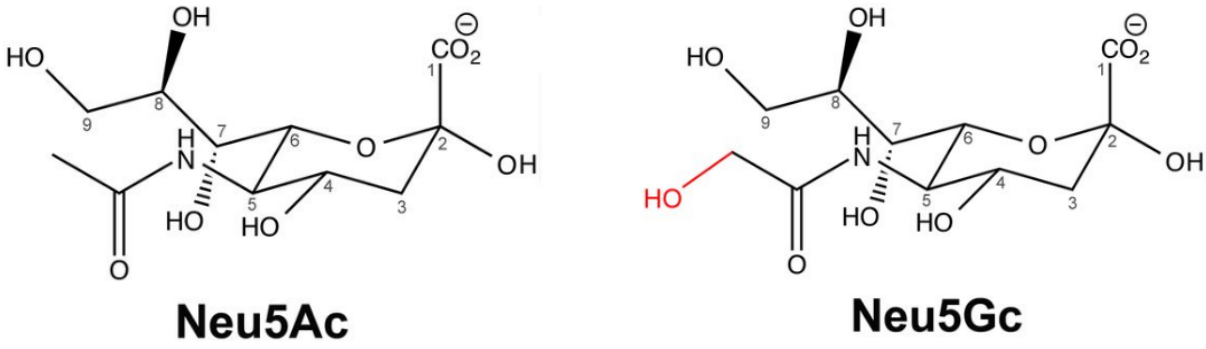

Figure 4. Chemical structure of Neu5Ac and Neu5Gc (Figure adapted from Stehle and Khan, 2014 (43)).

Sialylation refers to the terminal addition of sialic acid units to oligosaccharides and glycoproteins. Depending on the nature of the sialyltransferase involved in the transfer, sialic acids are attached to *N*-linked glycans at their Gal or *N*-acetylgalactosamine (GalNAc) residues by α-2,3- or α-2,6-linkage, or to other sialic acid moieties via α-2,8-linkage (Figure 5) (44).

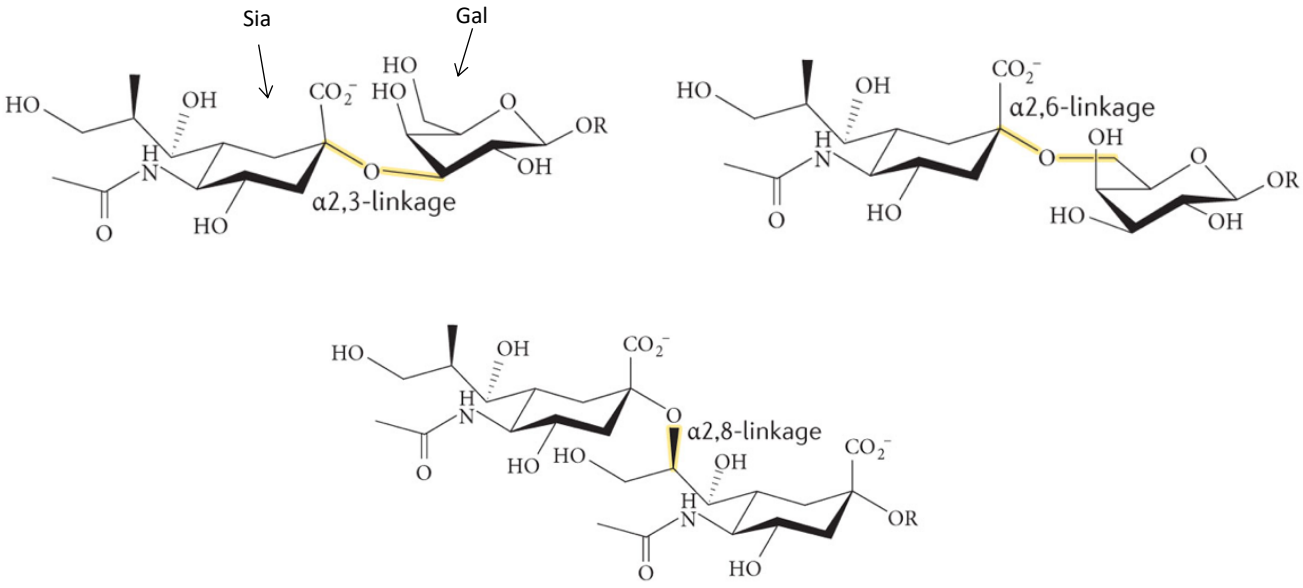

Figure 5. Different type of sialic acid linkages (Figure adapted from Stencel-Baerenwald *et al*., 2014, with minor modifications (45)).

Biological Importance of Sialylation

The presence of terminal sialic acid residues has a great impact on structure and function of glycoproteins and they play a crucial role in many physiological processes, including cell-cell recognition, communication, cell signaling and reproduction (36). Sialylation contributes to the functionality of hormones, such as follicle-stimulating hormone and human chorionic gonadotropin, by stabilizing them (46). Due to their hydrophilicity and negative charge, sialic acids contribute the biophysical features of several biological systems, including stabilization of erythrocytes and prevention of blood component aggregation (47). They also function as ligands for glycan-binding proteins, such as animal lectins (siglecs and selectins), virus lectins (hemagglutinins), and bacterial lectins (adhesins and toxins). The interactions of sialic acids with siglecs and selectins have been well-documented. The regulation of the immune response, immune cell functions, cell proliferation, and cell survival depends critically on the interactions of sialic acid-containing carbohydrates, or sialosides, with lectins from the siglec family (46). In contrast, sialic acids on the host cell also represent target structures for viral and bacterial lectins to establish infection.

While sialylation has many crucial roles in biological processes, they are also involved in tumor progression as well as immune escape. Aberrant levels and patterns of sialylation on cell surfaces are often associated with cancer. Particularly, tumor tissues exhibit hypersialylation more frequently than corresponding normal tissue because of abnormal expression of sialyltransferases (44). This overabundance of sialic acids is a key contributor to the progression of tumor and metastasis and is therefore an important target for cancer diagnosis and treatment. In addition, sialylation also facilitates cancer cells to evade immune monitoring, where highly coated sialyl glycans on tumor cells interact with siglecs to avoid immune surveillance (48).

More importantly, sialic acids are critical factors determining the half-life of glycoproteins in circulation. Masking the penultimate Gal or GalNAc of the *N*-glycan, sialic acid prevents glycoproteins from being recognized by asialoglycoprotein receptor (ASGPR), which is a lectin in hepatocytes that recognizes terminal Gal or GalNAc on non-sialylated glycoproteins and rapidly

eliminates them from the blood circulation. This last point will be explained in more details in the "Asialoglycoprotein Receptor" section.

Human Sialyltransferases

In human, sialylation of *N*-glycans is accomplished by twenty sialylatransferases (ST), which catalyze the reaction that transfer sialic acid residue onto a structure terminated with Gal or GalNAc residues via α-2,3- or α2,6-linkage, or onto another sialic acid through α-2,8-bond to form polysialic acid (PSA) (49). According to the type of linkage formed and the nature of the sugar acceptor, they are classified into four groups, including six β-galactoside α-2,3-sialyltransferases (ST3Gal I-VI), two β-galactoside α-2,6-sialyltransferases (ST6Gal I-II), six GalNAc α-2,6-sialyltransferases (ST6GalNAc I–VI), and six α-2,8-sialyltransferases (ST8Sia-I–VI). Among them, ST3Gal III and IV, along with ST6Gal I use type II disaccharide (Galβ1-4GlcNAc), which is of our interest, as an acceptor substrate on *N*-glycans to catalyze the formation of different types of glycosidic linkages. On the other hand, ST8Sia catalyze the addition of Sia residues in a α-2,8-linkage on the α-2,3-, α-2,6- or α-2,8-sialylated glycoproteins, which are found almost exclusively in the brain (50). Therefore, we will be focusing only ST3Gal III, ST3Gal IV and ST6Gal I for the rest of this section.

α-2,3-sialyltransferases (ST3) catalyze the covalent linkage between C2 of the Sia and C3 of the preceding Gal residue in a α-2,3-linkage (Figure 4). ST3Gal III and IV are broadly expressed in mammals. Although ST3Gal III acts preferentially on type I disaccharide (Galβ1-3GlcNAc), it can also catalyze the sialylation of type II disaccharide (Galβ1-4GlcNAc), however with lower catalytic efficiency. It has been demonstrated that the *ST3Gal III* gene is substantially expressed in skeletal muscle but not in the placenta, indicating the possibility of the existence of a second enzyme with a similar selectivity (51). Indeed, ST3Gal IV is found to be abundantly expressed in placenta (52). Unlike ST3Gal III, ST3Gal IV uses preferentially Galβ1-4GlcNAc disaccharide as an acceptor to add Sia residues on either glycolipids or glycoproteins.

Similar to ST3Gal IV, ST6Gal I shows a preferential activity towards type II disaccharide on *N*-glycans and is unable to use type I disaccharide as an acceptor substrate. As a result, it competes with ST3Gal IV for the same disaccharide Galβ1-4GlcNAc acceptor substrate (53). In

contrast to α-2,3-sialyltransferases, ST6Gal I transfer Sia in a α-2,6-linkage, which is a more common bond found in plasma glycoproteins (54). Interestingly, it has been previously shown that terminal Gal residue on the α-1,3-Man branch of the complex-type *N*-glycans is sialylated more efficiently than the Gal moiety on the α-1,6-Man branch by ST6Gal I (55). However, the reason for this selectivity is still unclear.

Metabolic Pathway of Sialic Acids

In addition to sialyltransferases, sialic acids are another key component in the biosynthesis of sialic acid-containing oligosaccharides and glycoproteins. Sialic acid metabolic pathway requires the collaborative action of specific enzymes that catalyze the biosynthesis, activation, and transfer of sialic acids to glycoproteins. In humans, sialic acid synthesis starts in the cytosol involving three enzymes in a four-step process (Figure 6) (48) . The first two steps are catalyzed by a bifunctional enzyme called UDP-GlcNAc 2-epimerase/ManNAc-6-kinase (GNE), where uridine diphosphate GlcNAc (UDP-GlcNAc) is converted to *N*-acetylmannosamine (ManNAc), followed by ManNAc-6-phosphate (ManNAc-6-P). The latter is then transformed to Neu5Ac with the help of Neu5Ac 9-phosphate synthase (NANS) and Neu5Ac-9-phosphate phosphatase (NANP), respectivey. The Neu5Ac synthesized in the cytosol is subsequently transferred to the nucleus in order to get activated by the cytosine 5'-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) synthetase (CMAS) to form CMP-Neu5Ac. Finally, activated CMP-Neu5Ac leaves nucleus and is transported by CMP-sialic acid transporter (CST) into Golgi apparatus to be used by sialyltransferases for the synthesis of glycoproteins, which are subsequently secreted or delivered to cell surface. Additionally, in some organisms, CMP-Neu5Ac is converted to CMP-Neu5Gc by CMP-Neu5Ac hydroxylase, which is inactive in humans as mentioned previously.

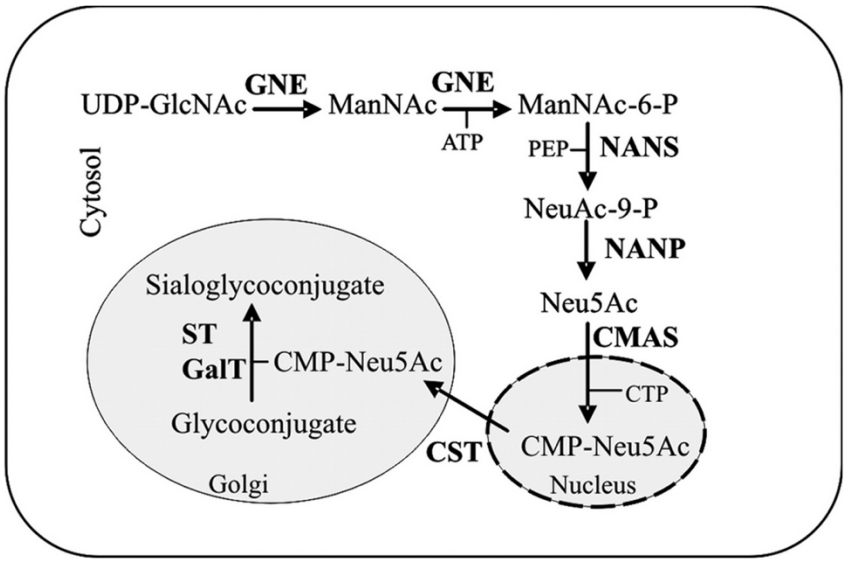

Figure 6. Metabolic pathway of sialic acids in human (Figure adapted from Castilho *et al*., 2010 (56)).

Alpha-1-antitrypsin as a Glycoprotein Model

Expression Pattern

Alpha-1-antitrypsin is one of the most abundant serum serine protease inhibitor that belongs to the serpin superfamily, which is the largest superfamily of protease inhibitors involved in many critical biological processes, including blood coagulation, fibrinolysis, programmed cell death, development and inflammation (57). A1AT is mainly secreted into circulation by hepatocytes. However, it can be also produced locally, to a much lesser extent, by monocytes, macrophages, pulmonary alveolar cells and intestinal epithelial cells (58-60). In healthy individuals, A1AT is circulating in the blood with a physiological concentration and half-life of 20–48 µM (1.5-3.5 g/L) and 3-5 days, respectively (61, 62). This concentration can increase rapidly up to 2–5 fold during acute phases of inflammation and infection (63). Only a small portion of the circulating A1AT is delivered into any given body compartment while one-third of circulating A1AT gets degraded daily (64). Thanks to its small size and polar properties, A1AT diffuses easily into most tissues, including lung and kidney, and is present in almost all body fluids, such as saliva, tears, breast milk, urine, semen and bronchoalveolar lavage fluid, in which A1AT concentration is

roughly equal to that in serum (65, 66). In the event of inflammatory bowel disease, A1AT can also be detected in higher concentrations in faeces. Although local synthesis may influence A1AT concentration in these tissues and body fluids, it is generally believed that in each case, A1AT is obtained from serum.

Gene Structure and Variations

A1AT is encoded by *SERPINA1* gene, which is ~12.2 kb in length and located on the long arm of human chromosome 14q31–32.3 (64). It is organized in seven exons and six introns (67). These exons are numbered as Ia, Ib, Ic, II, III, IV, and V, where Ia and Ib contain promoter regions for monocytes and macrophages, whereas Ic bears specific promoter for hepatocytes, resulting in variability of *SERPINA1* transcripts (68). The first three exons plus a short 5′ fragment of the fourth exon give rise to the 5′ untranslated regions of A1AT. Most of the fourth exon (exon II) along with remaining three exons (exons III, IV, and V) code for the protein sequence of A1AT, where the active site of the mature protein is encoded by the seventh exon (exon V). Different from hepatocytes, primary transcript in macrophages contains the first two exons and a short 5′ segment of the third exon, leading to a slightly longer mRNA transcript (69).

SERPINA1 gene has two alleles at a single locus, which are transmitted by autosomal codominant inheritance where each allele contributes to the phenotype (70). It is highly polymorphic with approximately 123 single nucleotide polymorphisms (SNPs) listed, resulting in over 100 genetic variants, which can affect both protein levels and activity of the protein (71, 72). The variants are categorised in alphabetical order based on the PI, or protease inhibitor, classification system as determined by differences in electrophoretic migration at acidic pH. Differences in this migration pattern depend primarily on variations in protein charge resulting from amino acid alterations. While the most common normal A1AT variant, designated “M”, results in a protein with a medium rate of migration, “Z” being the most common deficiency allele migrates further under the same conditions. When an individual inherits one copy of the M allele and one copy of the Z allele, it would be then referred to as “Pi*MZ” according to PI system. Compared to M variant, which are associated with serum concentration and activity of A1AT within the normal range, individuals who are Pi*MZ heterozygotes have a moderately reduced

level of alpha-1 antitrypsin in their blood (73). In contrast, Pi*Z individuals are severely deficient in A1AT, which is associated with clinical disease, with a serum concentration equivalent to 10-15% of that of the normal individuals due to a point mutation (Glu342Lys) resulting in an accumulation of the misfolded protein in hepatocytes that causes A1AT deficiency in the circulation (64). Another most common variant associated with deficiency is designated “S” (50-60% of normal concentration), however, it is generally considered to be less severe than the Z variant. In addition to deficiency variants, a dysfunctional variant of A1AT (Pittsburgh) has been identified, in which a single amino acid substitution at its active site (Met to Arg at residue 358) converted A1AT from an elastase inhibitor to a thrombin inhibitor. Finally, some individuals inherit null alleles resulting in protein levels that are not detectable in serum, and they seem to have worse lung function than Pi*Z subjects.

Protein Structure

The translation of A1AT results in a total of 418 amino acids, which includes signal peptide of 24 amino acids at *N*-terminus (74). With an overall molecular weight of 52 kDa, the fully processed, mature A1AT protein consists of 394 amino acids with three carbohydrate side chains. It has one cysteine (Cys) and nine methionine (Met or M) residues, which are potentially available for interaction with oxidants. Crystallographic analysis revealed that A1AT is a globular, highly ordered protein possessing a tertiary structure of nine α-helices, three β-pleated sheets and a mobile reactive center loop (RCL), which encompasses residues 344–368 and is responsible for the functional capacity of the inhibitor as well as its specificity (Figure 7) (13). Three internal salt bridges are implicated in protein folding and polymerization. In contrast to majority of proteins, A1AT is naturally folded in a metastable structure (75). As this is not the most stable form thermodynamically, the native protein is susceptible to conformational changes and aggregation. Similar to other serpin members, A1AT can intramolecularly transform into an inactive, more stable latent state, though biological activity can be recovered through denaturation and refolding.

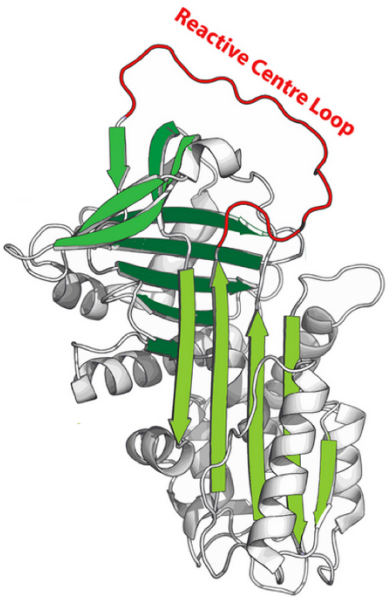

Figure 7. Metastable structure of A1AT (Figure adapted from Maas and de Maat, 2021 (76)).

β-*sheets are highlighted in green, α-helices are shown in grey and the reactive center loop (RCL) is highlighted in red.*

Function of A1AT

Anti-protease Activity

The main biological function of A1AT is to block the activity of serine proteases, including trypsin, proteinase 3, cathepsin G, but more importantly neutrophil elastase (NE) in the lung, to maintain protease/anti-protease balance (77). During inflammation, neutrophil elastase, a highly active proteolytic enzyme, is released by activated neutrophils as part of the defense mechanism against infectious agents and aids in the degradation of pathogens as well as products of inflammation (78). Under normal conditions, A1AT acts as a suicide inhibitor and inhibits the NE and other serine proteases at its RCL whose peptide sequence, more specifically methionine (Met) 358 residue, serves as a pseudo-substrate for the serine proteases (57, 79), as depicted in Figure 8. Following the interaction of two molecules, A1AT forms a rapid and strong association with NE having a binding strength several orders of magnitude greater than other serine proteases (65). Target protease then cleaves the RCL of A1AT at Met 358- serine (Ser) 359, releasing stored potential energy and allowing a conformational change of both molecules where

the RCL is inserted as an additional strand within the central β-sheet of A1AT while irreversibly trapping and dragging the target protease to the opposite end of the serpin molecule by 70 Å (80). Because of this translocation, the active site of the serine protease gets distorted and catalytically inactivated. Moreover, a novel binding pentapeptide domain is exposed in the carboxyl terminal end of A1AT due to cleavage of the RCL at Met 358 (81). The resulting inactivated A1AT-protease complex is subsequently recognized and cleared from the circulation through engagement of the newly exposed binding site with hepatocyte serpin enzyme complex (SEC) receptors (82).

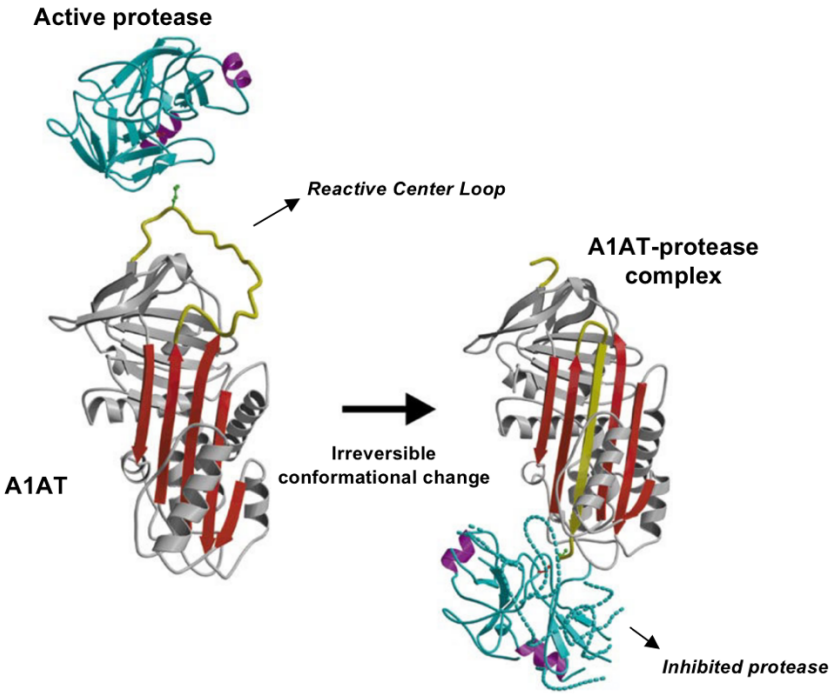

Figure 8. Mechanism of inhibition of an active protease by A1AT (Figure adapted from Huntington *et al.*, 2000 (83), with minor modifications).

A1AT-β-sheets are shown in red, reactive center loop in yellow.

Other Activities

In addition to its primary role in protease inactivation, A1AT has broader biological functions, involving stimulation of fibroblast growth and procollagen synthesis (84), upregulation of human B cell differentiation into immunoglobulin E- (IgE) and IgG4-secreting cells (85),

inhibition of apoptosis through interaction with the proteolytic cascade of enzymes (86, 87), prevention of human immunodeficiency virus type 1 (HIV-1) viral cell entry (88) and suppression of bacterial proliferation during infections (89). Moreover, independent of its anti-protease activity, A1AT has been shown to exhibit both anti-inflammatory and immunomodulatory activities, such as inhibition of cytokine production (e.g. IL-8, TNF-alpha) (90, 91), ability to regulate neutrophil chemotaxis (92) and activation of phosphatases to abrogate inflammatory responses in the lung (93). Finally, a recent study identified A1AT as a new inhibitor of transmembrane serine protease 2 (TMPRSS2), an endogenous serine protease that is involved in the cellular entrance of various coronaviruses, including severe acute respiratory syndrome (SARS)-coronavirus (CoV)-2, as it blocks SARS-CoV2 cellular entrance and prevents the primary clinical consequences of severe COVID-19, such as acute inflammation and acute respiratory failure (94).

Post-Translational Modifications of Circulating A1AT

N-Glycosylation of A1AT

Human A1AT is a glycoprotein with a ~15% carbohydrate content by weight (95). It is *N*-glycosylated at three Asn residues (Figure 9), where Asn 46 and 83 are encoded within exon II, and Asn 247 encoded within exon III. Due to multiple parameters including genetic regulation, availability of nucleotide sugars as well as the time spent in ER and Golgi, different glycan groups at these three sites can be observed in healthy individuals (96). When *N*-glycans released from A1AT were analysed using matrix assisted laser desorption/ionization (MALDI)-time-of-flight (TOF)-mass spectrometry (MS), the results leads to the detection of mostly bi- and tri-antennary complex type species, which have a tri-mannose fork core containing GlcNAc, Gal and a terminal negatively-charged Neu5Ac (97). Due to the different numbers of sialic acids residues on the *N*-glycans, isoelectric focusing further revealed eight different charge isoforms of A1AT (numbered M1 to M8), with isoforms 4 (M4) and 6 (M6) being the most abundant ones. While M4 isoform contains di-sialylated bi-antennary (A2G2S2) and tri-sialylated tri-antennary (A3G3S3) complex-type N-glycans structures in a molar ratio of 2:1, M6 isoform is mainly occupied with A2G2S2 complex-type glycans (see annex 2 for the nomenclature of *N*-linked glycan structures). More

precisely, site-specific analysis of A1AT *N*-glycans using liquid chromatography with tandem MS (LC-MS/MS) showed that Asn 46 and Asn 247 sites predominantly contain α-2,6-di-sialylated bi-antennary structures, A2G2S2 (6,6), (91.3 and 99.3 %, respectively), whereas core-fucosylated glycans, FA2G2S2 (6,6), is less abundant (8.6 and 0.7 %, respectively) (98). On the other hand, Asn 83 site exhibits most variation of complex-type *N*-glycan structures, bearing A2G2S2 (6,6) (52.5 %) species with possible core-fucosylation (1.5 %) as well as α-2,3-6-tri-sialylated tri-antennary species, A3G3S3 (6,3,6) (29.5 %) with possible antennary fucosylation, A3FG3S3 (6,3,6), (16.7 %). In contrast to bi-antennary glycans, which contain α-1,6-linked core-fucosylation, tri-antennary structures present a Lewis^X (Le^X)-type fucose on the β-1,4-linked GlcNAc of the α-1,3-arm. In addition to bi- and tri-antennary glycan profiles, very little tetra-sialylated tetra-antennary structures were also detected with potential antennary fucosylation in Asn 83 site.

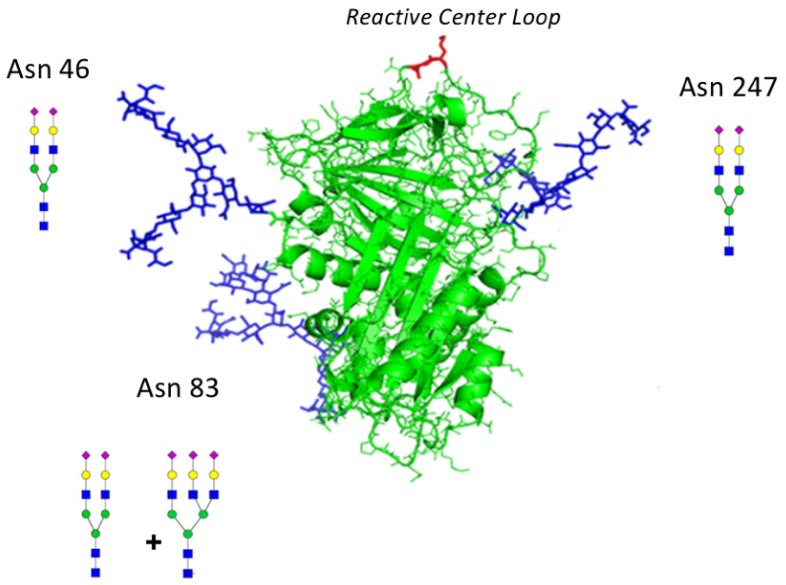

Figure 9. Molecular model of glycosylated A1AT and its corresponding *N*-glycan structures (Figure adapted from McCarthy *et al*., 2014, with minor modifications (99)

S-Nitrosylation of A1AT

Proteins can be nitrosylated by S-nitrosoglutathione or reactive nitrogen species directly interacting with protein thiol residues to attach covalently a nitric oxide (NO) group to cysteine

(Cys) residues (100). In the inflammatory environment, the sole Cys residue in A1AT (Cys232) is readiy S-nitrosylated (S-NO-A1AT) without affecting its anti-protease activity (101). Although two enzymes, involving S-nitrosoglutathione reductase and thioredoxin reductase, are responsible for the reverse process (protein de-nitrosylation), the S-NO-protein can also self-induce de-nitrosylation by transferring the NO molecule to cellular targets with free thiols, as seen in S-NO-A1AT exhibiting bacteriostatic activity enabled via transnitrosylation (101). In addition, Kaner *et al.* reported that S-NO-A1AT triggers a considerably higher anti-bacterial response in macrophages compared to native A1AT (102). During infection, A1AT becomes S-nitrosylated to reduce the bacterial burden through inducing an anti-bacterial and a pro-inflammatory phenotype in resting macrophages, including the production of inflammatory cytokines and the activation of inducible nitric oxide synthase. The transfer of NO from S-NO-A1AT to a free Cys residue on cellular targets is thought to provide this pro-inflammatory action. However, the precise amount of S-NO-A1AT created *in vivo*, as well as the ratio between A1AT/S-NO-A1AT at infection site, is still not known.

Carbamylation of A1AT

Protein carbamylation is a PTM that is influenced by inflammation, nutrition, smoking, and environmental variables in addition to exposure to the urea dissociation product cyanate (103). The addition of a "carbamoyl" moiety (-$CONH_2$) to α-amino groups of proteins or to ε-amino groups of Lys residue side chains is the end result of the carbamylation reaction (104). The 34 lysine residues within the A1AT protein thus serve as an ideal target for carbamylation. Notably, Verheul *et al.* reported autoantibodies against the carbamylated form of A1AT in the synovial fluid of the patients with rheumatoid arthritis (105). These antibodies may be able to predict the development of rheumatoid arthritis in patients since they can be observed years before the disease ever manifests. As a result, the development of carbamylated A1AT as an antigenic biomarker for rheumatoid arthritis is currently ongoing.

Oxidation of A1AT

Additionally, the A1AT molecule can have several other potential modifications including methionine oxidation. Met residues within A1AT are prone to oxidation by both endogenous

sources, involving activated inflammatory cells, or exogenous sources, such as air pollution and cigarette smoke (106). Notably, oxidation of active site, surface exposed Met 351 and 358 residues found on RCL (essential for the formation of the proteinase-inhibitor complex) to methionine sulfoxide leads to inactivation of A1AT, which results in significant loss of inhibitory activity of A1AT against neutrophil elastase (NE) (107). Since oxidized A1AT remains in the bloodstream in a functionally inactive form, it fails to promote further upregulation of A1AT synthesis (81). Subsequently, the imbalance between protease/anti-protease, influenced by the oxidation of A1AT, in the lungs causes enzymatic degradation of lung connective tissues and ultimately chronic obstructive pulmonary disease (COPD) development, which will be explained in more details in "A1AT Deficiency" section. In comparison to non-smokers with COPD or healthy controls, smokers with COPD were shown to have higher serum levels of oxidized A1AT (108). Furthermore, bronchoalveolar lavage fluid from smokers, but not from non-smokers, contained partially inactivated oxidized A1AT, which is the major determinant of an accelerated decreased functional activity of lung A1AT (109). However, previous experiments using site-directed mutagenesis to replace M351 and/or M358 with valine (V) residues, revealed maintenance of anti-elastase activity under oxidative stress, indicating a potential novel approach for treating COPD (107, 110, 111). Interestingly, despite losing its elastase inhibitory effect, oxidized A1AT still retains some anti-inflammatory characteristics by modulating other inflammatory mediators. Churg *et al.* showed that administration of oxidized A1AT in a smoke-exposed mouse-model decreased neutrophil influx and lowered plasma tumor necrosis factor α (TNFα) levels, which partially prevented the development of emphysema (112). Collectively, the current findings imply that oxidative changes reduce A1AT's ability to inhibit serine proteases while preserving some of its anti-inflammatory effects. As a result, A1AT without anti-protease activity can effectively provide anti-inflammatory functions. This suggests that A1AT's anti-inflammatory and anti-protease properties can be completely independent of one another.

Biological Importance of A1AT Glycosylation

Although glycosylation is not required for the anti-protease activity of A1AT as opposed to its immune-modulatory functions, it is crucial for its stability, correct protein folding, interaction with other proteins, and in particular for its *in vivo* half-life (13, 99, 113). Despite

retaining its anti-elastase activity, recombinant non-glycosylated A1AT protein produced by bacteria exhibits a significantly shorter half-life when compared to plasma-derived glycosylated A1AT, and is, therefore, therapeutically ineffective for intravenous augmentation therapy (114, 115). Notably, terminal sialic acid residue found on native A1AT is known to contribute to an extension of A1AT serum half-life through preventing its interaction with ASGPR (116), which will be explained in more details in the "Asialoglycoprotein Receptor" section. In addition, lack of glycosylation led to an increase in aggregation of the *Escherichia coli (E.coli)* expressed A1AT upon heat deactivation (117) or incubation with denaturant solution (118), demonstrating the importance of glycosylation on the stability of A1AT. Finally, appropriate glycosylation has been shown to prevent A1AT from polymerization as well as from proteolysis by masking protease cleavage sites, and therefore prolongs the circulation time of the protein (99).

A1AT Deficiency

When the serum concentration of A1AT drops below 11 μM, it leads to a clinical disease associated with A1AT deficiency (A1ATD), which is a genetic disorder that affects approximately 3.4 million individuals worldwide (13, 71). Although A1ATD was first reported in the 1960s, it is underrecognized by the medical community. Respiratory and hepatic diseases are among the main clinical manifestations of A1ATD (Figure 10). Individuals who are homozygous for the mutant Z allele or Pi*SZ are at risk for the development of liver disease due to accumulation of abnormally folded Z protein, which cannot be secreted out from ER, thereby resulting in retention within the hepatocyte (119). This misfolded protein then forms polymers that cause ER stress, hepatocyte damage, fibrosis and ultimately cirrhosis. In addition to hepatic disease, there is also an association of the Z variant of A1AT with asthma (120), pancreatitis (121) and vascular aneurysms (122). However, here, we will be primarily focused on respiratory manifestation related to A1AT deficiency.

Along with the liver, the lung is another most commonly affected organ in A1AT deficiency, in which chronic obstructive pulmonary disease (COPD), more specifically pulmonary emphysema, is the common respiratory manifestation due to an imbalance between protease and inhibitor (78). In the event of A1ATD, unrestrained NE starts to break down elastin,

fibronectin along with collagen fibers in the extracellular matrix component of the lung, thereby causing a decrease in the elasticity of the lung as well as lung parenchyma degradation and disruption of the epithelial barrier, which ultimately leads to emphysema and chronic inflammation (123). Early symptoms comprise dyspnea, which is experienced by 84 % of patients, exercise intolerance, chronic cough and wheezing (72). Therefore, A1AT deficiency is commonly misdiagnosed initially as asthma. When diagnosing patients with A1AT-deficiency-associated COPD, occupational and environmental exposures, including smoke and dust must be taken into consideration. Importantly, the clinical effects of A1ATD are significantly impacted by smoking since it worsens the imbalance between protease and anti-protease by inactivating A1AT. Nearly all patients who present earlier are smokers, and quitting smoking can largely stabilise the disease. The severity of COPD can be assessed by pulmonary function tests, involving spirometry, lung volumes, and carbon monoxide diffusing capacity (124).

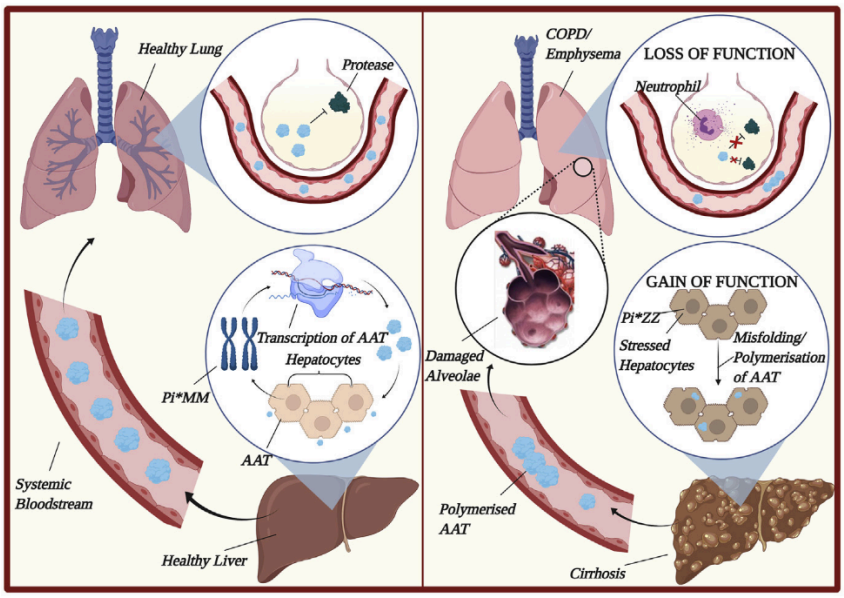

Figure 10. Pathogenesis of A1ATD-related disease. (Figure adapted from Remih *et al.*, 2021 (125))

Left panel presents individuals without A1ATD, right panel shows individuals with A1ATD.

Development of Therapies for A1ATD

Augmentation Therapy

Currently, the only approved treatment for patients with A1AT deficiency is to receive weekly intravenous injection of plasma-purified A1AT in order to replace the missing or deficient A1AT protein in the blood while slowing down the progression of pulmonary emphysema, reducing exacerbations and enhancing quality of life (13). This augmentation therapy is only intended for patients with pulmonary emphysema and has no effect on liver disease associated with this deficiency. As shown in Table 1, various products, which differ in purity and activity, are approved and accessible in the US and EU. According to a study that compared four plasma-derived A1ATs commercially marketed in the EU, Respreeza® has the highest level of purity and the most specific activity (126).

The augmentation therapy was first approved by the US Food and Drug Administration (FDA) in 1987 (127). Both European Respiratory Society (ERS) and American Thoracic Society (ATS) recommend the augmentation therapy at a dose of 60 mg/kg of body weight to maintain a threshold level of A1AT (11 µM), which has been calculated using the plasma levels of individuals who are heterozygous for Z-mutant A1AT and do not develop emphysema (72, 78). Without considering its efficacy, the therapy was approved based on the pharmacokinetics of A1AT. The Randomised, Placebo-controlled Trial of Augmentation Therapy in Alpha 1 Proteinase Inhibitor Deficiency (RAPID) programme provided the first conclusive evidence of the clinical efficacy of A1AT therapy and emphasised the significance of early A1AT deficiency detection and treatment to preserve functional lung tissue while decreasing the severity and frequency of lung infections (128). Higher doses and longer intervals (150 or 180 mg/kg every 3 weeks, 250 mg/kg every 4 weeks) have also been tested, however, these doses failed to keep serum A1AT concentrations within the dose window (129). Because of the weekly intravenous infusions over a lifetime, alternative methods of administration, such as self-administration or at-home administration, were investigated. Indeed, Respreeza® is the only drug that is authorised for self-administration in Europe for augmentation therapy, which allows patients to manage their disease in their own environment, saving work time, hospital visits and travel time (128).

Table 1. Commercially available purified A1AT preparations (Adapted from Cortes-Lopez and Barjaktarevic, 2020 (130))

Trade name	Manufacturer	Formulation	Regulatory Approval
Prolastin®-C	Grifols	Lyophylized	USA, Canada, Argentina and Colombia
Prolastin®	Grifols	Lyophylized	European Union
Prolastin®-C Liquid	Grifols	Liquid	USA
Aralast-NP	Takeda	Lyophylized	USA
Zemaira®	CSL Behring	Lyophylized	USA
Glassia®	Takeda/Kamada	Liquid	USA and Israel
Alfalastin®	LFB	Lyophylized	France
Respreeza®	CSL Behring	Lyophylized	European Union

Inhaled A1AT

In addition to augmentation therapy, inhaled A1AT was explored as an alternative administration method. The fact that inhaled A1AT does not need intravenous access makes it more advantageous over augmentation therapy (131). Additionally, inhaled A1AT therapy involves delivering A1AT directly to the lungs through an inhaler, which allows for the targeted treatment of the lungs. Hubbard *et al*. made the initial attempt to use inhaled A1AT in 1989, where a dose of roughly 100 mg of A1AT generated from human plasma was given to the patients suffering from A1ATD by aerosol every 12 hours for seven days (132). Following this short-term aerosol administration to patients, A1AT levels were increased and the anti-elastase activity in the lower respiratory tract restored to normal. However, despite its efficacy, clinical studies are still needed to determine optimal inhalation devices and A1AT doses.

Other Therapies

For the treatment of A1ATD, additional alternative strategies have also been developed and assessed in pre- and/or clinical studies, such as orally bioavailable neutrophil elastase

inhibitors, A1AT silencing to prevent accumulation of mutant A1AT within liver, chemical chaperones to promote proper folding along with secretion, and autophagy enhancers to degrade aggregated protein (123, 125). Some of these products have undergone clinical trials, but none of them have received clinical application approval. Recent advances in clustered regularly interspaced short palindromic repeats (CRISPR) have prompted scientists to consider the possibility of using this genome editing tool, in which an adeno-associated virus (AAV) vector that encodes an A1AT guide RNA and a homology-directed repair template in order to correct the gene mutation that causes A1AT deficiency while partially restoring the serum level of normal A1AT (133). Although it is a significant development that might result in a treatment for A1ATD, further studies and trials shall be carried out before it is used in clinical settings. In the case of severe disease, lung transplantation is another option that could be offered to the selected patients (134). Finally, several recombinant forms of A1AT have been developed, which will be discussed in more details in the "Production of Recombinant A1AT" section.

Unmet Needs of Augmentation Therapy

Although augmentation therapy has been available in USA and Canada, it is still not licenced in some countries, such as UK, due to lack of evidence of the clinical effectiveness, which has been a subject of a great debate (135). This is mainly due to the fact that A1ATD is a rare disease and it is highly challenging to enrol enough patients for clinical trials with adequate sample sizes. Another problem is that this treatment can be expensive with a cost per patient each year between US $60,000 and US $250,000, which might be financially draining for patients (136). Moreover, with such extended, high-dose therapy, there are some concerns about risk of contamination, however very low, with pathogens, as well as batch-to-batch heterogeneity of pooled human plasma-purified product in terms of glycosylation, purity and activity (13, 127, 137). Finally, the plasma supply itself is a limited source and patients can find augmentation therapy inconvenient as they require weekly lifetime infusions. Thus, these issues, underlying the need to identify alternative and cost-effective sources of this protein, has led researchers to look into new and more convenient approaches, including recombinant approaches, for treating A1ATD in order to improve consistency of supplies that are able to meet future therapy demands.

Production of Recombinant A1AT

For three to four decades, there has been intense research into the producing functionally active A1AT with anti-elastase activity similar to that of human plasma A1AT using plasmid constructions and/or retroviral vectors, and recombinant DNA technologies. Different types of experiments were conducted to produce rA1AT protein using various prokaryotic and eukaryotic expression systems, including bacteria, yeast, insect cells, plant cells, transgenic animals, and mammalian cell lines. Nevertheless, to date, no therapeutic product has been approved from these sources due to the great difficulty in producing it with natural glycosylation pattern to ensure adequate tissue penetration as well as acceptable plasma half-life values, and/or the high quantities needed (138). In addition, further studies are required to show the safety and efficacy of such products. Meeting these requirements will have a major role in bringing the new alpha-1 antitrypsin products to the market and obtaining regulatory approval for them.

E.coli

Recombinant A1AT has been expressed widely in different strains of *E. coli* due to their inexpensive and relatively quick production abilities compared to other expression platforms (139-141). One of the first studies of a human A1AT expression was performed by Bollen *et al.* in 1984 in a K-12 strain where the yield was presented as 3.4 mg/L (142). However, the same study showed that the recombinant A1AT was expressed as an inactive form. To improve activity and yield, several research groups (Kwon et al., 1995, Bottomley and Stone 1998, Karnaukhova et al., 2004) used BL21 (DE3) strain to express the target protein and all of them has expressed active protein with yields ranging from 5 mg/L to 20 mg/L (13, 143, 144). However, *E. coli* do not have internal organelles including the Golgi and ER, and thus, is not capable to biosynthesize complex heterologous proteins, such as proteins requiring post-translational modifications and correct protein folding, which is vital on protein's activity (5). Since glycosylation is very important for A1AT's pharmacokinetic properties (i.e. half-life), *in vitro* stability and its correct folding to prevent aggregation, it can be concluded that *E. coli* is not the best expression platform to express recombinant A1AT for therapeutic purposes (13). Nevertheless, the extension of non-glycosylated recombinant A1AT's half-life through conjugation with polyethylene glycol (PEG) appears to start

a new chapter in the development of rA1AT for therapeutic purposes. For instance, Cantin and co-workers showed that conjugating 20 or 40 kDa PEG to Cys extended the half-life of *E.coli* produced A1AT in plasma and lung without altering its anti-elastase activity compared to non-PEG-conjugated rA1AT (115). However, it is important to note that there is a high risk of transmission of toxins and infectious particles for patients with the use of bacteria-derived A1AT since many bacteria have a high endotoxin content that may be harmful to humans (145).

Yeast

Yeast expression system has been used frequently for the expression of heterologous proteins in both academic research and industry (146-148). There are several advantages of protein expression in yeast expression platforms, including easy to handle, well-characterized, scalable, cost-effective production and ease of growth. In addition, purification process is simpler with this expression platform as yeasts do not produce endotoxins unlike most bacteria (13). On the other hand, products produced in yeast might cause allergic reactions to patients due to host cell proteins contaminants.

Yeast expression platform can provide some PTMs of protein, such as glycosylation. Consequently, expression of the human A1AT has been extensively performed in *Saccaromyces cerevisiae* both at laboratory levels and a larger scale production by different groups (14, 149-151). In the study of Tamer and Chisti, they reported an A1AT yield as high as 1.23 g/L in fed-batch culture (14). Although maximum A1AT concentrations can be attained using *Saccaromyces cerevisiae*, in contrast to human glycosylation, *N*-glycosylation in yeast results in high level of mannose content, referred to as “hypermannosylation” which causes a high-level degree of glycoprotein heterogeneity compared to native form (13, 152). For instance, Kang *et al.* observed that the glycosylation profile of rA1AT expressed in *S. cerevisiae* was heterogeneous consisting of unglycosylated, core-glycosylated and hypermannosylated forms (153). This improper glycosylation patterns create two major issues during intravenous therapy in terms of efficacy as recombinant protein became less effective due to fast clearance from the blood, and safety as it could create possible immune response that could be resulted by non-human glycans on recombinant protein. A study conducted by Casolaro *et al*. demonstrated that plasma-purified

human A1AT had significantly different PK profile compared to recombinant yeast-expressed A1AT protein (154). Although they had comparable anti-elastase activity, the latter had significantly lower *in vivo* half-life in their PK studies using primates. Moreover, 38 % of yeast-produced rA1AT was excreted in the urine within 3 hours, whereas plasma-purified A1AT was not detectable at all in the aforementioned study (154).

Insect Cells

Insect cells have been used for heterologous gene expression as they are capable of producing recombinant proteins with complex glycans, but with structures quite different from the human ones. They produce *N*-glycan precursors that are trimmed, creating high mannose or paucimannose structures (155). Moreover, recombinant proteins produced by insect cells lack sialic acid residue on their glycans (5). The baculovirus expression system (BES) is mainly used to express recombinant proteins in insect cells and there are very few studies performed to express rA1AT using this platform. In the work of Sandoval *et al*., BES system was used to express mutant A1AT fusion protein linked to the C-terminus of a human insulin-like growth factor analog (156). The substitution of Met 351 to glutamic acid (E) in the fusion protein resulted in an increased production in insect cells and in a more effective anti-elastase activity as compared to the wild-type protein. However, there are not much data on glycosylation as well as its effect on PK of the heterologous proteins obtained from these insect cells (156). Morifuji *el al.* then reported the first detailed *N*-glycosylation analysis of rA1AT purified from silkworm-BES where $Man_2GlcNAc_2$ and $Man_3GlcNAc_2$ lacking sialic acid residues were found to be major glycan structures, which differs from that of human A1AT (157). Although this differences of *N*-glycan between insect-produced rA1AT and human A1AT had no effect on their activity and stability, it is again unknown whether the insect-type *N*-linked glycans have a significant effect on the half-life of rA1AT in blood.

Transgenic Plants

Transgenic plant expression platform is one of the prominent choices to express recombinant therapeutic proteins as it allows to control and/or modify post-translational modifications compared to bacterial and yeast expression platforms (158). A1AT production has been attempted in different transgenic plant cell suspension culture, including rice cell

suspension culture (159-161), tobacco cell suspension culture (111, 138, 162), tomato plants (163, 164) and tobacco leaf chloroplasts (165). However, plant expression system has its own limitations, such as low expression levels and/or incorrect glycosylation resulting from plant-specific glycans with core α1,3-fucose and β1,2-xylose, which are completely absent in human cells (5). For instance, Jha *et al.* reported that the yield of rA1AT purified from transgenic plants is lower than that obtained from *E. coli (163)*. In the work of Terashima *et al.,* the glycosylation pattern of the rA1AT expressed by rice cell suspension culture was found to be different than that of the plasma-derived protein (159). Castilho *et al.* observed that rA1AT secreted by *N. Benthamiana* was truncated at both the N- and C-termini although it was still as active as human plasma-derived A1AT in anti-elastase inhibitory activity (138). Huang *et al.* indicated that the rice-derived human rA1AT was as active as the native protein derived from human plasma regarding its binding capability to the elastase *in vitro* (160). However, they demonstrated that both proteins had extreme differences in terms of PK behavior through an *in vivo* study performed in rats where rice-derived rA1AT had significantly lower half-life compared to the plasma derived protein. The authors from the same study explained the rapid serum clearance of plant-derived rA1AT might be due to the fact that plants are incapable of adding sialic acid to the glycan termini of their glycoproteins. Another challenge that would arise is that differences in the glycosylation pattern would lead to potential induction of immune response upon administration of the therapeutic agent into the human (5). Extensive glycoengineering strategies are needed to be employed to circumvent the attachment of plant-specific glycans to lower the risk of immunogenic reactions that may be elicited by nonhuman glycans.

Transgenic Animals

Genetic manipulations on animals have been implemented to synthesize human proteins for the past forty-five years (166-169). Recombinant human A1AT has been produced from several different transgenic animal sources, including mice (170, 171), rats (172), rabbits (173), sheep (174, 175) and goats (176). Moreover, several companies and research groups have achieved to perform large scale production of A1AT in dairy animals including in sheep (by PPL Therapeutics, Scotland, UK in partnership with Bayer Biologicals, CT, USA) (168, 174), and in goats (by Genzyme Transgenics Corporation, MA, USA) (176). Nevertheless, all these works did not

result in the therapeutic licensed product on the market. Harris *et al.* reported that human A1AT expressed from transgenic sheep milk can be produced with high level of purity (177). In another study, transgenic A1AT derived from sheep milk was utilized in a Phase II clinical trials to study its safety and efficacy for the treatment of cystic fibrosis patients through delivering it in aerosolized form, and it was reported that the study was success without any side effects (178). Conversely, Spencer *et al.* observed systemic antibody responses to native sheep A1AT and α1-antichymotrypsin that were present at very low concentrations in a sheep-derived transgenic human A1AT formulation (179). Since there was an observation of immune response in the study, there are still uncertainties and hurdles present in the development of recombinant A1AT from transgenic animals.

Mammalian Cells

Mammalian cells possess the metabolic machinery to express large and complex recombinant proteins that closely resemble or are fully compatible with humans. Therefore, these host cells, involving CHO, baby hamster kidney cells (BHK), human embryonic kidney cells 293 (HEK293) and HT-1080 human cells, NS0 myeloma and Sp2/0 hybridoma mouse cell lines, have been one of the most popular expression platforms utilized for expression therapeutic proteins in both academia and industry (5, 180). Many of biotherapeutic glycoproteins produced in these cells have been approved by FDA and the European Medicines Agency (EMA) for clinical use in humans (181). These include several monoclonal antibodies (mAbs) (e.g. Rituximab, Siltuximab, Pertuzumab and Cetuximab), human DNAse (Pulmozyme®), Epoetin delta (Dynepo ®), Factor VIIa (NovoSeven®), etc…

In order to produce recombinant A1AT comparable to the plasma-derived counterpart and avoid immunogenicity, various human expression systems were used by different groups. In 2011, Blanchard *et al.* released the first report on physiologically active A1AT generated in the human neuronal cell line AGE1.HN® (182). Despite some similarities to the native equivalent, structural analysis of the A1AT *N*-glycan profile resulted in higher level of core- and Le^X -fucosylation most likely referable to features of neuronal *N*-glycosylation. Moreover, A1AT expressed in AGE1.HN® presented di-, tri- and tetra-antennary *N*-glycan structures with α-2,3-sialylation as opposed to

human plasma A1AT bearing α-2,3-sialylation only in tri-antennary *N*-glycan structures. Human primary embryonic retinal cell line, PER.C6, is another cell host that provides a reliable source of functionally active A1AT. However, poor sialylation of recombinant proteins expressed by this cell line reflects the fact that the endogenous sialyltransferases cannot provide sufficient capping activity to meet the demands of highly overexpressed recombinant proteins (183). To overcome poor sialylation in these cells, Ross *el al.* co-expressed rA1AT along with human α-2,3-sialyltransferase (183). While they achieved high levels of α-2,3-capped glycans on the rA1AT, this is in contrast to that of plasma A1AT, in which α-2,6-sialylation is the main primary glycan modification. Nevertheless, PER.C6 cell-derived rA1AT was found to be fully active *in vitro* and had similar pharmacokinetic profiles *in vivo* as plasma-derived A1AT. Aside from AGE1.HN® and PER.C6 cell lines, HEK293 and HEK293T were also used as host cells for the production of rA1AT (113, 184). However, the glycan structures of rA1AT produced by these cells appeared to be more heterogenous, with varying levels of fucosylation, sialylation and terminal GalNAc, than that of native A1AT(184). Although it might be considered that human cells are the best choice for the production of human glycoproteins, it is worth to note that they carry a potential risk for the transmission of the human pathogens due to lack of species barrier (180).

On the other hand, CHO cells are by far the most commonly used host cells for the commercial therapeutic protein production purpose as they are capable of making human-like PTMs as well as producing safe and effective glycoprotein therapeutics with high yields (185). These cells are suitable for large-scale industrial suspension culture and can be adapted to grow in various serum-free and chemically defined culture media (5). Additionally, they are resistant to infection by human viruses, which reduces the biosafety risks associated with commercial production. Indeed, a Phase 2 clinical trial using CHO cell-derived oxidation-resistant recombinant human A1AT-Fc fusion protein (INBRX-101) has recently been initiated (ClinicalTrials.gov Identifier: NCT05856331). However, no details on the glycan profile of this recombinant A1AT-Fc fusion are reported.

Previous studies showed that despite having nearly similar *in vitro* biological activities, the glycosylation pattern of rA1AT expressed in CHO cells is considerably different than that of native A1AT (186-189). Notably, CHO-derived A1AT bears heterogeneous complex-type *N*-glycans with

bi-, tri- and tetra-antennary structures containing core-fucose sugars (186). Due to lack of β-galactoside α-2,6-sialyltransferases expression, sialic acids found in CHO-derived A1AT were exclusively α-2,3-linked in contrast to the native A1AT, which contained predominantly α-2,6-linked sialic acids (186). Additionally, production in CHO results in incomplete sialylation and CHO-derived A1AT was found to have traces of non-human Neu5Gc at 1.3% of total sialic acids (186, 190). It is therefore indispensable to develop an engineered CHO cell line that can provide humanized glycosylation patterns crucial for the therapeutic success of recombinant A1AT. Indeed, a recent study, and this is the area that interests us here, highly engineered CHO cells to produce rA1AT with *N*-linked glycan structures resembling those in plasma-derived A1AT (188). However, the titers reported in the aforementioned study (~ 120 mg/L) were not suitable for industrial purposes and the circulatory half-life time of rA1AT remain to be investigated. This modification of *N*-linked glycan structure in CHO cells will be discussed in more detail in the following section.

Modification of Glycan Composition and Structure

The development of therapeutic proteins has provided new treatment options for a wide range of diseases and has significantly improved patient outcomes. In this regard, a process known as "glycoengineering" is an emerging strategy that involves modifying protein-associated carbohydrate moieties to change the pharmacokinetic, biochemical, or functional characteristics of the corresponding therapeutic glycoproteins (191). Major targets of these engineering techniques include increasing molecule stability or solubility, preventing glycan antigenicity, reducing structural heterogeneity to make pharmaceutical production and quality monitoring easier, and more importantly enhancing serum half-life through maximizing sialylation in order to reduce injection frequency while increasing convenience and improving patient compliance (191, 192). Currently, mammalian expression systems, mainly CHO cells, are widely used as a workhorse for the therapeutic protein production as they are capable of making human-like PTMs that are compatible with the human immune system as well as producing safe and effective glycoprotein therapeutics with high yields (185), and thus will be our main focus for the glycoengineering.

Cell-Based Glycoengineering

In the past thirty years, a wide range of techniques have been developed to engineer host cell lines, including CHO cells to achieve the production of proteins with desirable glycosylation patterns. These techniques primarily involve generating a genetically modified organism by using gene knockout (KO) or knockdown along with knock-in or over-expression technologies, to alter the type and concentration of cellular glycosidases and glycosyltransferases, thereby changing the glycosylation patterns of relevant proteins expressed in those cells (193). The relevant cell-based glycoengineering strategies in CHO cells will be discussed in the following sections.

Overexpression of sialyltransferases

Overexpression of sialyltransferases to improve sialylation of a variety of glycoproteins in CHO cells has been a topic of great interest. The benefit of introducing these glycosylation genes in the host cell is not only to increase the pre-existing capacities of the cell, but also to introduce new skills into it. For instance, CHO cells possess only α-2,3-sialyltransferases, but not α-2,6-sialyltransferase, resulting in glycoproteins having only α-2,3-sialylation linkages (6, 194). However, human glycoproteins contain both α-2,3- and α-2,6-linked sialic acids (195). As a result, many groups have tried overexpressing α-2,6-sialyltransferase to overcome this deficiency in CHO cells.

One of the first studies of a ST6 sialyltransferase overexpression was carried out by Minch *et al.* in 1995 in a CHO line expressing recombinant human tissue plasminogen activator (tPA) where rat ST6 was introduced with the aim of producing humanized glycoproteins (195). As a consequence of this ST6 incorporation, the authors observed sialylated glycans displaying a mixture of α-2,3- and α-2,6-linkages that are similar to human glycans compared to parental cell line bearing only α-2,3-sialylated glycans. This distribution of α-2,3 and α-2,6 sialic acids is the result of competition between endogenous ST3 and introduced ST6 for galactosylated *N*-glycans (196). Similarly, Bragonzi *et al.* demonstrated that rat ST6 transfected into CHO cells yielded human interferon-gamma (hIFN-γ) with 40 % α-2,6- and 60 % α-2,3-sialic acid residues. Subsequently, the authors from the same study observed that IFN-γ with a mixed sialylation showed an improved half-life with a comparable activity compared to IFN-γ produced by parental

CHO cell line displaying exclusively α-2,3-sialylation (197). Another group discovered that the expression of α-2,6-sialyltransferase in CHO cells led to an increased percentage of tri- (by 8%) and tetra- (by 16%) sialylated recombinant thyroid-stimulating hormone (198). Moreover, authors from the same study included that the hydrophobicity and the bioactivity of the protein were unaffected by this increase in sialylation. On the other hand, altering the sialylation pattern of the recombinant proteins is not necessarily beneficial all the time. For example, Zhang *et al.* demonstrated that parental CHO produced-recombinant EPO bearing exclusively α-2,3-sialylation was more biologically active than the one with a mixture of α-2,3- and α-2,6-sialylation produced by CHO cells stably expressing human ST6Gal I (199). Therefore, the impact of different type of sialylation on the bioactivity must be validated separately for different recombinant proteins.

Another way to improve sialylation is to overexpress sialyltransferases along with β-1,4-galactosyltransferase (GT) in CHO cells since galactose is required for sialylation. Weikert *et al.* showed that the co-expression of GT with ST3Gal III in CHO cells expressing a fusion protein composed of the tumor necrosis factor receptor binding domain fused to the Fc domain of an IgG1 (TNFR-IgG) or a modified tPA caused a significant decrease in GlcNAc-terminated *N*-glycans and an effective increase in terminal sialylation, which resulted in a longer residence time in a rabbit model (200). Jeong *et al.* reported that overexpression of both GT and α-2,3-sialyltransferase yields even higher sialic acid content of EPO compared with α-2,3-sialyltransferase overexpression alone in CHO cells (190). Similarly, a simultaneous transfection of CMP-sialic acid transporter (CST), CMP-Sia synthetase (CMAS) and α-2,3-sialyltransferase also led to an increase in EPO sialylation (201), which will be discussed in more detail in "Overexpression of CMP-Sia Transporter and Synthetase" section.

Overexpression of CMP-Sia Transporter and Synthetase

In addition to sialyltransferase overexpression, other approaches have been performed to enhance the sialylation machinery of CHO cells. In a study conducted by Wong *et al.,* transfection of CMP-sialic acid transporter (CST) gene in CHO cells resulted in two to three times more CST, which is required for transporting CMP-Sia from the nucleus to the Golgi, at the protein level than the parental cell line, leading to a 4–16% increase in site sialylation of the produced

IFN-γ (202). Similarly, a simultaneous transfection of CST, CMP-Sia synthetase (CMAS) and α-2,3-sialyltransferase effectively increased the intracellular CMP–Sia levels in CHO cell lines and enhanced the sialic acid content on glycans for human EPO (201). Interestingly, the same group also discovered that the synergistic co-expression of α-2,3-sialyltransferase and CMAS did not cause a noticeable increase in EPO sialylation, despite an elevation in CMP-Sia levels, unless CST was also co-overexpressed. Based on these observations, it could be concluded that overexpression of the CST may represent a novel and powerful approach to improve sialylation during recombinant glycoprotein production (203).

Inhibition of Sialidase Activity by RNA Interference

Sialidases (neuraminidases) are exoglycosidases, which cleaves the terminal sialic acid residues on the glycans (204). In CHO cells, four sialidases (Neu 1-4) have been discovered and their activity is confined to several subcellular compartments: Neu1 and Neu4 are found in the lysosome, Neu2 in the cytoplasm and Neu3 in the plasma membrane (205). Sialidase cleavage occurs in cell culture as the viability declines, leading to the asialylation of recombinant glycoproteins. To prevent the degradation of protein sialylation by sialidases, some groups have attempted to alter the synthesis of sialidases by using non-coding RNAs, such as microRNA (miRNA), short hairpin RNA (shRNA), or small interfering RNA (siRNA). For instance, Ngantung *et al*. obtained a 60% reduction in sialidase activity by degrading sialidase mRNAs using siRNA compared to the parental CHO, and sialic acid content of hIFN-γ was sustained at a relatively high level even during cell death phase (204). Similarly, Zhang *et al.* inhibit various sialidases using shRNA or siRNA in CHO cells in order to increase sialic acid content of a recombinant hIFN-γ (206). In their study, they have found out that protein sialylation was enhanced when host cells were at both the stationary and death phases following silencing the gene expression of Neu3 compared to Neu2 sialidase silencing where sialic acid content of recombinant glycoprotein was improved only in the death phase. Finally, Ferrari *et al.* developed a CHO cell line expressing sialidase antisense RNA and observed a 40% reduction in sialidase activity over the culture duration, which resulted in an increase in sialic acid content ranging from 20 to 37% compared to the control culture (207).

CRISPR/Cas9 Genome Editing Technology

Aside from enhancing sialylation, engineering CHO cells would be highly informative to evaluate the impact of sialic acid linkage specificity on protein's half-life and its activity. The availability of genomic sequences has made CHO cells possible to engineer with precision using engineered nucleases, such as zinc finger nucleases (ZFNs), transcription activator like nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR) system, which are now used as methods of choice in academic and industrial settings (208). While ZFNs are artificial restriction enzymes composed of a customizable zinc finger DNA-binding domain and a Fok1 endonuclease domain, which must dimerize for activity, TALENs are dimeric transcription factor nucleases, consisted of 33–35 amino acid modules, that can each target a single nucleotide (205). Due to their complexities and lower efficiency, more efficient genome editing technologies like CRISPR-Cas9 have become more popular since it is more practical, easy to use and amenable to other applications for achieving targeted modifications in CHO cells and other cell lines (209). Therefore, we will be focusing only CRISPR/Cas9 technology for the rest of this section.

CRISPR/Cas9 represents one of the newest and the most rapidly expanding methods for modifying the genome in CHO cells. It is a prokaryotic defense mechanism that is widely found in bacterial and archaeal genomes to provide protection against invading viruses as well as plasmids (210, 211). CRISPR/Cas9 genome editing system has two key components including a chimeric single guide RNA (sgRNA) along with the DNA endonuclease Cas9 (Figure 11) (212). More precisely, 20 nucleotide sgRNA carries Cas9 to the desired genomic site where Cas9-mediated DNA cleavage at the target site is triggered by a protospacer adjacent motif (PAM) situated downstream of the target sequence (212). Following, Cas9-induced double-stranded breaks (DSBs) is repaired by the error-prone non-homologous end joining (NHEJ) DNA repair pathway, which introduces mutations to disrupt the function of the targeted genes (212). Compared with ZFNs and TALENs, this engineering approach has the advantage that only the gRNA sequence needs to be changed to target new genes (209).

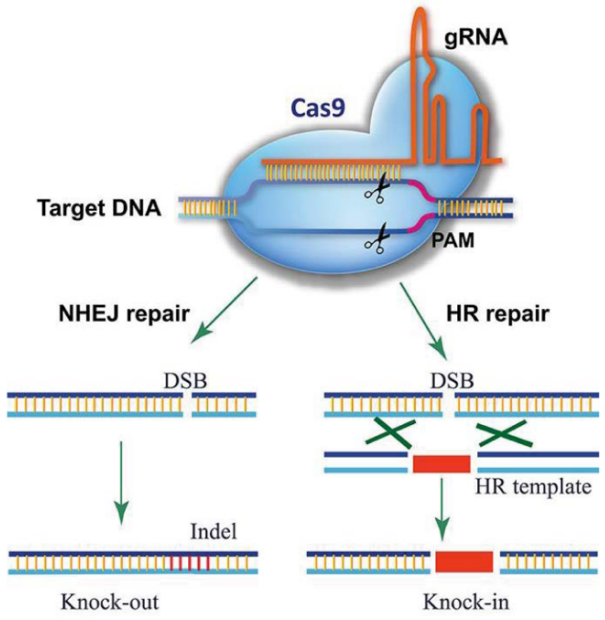

Figure 11. CRISPR/Cas9 gene editing mechanism (Figure adapted from Razzaq and Masood, 2018 (213)).

In a study performed by Chung *et al.*, using CRISPR/Cas9, the impact of altering protein sialylation on PK of human butyrylcholinesterase (BChE) was examined by knocking out two α-2,3 sialyltransferase genes, *ST3Gal* IV *and ST3Gal VI,* to minimize α-2,3-sialylation, followed by overexpression of the ST6Gal I (214). The same authors observed that *in vivo* clearance for α-2,6-linked BChE was greater than that for α-2,3-linked BChE in mice, although this difference in pharmacokinetic analysis did not reach statistical significance.

Likewise, Chung *et al.* combined amino acid substitution of the IgG fragment crystallizable (Fc) region with CRISPR-Cas9-mediated KO of *ST3Gal IV* and *ST3Gal VI*, and overexpression of ST6Gal I in CHO cells (215, 216). By combining these three approaches, the authors showed that more than 77% of IgGs produced were almost exclusively α-2,6-sialylated.

When a distinct glycan structure is a prerequisite for a therapeutic protein, the heterogeneity of CHO *N*-glycans is especially undesired. Amann *et al*. achieved this by CRISPR-Cas9-mediated KO of ten glycosylation gene, involving *ST3Gal III*, *ST3Gal IV* and *ST3Gal VI*, in CHO-S cells, followed by overexpressed ST6Gal I with the aim of mimicking glycosylation/sialylation profile of human A1AT and C1 esterase inhibitor (C1INH) (188). Both A1AT and C1INH expressed

in this glycoengineered CHO cell line carry homogeneous glycostructures similar to the human plasma-derived proteins although the titers obtained from these cell lines were very low.

In addition, a KO of *FUT8,* which controls *N*-glycan core fucosylation, has also been generated to enhance ADCC using CRISPR in CHO cells since afucosylation can be a desirable glycan characteristic in mAbs to trigger immune responses (217). ADCC is an essential mechanism of antibody therapeutics. Through the antigen binding fragments (Fab), antibodies recognize and bind to surface antigens of target cells (such as cancer cells), and interact with Fc receptors (FcR) on effector cells (e.g. natural killer cells) via the Fc portion (193). Following the interaction of Fc with FcR, immune effector cells are activated and release cytotoxic chemicals to kill target cells. The ability of antibodies to bind more strongly to Fc receptors can be increased by changing the glycosylation of the IgG's Fc region. Therefore, mAb fucosylation has been the main focus in biopharmaceutical glycoengineering, and as of 2018, three afucosylated antibodies have been approved by FDA, although more are undergoing clinical trials (218).

In contrast, CRISPR technology has also been used for the activation of an endogenous gene in CHO cells. The first application of targeted CRISPR-based activation was performed by Marx *et al.* where they targeted the silenced *ST6Gal I* gene in CHO cells using deactivated Cas9 (dCas9), which lacks endonuclease activity but retains selective DNA binding ability, as specified by sgRNA (219). The activation of *ST6Gal I* gene was achieved by targeting dCas9 fused to the catalytic domain (CD) of the ten-eleven translocation methylcytosine dioxygenase 1 (TET1) to its methylated promoter region. Demethylation of the promoter by TET1 resulted in strong activation of ST6Gal I in up to 60% of the cells. Moreover, they reversed this effect by targeting CD of DNA methyltransferase (DNMT) to re-methylate the same promoter, leading to 5.4-fold reduction of ST6Gal I mRNA expression in ST6Gal I-expressing cells. Likewise, *ST6Gal I* along with another silenced gene, *Mgat3* that is responsible for bisecting GlcNAc, were upregulated in a study published by another group where the authors fused dCas9 with one of the most potent transcription activator domains VPR, composed of three activation domains VP64, p65 and Rta (220). Consequently, the authors were able to detect modest amounts of glycans with bisecting GlcNAc and α-2,6 sialic acid in engineered CHO cells compared to control cells.

In vitro Glycoengineering

In vitro glycoengineering has emerged as a way to circumvent the primary limitation *of in vivo* engineering, which requires optimization of expression systems for each specific protein and their respective glycan type through glycan biosynthetic pathway engineering (221). Usually, recombinant proteins produced through *in vivo* technologies carry highly heterogeneous and/or incomplete *N*-glycans. In contrast, *in vitro* glycoengineering approach is very useful and efficient in producing homogeneously glycosylated glycoproteins since it is independent from the production cell line as well as the production process (222).

Several strategies have been developed to enhance glycoprotein homogeneity, such as chemoenzymatic method, which takes advantage of the transglycosylation activity of several endoglycosidases and their glycosynthase mutants using glycan oxazolines as their substrates (223, 224). With this method, glycosylation remodeling is performed via trimming off the heterogeneous *N*-glycans by an endoglycosidase, leaving the initial GlcNAc at the glycosylation site, and then transferring a chemically modified intact *N*-glycan oxazoline donor onto the glycan through an endoglycosidase-catalyzed transglycosylation reaction, producing a homogeneously *N*-glycosylated proteins. By using this strategy, Huang *et al.* transformed rituximab (a therapeutic mAb) from mixtures of G0F, G1F, and G2F glycoforms to well-defined homogeneous glycoforms, involving a fully sialylated glycoform that may gain anti-inflammatory activity, a afucosylated glycoform that showed optimal ADCC activity, and an azido-tagged glycoform that can be further transformed into other glycoforms (223).

Additionally, the glycosynthase-based approach could be used to accomplish site-selective enzymatic glycoengineering, which entails attaching various glycans to different glycosylation sites. In an example of such study by Giddens *et al.,* three endoglycosidases (Endo-S, Endo-S2, and Endo-F3), α-1,6-fucosidase from *Lactobacillus casei,* and endoglycosidase mutants were used in combination to successfully remodel the Fc and Fab glycans of mAb independently with different *N*-glycan structures (225). They discovered that the antibody with sialylated *N*-glycans on the Fab fragments and afucosylated galactosylated ones on the Fc fragments had significantly increased ADCC activity and improved binding ability to the FcRIIIa receptor. By taking advantage

of the substrate specificity of different endoglycosidases and their glycosynthase mutants, this method permits independent manipulation of the *N*-glycans at different glycosylation sites, enabling high uniformity and precise control over the glycoform.

Another alternative approach to deal with the inconsistency and heterogeneity in glycosylation of glycoproteins is to treat glycan structures from their terminal ends and complete the elongation of the glycan *in vitro*, by bringing together the glycosylated protein with the terminal glycosyltransferases and the corresponding activated sugars. A previous study described an *in vitro* regalactosylation and resialylation method of TNFR-IgG using a combination of bovine β-1,4-galactosyltransferase and rat α-2,3-sialyltransferase in the presence of UDP-Gal, CMP-Sia, and $MnCl_2$, which resulted in an increase in the terminal sialylation level by ~20-23 % as well as decrease in heterogeneity (226). Another group used Cst-II, a bacterial sialyltransferase with the ability to catalyze α-2,8 sialylation, enables effective polysialylation on rA1AT *in vitro*, leading to a noticeably extended half-life in mice compared to native A1AT bearing α-2,6 sialylation without compromising its specific function (50).

Despite all of the advantages, *in vitro* glycoengineering has the disadvantage of being more labor-intensive and less useful in large-scale production (193). Moreover, many crucial steps for glycoprotein synthesis have not been effectively optimized, and the most essential starting materials are not readily available in the market. This technique is therefore, for the moment, reserved for the preparation of small quantities, which are very useful for conducting tests on the impact of a sugar on the biological functions of a glycoprotein and its structure.

Protein Glycoengineering

Besides altering specific glycan structures, glycoengineering can also be carried out by changing the number of glycosylation sites through the introduction of additional glycosylation motifs into the existing amino acid sequence. In this way, the hyper-glycosylated variant of recombinant human erythropoietin (EPO), known as darbepoetin alfa, showed a considerable extended serum half-life when compared to the unaltered recombinant equivalent due to the introduction of new *N*-glycosylation sites by changing amino acid residues via site-directed mutagenesis, which exhibited a biologically active molecule with an increased molecular weight

and greater negative charge (227, 228). Similarly, Lush *et al.* aimed to reduce glomerular filtration of A1AT by increasing its negative charge through the introduction of additional *N*-glycosylation site at position Asn123, which resulted in a 62% increase in its serum half-life (116). Other strategies have been also attempted to modify the protein glycosylation where well-known glycosylation domains are transferred from one protein to the N- or C-terminus of a target protein. For instance, Grabenhorst *et al.* transferred a human interferon-β *N*-glycosylation domain to the N- or C-terminus of interleukin-2 and yielded proper *N*-glycosylation compared to the wild-type variant of interferon-β (53). However, the introduction of new *N*-glycosylation sites into the coding sequence of a protein might have negative consequences on the protein folding, molecular stability, or biological activity since the expressed *N*-glycans or the mutated amino acids may alter the steric properties or the three-dimensional structure of the protein. To overcome this, Kaup *et al.* designed unique peptide tags called GlycoTags with an unnatural *N*-glycosylation sites, and effectively linked them to the C-termini of human EPO or A1AT without changing the sequence of the target proteins (229). Nevertheless, the effect of GlycoTags addition on the half-life of the target proteins was not investigated in the aforementioned study.

Metabolic Glycoengineering

Metabolic glycoengineering, also referred to as metabolic oligosaccharide engineering, is another widely used technique, which was developed almost thirty years ago. Several approaches have been made where protein glycosylation can be altered to enhance sialylation by changing the concentrations of media components, nucleotide sugars, metal co-factors etc. and will be discussed in the following section.

Glucose and Glutamine

Glucose and glutamine are the two main sources of energy for cells in the culture medium and can play a role in the glycosylation control. Proper glycosylation requires an adequate supply of energy and nucleotide sugars, which are the substrates for glycan synthesis (230). When glucose or glutamine concentrations are limited, it can result in a decreased availability of these precursors, leading to incomplete or altered glycosylation patterns (231). Indeed, a previous study reported that very low glutamine (<0.1 mM) or glucose (<0.7 mM) concentrations led to

decreased sialylation and increased presence of minor glycan species consisting of hybrid and high-mannose types, indicating the importance of optimizing glucose and glutamine levels in order to ensure proper glycosylation and the desired glycan structures on target molecules (232). Similarly, Hayter *et al.* demonstrated that glucose-limited continuous cultures of CHO cells produced recombinant hIFN-γ without glycosylation (233). In contrast, continuous cultures of BHK-21 cells expressing a human IgG-IL2 fusion protein at low glucose and glutamine concentrations did not differ from a non-nutrient-limited culture in terms of the oligosaccharide profile (234).

Nucleotide Sugar Precursors

The availability of CMP-Neu5Ac is crucial for proper sialylation as it serves as the activated form of sialic acid and is utilized by sialyltransferases to transfer sialic acid residues onto glycan chains (48). Both ManNAc and cytidine triphosphate are necessary for the biosynthesis of CMP-Neu5Ac, and therefore ultimately for sialylation. Although addition of ManNAc, a sialic acid precursor, in cell culture medium was previously shown to enhance sialylation of recombinant proteins, involving A1AT, IFN-γ and EPO (187, 235-237), Baker *et al.* reported that increased CMP-Neu5Ac content induced by ManNAc supplementation had no effect on the overall level of tissue inhibitor of metalloproteinases 1 (TIMP-1) sialylation (238). Moreover, combined feeding of ManNAc and cytidine triphosphate did not lead to a synergistic increase in IFN-γ sialylation either despite elevated levels of CMP-Neu5Ac (239). However, ManNAc is an expensive supplement and its application is not the first choice to improve sialic acid concentrations in a large-scale production process (203).

Sodium Butyrate

Frequently, small molecules are added to the culture medium to trigger a desired cellular behaviour, including high specific productivity. Sodium butyrate (NaBu) is one of the most commonly used chemical additives in mammalian cell culture, and is well-known for increasing specific productivity, modulating sialylation as well as reducing growth rate by upregulating apoptosis mechanisms (240, 241). While specific protein productivity in CHO cells cultured at various NaBu concentrations increased, the product quality of number of recombinant

glycoproteins, including human thrombopoietin, EPO and GP1-IgG immunoadhesion chimeric protein, decreased overall due to reduced sialylation (241-243). In contrast, NaBu added early to the culture medium and at a final concentration of 1 mM was shown to increase sialylation along with *N*-glycan site-occupancy, demonstrating the effect of the concentration and NaBu addition timing on the product quality of recombinant glycoproteins expressed in CHO cells (244, 245).

DANA

The level of sialylation gradually declines over time during recombinant protein production as cell lysis occurring towards the end of batch or fed-batch processes promotes the release of sialidases (neuraminidases) into the medium (206, 246). Therefore, the sialidases present in the medium are an interest of an inhibition strategy using a sialic acid analogue, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA). Indeed, previous groups showed that the undesirable degradation of sialylation by sialidase following cell death was minimized through the addition of sialidase inhibitor, DANA, to the CHO culture supernatant (206, 246, 247). However, it is worth noting that adaption of this method for an industrial-relevant process is limited due to the high cost incurred for large-scale process. (204, 207). Thus, some researchers have attempted to inhibit various CHO sialidases, such as Neu2 and Neu3, using shRNA or siRNA in order to enhance the sialylation level of recombinant hIFN-γ (204, 206).

Manganese

Manganese (Mn^{2+}) serves as a co-factor for β-1,4-galactosyltransferase, which catalyzes the addition of terminal galactose to GlcNAc of elongating *N*-glycans. Mn^{2+} supplementation leads to an enrichment in bi-antennary complex glycans rich in galactose, followed by an increase in sialylation (248).

Others

In addition to most commonly used media additives that were listed earlier, several other compounds, involving dimethyl sulfoxide (DMSO), glycerol (240), dexamethasone (249), amino acid cocktails (248), sucrose and tagatose (26), are also tested in an attempt to modulate glycosylation and increase protein quality.

Impact of Cell Culture Parameters and Process Strategies

The effect of process environment changes on process performance is one of the most important concerns when cultivating mammalian cells. Directed shifting of process parameters, on the other hand, can be employed as an optimisation tool to enhance product quality and yield. Process parameter changes can be carried out quickly and easily without the need for reagent addition or even genetic modification of the host cell line. However, effective application of altering process conditions necessitates a thorough comprehension of the physiological changes provoked within the cells. The most commonly used cell culture parameters as well as process strategies are introduced in detail within the following sections.

Culture Mode

Batch, fed-batch, and perfusion are the three most popular modes for the cell culture production of biologics. Batch production is the process of cultivating cells with a known initial concentration of feed source and medium (250). This system is not subjected to any additional nutrient additions or removals and the protein of interest is harvested at the end of culture following the first signs of cell death. On the other hand, by feeding culture with nutrient supplements at regular intervals, most commonly every 24 to 48 hours, fed-batch culture enables extended culture life and high viable cell densities, resulting in high recombinant protein productivity and yield compared to simple batch culture (251). Similar to batch mode, cellular waste, such as lactate and ammonia, accumulate in the medium of the fed-batch production, which can have a significant impact on the final glycoform profile (252, 253). In fact, various studies have shown that sialylation quality changes significantly as a function of time during cell culture. Wong *et al.* observed a reduction in IFN-γ sialylation as the fed-batch culture progressed from exponential, stationary and death phase due to downregulation of genes involved in CMP-Neu5Ac synthesis as well as increase in activity of sialidases released by cell lysis toward the end of fed-batch culture in a CHO cell line (254). Similar results were reported for human recombinant antithrombin III (rhAT III) where significant loss of terminal sialic acid was observed as a consequence of an increase in sialidase activity throughout long-term batch cultivation (255).

Additionally, Rodriguez *et al.* also noted a remarkable decrease in the proportion of di-sialylated bi-antennary structure of IFN-beta during the course of a batch culture (240).

On the other hand, perfusion culture is suitable for achieving high cell concentration and reducing degradation of recombinant proteins as nutrients are continuously supplied and the used medium, which contains metabolic by-products and secreted target recombinant protein, is periodically removed while cells are kept inside the bioreactor (256). In order to minimize the impact of dynamic changes in medium components on protein glycosylation, perfusion culture is preferred over batch culture since steady state physiological conditions can be achieved with perfusion culture. Lipscomb *et al.* showed that the level of secreted alkaline phosphatase (SEAP) sialylation was higher in perfusion culture compared to fed-batch culture as a result of slow growing cells producing more completely glycosylated proteins (257). Goldman *et al.* also reported an increase in the proportion of tri- and tetra-sialylated *N*-glycans of CHO-produced hIFN-γ and concomitant decrease in mono-sialylated and neutral *N*-glycans during perfusion culture compared to batch culture (258). Nevertheless, perfusion production processes still account for less than 10% of all commercial mammalian cell culture processes in the industry because of difficulties in process development and process characterization, complex operation requirements with high demands on automation and modelling, and the design of manufacturing facilities (259), whereas fed-batch culture is the most widely used due to its simplicity of operation and process setup, scalability and cost-effectiveness (251).

pH

In addition to culture mode, cell culture parameters, including pH, can also have a significant impact on protein glycosylation. A study by Müthing *et al.* showed that a pH shift from 6.9 to 7.4 had an impact on the rate of specific growth, mAb synthesis as well as the glycosylation profile of IgG3 produced by hybridoma cells (260). In the same study, while maximum galactosylation levels were observed at pH 7.4, the highest sialylation was detected at pH 7.2. Similarly, Yoon *et al.* reported that sialylation of EPO increased with decreasing culture pH with an optimal range of 6.8–7.2 (261). Bory *et al.* demonstrated decreases in the extent of glycosylation of recombinant mouse placental lactogen-I (mPL-I) expressed in CHO cells at low

(below 6.9) and high (above 8.2) pH values (262). Finally, Trummer *et al.* showed that the loss of EPO-Fc sialylation occurred at reduced temperatures could be prevented by reducing the external pH to 6.9 (263).

Ammonia Accumulation

Ammonia is a toxic and inhibitory by-product to cells. It is mainly produced in cell cultures due to L-glutamine metabolism as well as spontaneous degradation and deamination of L-glutamine (264). Due to its negative impact on sialylation, ammonia accumulation has received a lot of attention (265). One of the main theories is that ammonia affects a variety of enzymes' activities during the glycosylation process by increasing the pH of endoplasmic reticulum (ER) and Golgi compartments (266). As ammonia levels increased, activity of numerous glycosyltransferases, such as galactosyltransferases and sialyltransferases, were downregulated (266, 267). A second proposed mechanism is that elevated ammonium perturbs the balance of intracellular nucleotide sugar pools, which results in elevated intracellular uridine diphosphate (UDP)-*N*-acetylhexosamines (UDP-GNAc), including UDP-GlcNAc and UDP-GalNAc, and according to Valley *et al.* increased UDP-GNAc is thought to prevent the availability of CMP-sialic acid in the Golgi (268). Contrarily, the study by Ha *el al.* discovered that increased expression level of sialidase with ammonium addition was partially responsible for the reduced sialic acid content (269).

Temperature

Similar to most mammalian cell cultures, CHO cells are typically maintained at 37°C throughout seed culture and the beginning of fed-batch production (270). Although reduced culture temperature (30°C-33°C) is known to significantly improve recombinant protein production by slowing down the cell cycle progression and allowing cells to shift from a proliferative to a productive mode, it seems that decreased temperature leads to lower levels of protein sialylation (5). As indicated by Trummer *et al.* EPO-Fc sialylation was reduced when the temperature shifted from 37°C to 30°C and 33°C by 40% and 20%, respectively, whereas no significant effect was found at high temperatures (>37°C) (271). Similarly, Ahn *et al.* observed that the proportions of acidic isoforms of EPO at 30°C was decreased by 24% compared to that at 37°C,

implying that lowering culture temperature negatively affected the degree of EPO sialylation (256). A study conducted by Sou *et al.* suggested that the main cause for this decrease in sialylation at low temperature was due to lower expression of glycosyltransferases that are responsible for *N*-glycosylation as well as shortage of substrates for glycosyltransferases (272). Additionally, protein sialylation was also shown to be dependent on the timing of the temperature shift. Borys *et al.* observed that sialic acid levels were lower when temperature was shifted later near the stationary phase of the culture compared to an early-temperature shift, near the end of the exponential growth phase (245). In contrast, there are some reports showing no change or increase in sialic acid content of recombinant proteins at low culture temperature. For instance, according to Bollati-Fogolín *et al*., decreasing the culture temperature to 33°C in CHO cell line producing recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF) had no significant impact on its site-occupancy and sialylation level (273), whereas Kaufmann *et al.* showed that production at 30°C resulted in a significant increase in the degree of sialylation of CHO-produced SEAP (274). In another study, the sialylation of EPO produced at 33°C was comparable to or even better than that at 37°C (275). In any case, the studies presented here were conducted under different conditions, which might explain the differences in the outcome of a temperature shift on protein glycosylation.

Dissolved Oxygen (DO)

The effect of dissolved oxygen (DO) on the glycosylation of different recombinant proteins has been studied by different groups. Chotigeat *et al.* observed a positive correlation between sialyltransferase activity in CHO cells and DO level, resulting in an increase in human follicle stimulating hormone (hFSH) sialylation at 90 % DO compared to 10 % DO (276). In contrast, a study conducted by Trummer *el al.* reported that variations in DO from 10 % to 90 % had no significant effect on EPO-Fc sialylation (271). On the contrary, they observed a loss in EPO-Fc sialylation at 100% DO. Similarly, Lin *et al.* showed no change in the *N*-glycosylation pattern of recombinant tissue-type plasminogen activator (tPA) produced by CHO cells under a wide range of DO concentrations (277). A research conducted by Restelli *et al.* demonstrated that EPO sialylation did not exhibit variations with changes in DO concentration whereas maximum core-fucosylation was observed at 50% and 100% DO and the proportion of fucosylated structures

decreased at lower or higher DO concentrations of 3%, 10%,and 200% in CHO cells (278). However, the exact mechanism by which DO affects protein glycosylation is still not well understood.

Conclusion of Modification of Glycan Composition and Structure

The modification of glycan composition, particularly sialylation, in CHO cells represents a promising avenue for enhancing biopharmaceutical production and therapeutic efficacy. Through precise manipulation of sialylation, one can influence stability, immunogenicity, and more importantly half-life of the target protein through preventing its interaction with the ASGPR, thereby optimizing the therapeutic potential of the biologics. Nevertheless, the control of sialylation is a complex task since it is sensitive to variations in both intrinsic cellular processes and extrinsic environmental conditions. When the objective is to increase the level or to modify the type of sialylation of the target protein, several methods, including genetic engineering, protein engineering, metabolic pathway manipulation, culture parameter optimization etc.., are attempted to tailor glycan profiles. However, in the case of CHO cell line, which is the most used cell line in the pharmaceutical industry, the only way to introduce human-type sialylation remains still through the introduction of the α-2,6-sialyltransferase (*ST6Gal I*) gene into the cell.

Asialoglycoprotein Receptor (ASGPR)

Human asialoglycoprotein receptors (ASGPRs) are Ca^{2+} dependent C-type lectin, predominantly located on the basolateral membrane of parenchymal liver cells facing the sinusoids, and thus are having direct contact with the blood circulating through the liver (279-281). It has also been detected on extra-hepatic cells, including macrophages, intestinal epithelial cells, testicles, peripheral blood monocytes and some tumor cells of non-hepatic origin (282). Nevertheless, ASGPR expression on hepatocytes is significantly higher than that in other parts of the body. There are approximately 500,000 receptor subunits per cell expressed in human and rodent hepatocyte *in vivo* (283). It was first discovered by Ashwell and Morell as early as 1965 upon their observations of sialic acid residues removal from *N*-linked oligosaccharides on glycoproteins resulted in their fast clearance from the blood and degradation in the liver (281, 284-286). The role of ASGPR is to implement endocytosis and degradation of a large range of

asialylated glycoproteins possessing terminal galactose (Gal) or *N*-acetylgalactosamine (GalNAc) on their *N*-linked carbohydrate chain (2, 280, 285). The endocytosis mechanism of this receptor-bound asialoglycoprotein (ASGP) through coated pits as well as vesicles, and the pathway of these ligands to lysosomes where they are degraded, have been intensely investigated, and will be discussed in the "Receptor Mediated Endocytosis" section.

Structure of the Mammalian ASGPR

The functional mammalian ASGPR is characterized as a hetero-oligomer composed of two subunits that are encoded by two different genes (287). The two human homologous ASGPR subunits, , entitled human hepatic lectin (hHL)-1 for the major and hHL-2 for the minor subunit, share 59 % sequence homology with a molecular weight of ~45 kDa and 50 kDa, respectively (288). Similarly, two ASGPR subunits were isolated from mouse liver, and designated mouse hepatic lectin (mHL) 1 and 2. In contrast, three homologous ASGPR subunits were identified in rat liver, which are named rat hepatic lectin (rHL) 1, 2 and 3, and are ~42, 49 and 54 kDa in size, respectively (287, 289). These three rat ASGPR subunit proteins originate from separate genes. While rHL-1 comes from a single gene, both rHL-2 and rHL-3 are encoded by a distinct second gene. Although the polypeptide backbones of rHL-2 and rHL-3 proteins are the same, variations in their post-translational glycosylation lead to differences in their electrophoretic mobility (289).

The amino acid sequences derived from the cDNAs of the human, mouse and rat ASGPR gene products show significant similarities. The major subunits, however, are more closely related to each other than to their respective minor subunits across species (290). For example, mHL-1 and hHL-1 share 89 % and 80 % similarity with rHL-1, respectively, while mHL-2 and hHL-2 are 80 % and 62 % identical to rHL-2, respectively. All the ASGPR subunits are type II transmembrane proteins containing a cytosolic N-terminal domain, a single transmembrane segment, a stalk domain consisting of heptad repeats characteristic of α-helical coiled-coil structure, and a Ca^{2+}-dependent extracellular C-terminal carbohydrate recognition domain (CRD), which specifically binds to terminal Gal or GalNAc residues on the glycan (Figure 12) (284, 291).

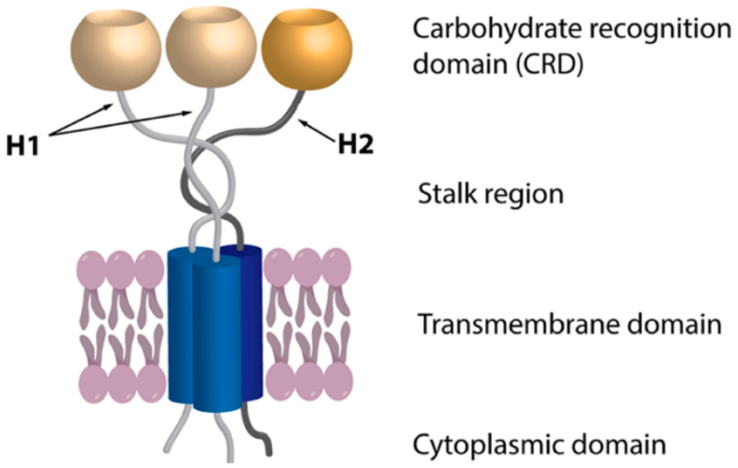

Figure 12. Schematic representation of the ASGPR, illustrating the hetero-oligomer composed of two HL-1 and one HL-2 subunit containing N-terminal cytoplasmic domain, transmembrane domain, extracellular stalk region and carbohydrate recognition domain (CRD) (Figure adapted from Huang *et al*., 2017 (292), with minor modifications).

Although it is generally agreed that the functional ASGPR is a hetero-oligomeric complex consisting of two types of subunits, there are still disputes on the subunit stoichiometry and size of the native ASGPR in hepatocytes. While Henis *et al.* reported that HL-1/HL-2 ratio as 3:1 (293), Bider *el al.* indicated that there is a formation of functional 2:2 HL-1/HL-2 hetero-tetramers (291). Nevertheless, literature data reports a dominant biological role of the HL-1 subunit of the ASGPR (294). For instance, a previous study showed that expression of the human HL-1 subunit alone in transiently transfected COS-7 cells resulted in high-affinity binding of asialofetuin (ASF), one of the most commonly used ligand for ASGPR (294). Similarly, Braiterman *et al.* found that rHL-1 polypeptide, when expressed in the absence of rHL-2, formed homo-oligomers that were carried to the cell surface where it showed high affinity for a highly galactosylated synthetic ligand despite losing its capacity to bind asialoorosmuccoid (ASOR), another high affinity ligand for ASGPR (295). The same group then suggested that the structure of the galactosylated ligand might also dictate whether both subunits are required for binding since synthetic ligand they used in their study contained 5-10 times more and differently organized Gal residues than does ASOR. On the other hand, Tozawa *et al.* suggested that assembly of both HL-1 and HL-2 subunits are required to obtain functional, high affinity receptors on the cell surface (296). The absence of the minor

ASGPR subunit in mice (mHL-2) caused by the disruption of the corresponding gene, resulted in substantial reduced expression of the major mouse subunit (mHL-1) in their livers, thereby preventing the clearance of ASOR in their circulation (296). Similar observation has been made as mHL-1 deficient mice demonstrated no quantifiable expression of mHL-2 and are unable to clear ASOR from the circulation. Interestingly, the same authors also reported that there was no accumulation of asialylated glycoproteins in the circulation or any phenotype abnormalities in the both mHL-1 and mHL-2 deficient mice despite severely impaired clearance of ASOR by ASGPRs.

Structure of the CRD of HL-1 Subunit

Crystallography study of the CRD of HL-1 subunit was first presented by Meier *et al.* in 2000 (297). It consists of a globular protein structure containing six long β-strands, two short, but conserved β-strands, and two α-helices. As a long-form CRD, it contains three conserved disulfide bridges, as opposed to short-form CRD with two disulphide bridges, and a prominent glycine-rich loop, which sticks out from the receptor surface as part of the sugar-binding site (Figure 13). The CRD of HL-1 is the first reported CRD structure to contain three Ca^{2+} ions as an integral part of the structure. When a Gal or GalNAc monosaccharide binds to the sugar-binding site, the 3- and 4-hydroxyl groups of the sugar directly interact with the Ca^{2+} at position 2 (Figure 13). Moreover, the sugar makes four more hydrogen bonds to residues Gln239, Asp241, Gln252, and Asn264 within HL-1.

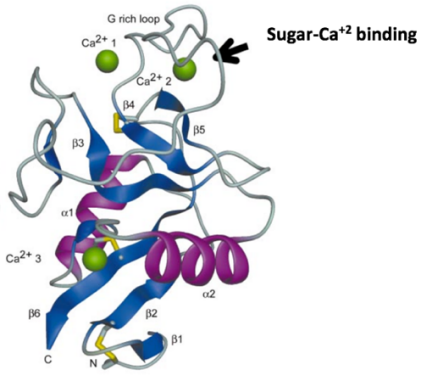

Figure 13. Ribbon diagram of the HL-1-CRD (Figure adapted from Meier *et al*., 2000).

β-strands are shown in blue, α-helices in magenta, the calcium ions in green and the three disulphide bridges in yellow.

In addition to the primary binding site, a shallow hydrophobic pocket on the surface of the CRD serves as a binding subsite for the N-acetyl substituent of GalNAc, which most likely explains why the receptor has a higher affinity for GalNAc than Gal (298).

Receptor Mediated Endocytosis

Receptor mediated endocytosis (RME) is a common process for the uptake of macromolecules by cells, and ASGPR is one of the prominent models of an endocytic transport receptor (299). Binding of ligand to ASGPR occurs in presence of Ca^{+2} at pH >6 (282). Upon ligand binding to ASGPR, the receptor-ligand (RL) complex accumulate in clathrin-coated pits, which subsequently pinch off from the plasma membrane to form larger early endosomes (280) (Figure 14). These formed endosomes are then transported to and fuse with an organelle where RL complexes dissociate from each other due to acidification before undergoing a sorting process (300). Following the sorting process, the ASGPR recycles back to the cell membrane while the ligand undergoes a lysosomal degradation. Even in the absence of ligands, it is known that the ASGPR is constitutively internalized and recycled back to the membrane. Therefore, at steady-state, only 40-60 % of the total cellular ASGPR are present on the cell surface and the remaining is distributed intracellularly (301). However, the rate of endocytosis is enhanced following ligand binding to extracellular domain of the receptor (302).

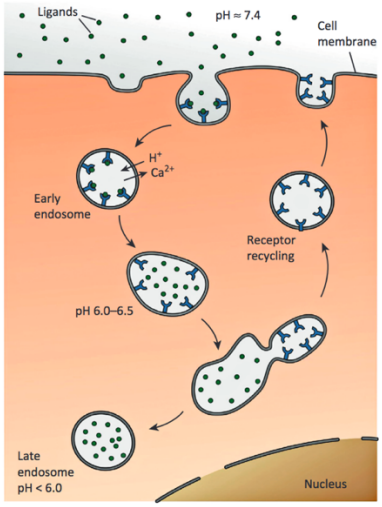

Figure 14. Receptor-mediated endocytosis via clathrin-dependent pathway following ligand binding (Figure adapted from Andersen and Moestrup, 2014 (303)).

Ligand Specificity and Affinity

Many studies have been conducted with both natural and synthetic carbohydrates to establish the structure-affinity relationship for the ASGPR. The ligand type (304), the valency (binding hierarchy of polyvalent ligands tetra- > tri- >> bi- >> mono-antennary) (305), the spacing of the carbohydrate ligands (306) and the size of glycosylated particles (limited by the perisinusolidal space to a diameter of ≤ 70 nm *in vivo*) (304) plays a crucial role in the binding and internalization of ligands by the hepatic ASGPR. The affinity and specificity of the ASGPR result from oligovalent interactions with its physiological ligands, which Lee *et al*. refer to as the "cluster glycoside effect" (305).

ASGPR is known to bind sugars with non-reducing terminal Gal or GalNAc, with GalNAc showing 10–50-fold higher affinity than Gal (282). In addition to terminal Gal or GalNAc, some studies have shown that α-2,6-sialylated glycans can also be recognized by mouse and rat ASGPR. Park *et al.* reported that ASGPR mediated the rapid clearance of glycoproteins bearing terminal Gal with α-2,6-Sia or GalNAc with α-2,6-Sia, the latter having a high affinity for the rat and mouse ASGPR, as opposed to the ones with α-2,3-linked Sia (307). Similarly, Steirer *et al.* observed that the ablation of the ASGPR in mice resulted in significant increases in the plasma concentrations of α-2,6-sialylated glycoproteins, indicating the important contribution of ASGPR to regulate relative concentration of plasma glycoproteins (308). Another recent study confirms that the linkage is relevant because a glycoprotein containing α-2,6-linked sialoglycans was cleared faster than that containing α-2,3-linked sialoglycans in mice, although this difference in pharmacokinetic analysis did not reach statistical significance (214). These studies suggest that the type of sialylation linkage can also have a great impact on the binding of glycoproteins to the ASGPR.

Aside from the specificity for terminal Gal/GalNAc residues as well as type of sialic acid linkage, binding to the ASGPR greatly depends on the valency of the ligand. Due to the cluster effect, the affinity of Gal binding increased 100-1000-fold from mono- to tri-antennary Gal structure. While the affinity of a single galactose residue for an individual receptor subunit is in the millimolar range, bi-, tri- and tetra-antennary asialylated glycoproteins exhibit dissociation constants, K_d, of 10^{-6}, 5 x 10^{-9}, and 10^{-9} M, respectively (309). However, it is important to note that

the binding requirement of the cell-surface receptor is largely satisfied by the tri-antennary structure since the fourth Gal moiety present in the tetra-antennary ligand does not noticeably increase the affinity (310). In addition to the valency of the ligand, Managit *et al.* reported that the higher surface density of Gal is associated with the higher binding strength to ASGPR (311). Similarly, Staud *et al.* showed that increasing Gal density of Gal-proteins, including Gal-bovine serum albumin, Gal-superoxide dismutase, Gal-chicken egg white lysozyme, resulted in an increase in hepatic uptake and gradual decrease in biliary excretion rate (312).

Sugar spacing has a substantial impact on the order of binding in branched Gal (30-20 Å > 10 Å > 4 Å) (282). It was revealed that only the terminal residues are essential for specific ASGPR recognition, and that the binding mechanism involves the simultaneous interaction of 2-3 sugar residues with 2-3 heterooligomeric receptor binding sites where the sugars are spaced 25 Å to 30 Å apart on the native receptor (Figure 15) (310). Likewise, Biessen *et al.* reported that the affinity of galactosides for the ASGPR significantly increased with increasing spacer length where galactosides with spacers of 20 Å possessed an at least 2000-fold higher affinity compared to that lacking the spacer (306). Therefore, for a simultaneous binding of sugars in the receptor's CRD, sugar residues should ideally be 20-30 Å apart.

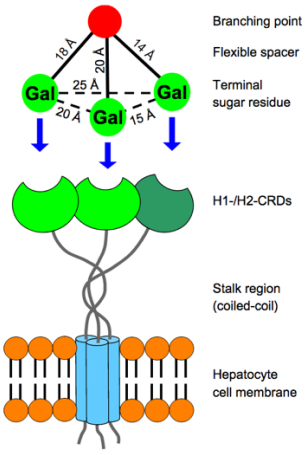

Figure 15. Binding model for ASGPR ligands in an optimal conformation to the heterooligomeric receptor consisting of HL-1 and HL-2subunits (Figure adapted from Khorev *et al.*, 2008 (310))

Dashed line demonstrates the distance between the C4 of each Gal moieties and filled line indicates the approximate distance between branching point and C6 of Gal residues.

Chapter 3: High-level Production of Wild-type and Oxidation-resistant Recombinant Alpha-1-antitrypsin in Glycoengineered CHO Cells

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Presentation of the Article

This chapter presents the research article entitled *" High-level production of wild-type and oxidation-resistant recombinant alpha-1-antitrypsin in glycoengineered CHO cells ",* which was published on May 4th, 2022 in *Biotechnology and Bioengineering Journal, volume 119, pages 2331-2344,* DOI: 10.1002/bit.28129.

In this work, we aimed to engineer a cumate-inducible CHO cell line in order to produce recombinant wild-type (WT) A1AT and a mutein version for increased oxidation resistance with human-like *N*-glycosylation pattern at high yields. To this end, using CRISPR/Cas9 technology, we made functional knock-outs of two glycosylation-gene targets, followed by an overexpression of human α-2,6-sialyltransferase in CHO cells. For the production of rA1AT, the strength of the inducible CR5 promoter was compared with five other constitutive promoters commonly used in industry. Within four weeks post-transfection, glycoengineered stable pools were capable of

producing over 2.1 and 2.8 g/L of recombinant WT and mutein forms of A1AT, respectively, with predominant A2G2S2 *N*-glycan structure, which is similar to that of plasma purified A1AT (Figure 16). Supplementation of *N*-acetylmannosamine to the cell culture media during production improved overall A1AT sialylation along with the proportion of A2G2S2 *N*-glycans, better mimicking native human A1AT glycosylation pattern. Finally, both rA1ATs showed comparable *in vitro* inhibitory activity with plasma-purified A1AT, and substitution of critical Met 351 and 358 residues with Val rendered A1AT mutein more resistant to oxidation without affecting its anti-elastase activity.

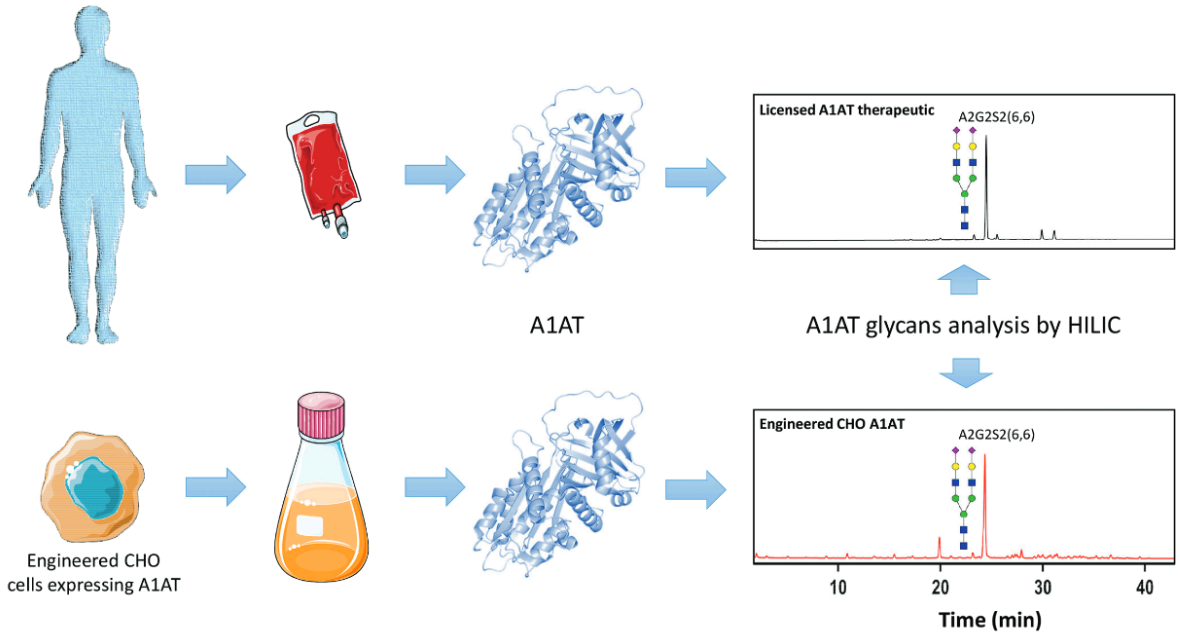

Figure 16. When assessed by HILIC, recombinant A1AT expressed by engineered CHO cells displayed an *N*-glycan profile similar to that of plasma-purified A1AT (Figure by courtesy of Dr. Yves Durocher).

Abstract

Alpha-1-antitrypsin (A1AT) is a serine protease inhibitor which blocks the activity of serum proteases including neutrophil elastase to protect the lungs. Its deficiency is known to increase the risk of pulmonary emphysema as well as chronic obstructive pulmonary disease. Currently, the only treatment for patients with A1AT deficiency is weekly injection of plasma-purified A1AT. There is still today no commercial source of therapeutic recombinant A1AT, likely due to significant differences in expression host-specific glycosylation profile and/or high costs associated with the huge therapeutic dose needed. Accordingly, we aimed to produce high levels of recombinant wild-type A1AT, as well as a mutated protein (mutein) version for increased oxidation resistance, with *N*-glycans analogous to human plasma-derived A1AT. To achieve this, we disrupted two endogenous glycosyltransferase genes controlling core α-1,6-fucosylation (*Fut8*) and α-2,3-sialylation (*ST3Gal4*) in CHO cells using CRISPR/Cas9 technology, followed by overexpression of human α-2,6-sialyltransferase (*ST6Gal1*) using a cumate-inducible expression system. Volumetric A1AT productivity obtained from stable CHO pools was 2.5- to 6.5-fold higher with the cumate-inducible CR5 promoter compared to five strong constitutive promoters. Using the CR5 promoter, glycoengineered stable CHO pools were able to produce over 2.1 g/L and 2.8 g/L of wild-type and mutein forms of A1AT, respectively, with *N*-glycans analogous to the plasma-derived clinical product Prolastin-C. Supplementation of *N*-acetylmannosamine to the cell culture media during production increased the overall sialylation of A1AT as well as the proportion of bi-antennary and disialylated A2G2S2 *N*-glycans. These purified recombinant A1AT proteins showed *in vitro* inhibitory activity equivalent to Prolastin-C and substitution of methionine residues 351 and 358 with valines rendered A1AT significantly more resistant to oxidation. The recombinant A1AT mutein bearing an improved oxidation-resistance described in this study could represent a viable biobetter drug, offering a safe and more stable alternative for augmentation therapy.

Key words: A1AT, CHO pool, CRISPR/Cas9, glycoengineering, *N*-glycosylation, oxidation, cumate inducible expression

Introduction

Alpha-1-antitrypsin (A1AT), encoded by the SERPINA1 gene, is an abundant serum protein secreted primarily by the liver in humans. A principal physiological function of A1AT is inhibition of elastase, a serine protease released from neutrophils, for protection of the lung's connective tissue during inflammatory responses (313). A large number (>100) of polymorphisms have been reported in the coding sequence of the SERPINA1 gene, resulting in diverse A1AT variants, which can affect both protein levels in circulation and/or activity of A1AT (71). In the event of A1AT deficiency, unrestrained neutrophil elastase activity breaks down elastin, causing a decrease in the elasticity of the lungs due to degradation of connective tissue (314, 315). Respiratory complications associated with A1AT deficiency, such as chronic obstructive pulmonary disease (COPD), affects more than 3 million people around the world (71). In addition to respiratory diseases, A1AT deficiency is associated with liver dysfunction caused by aggregation and polymerization of abnormal A1AT mutants in hepatocytes (119). Currently, the only treatment available for patients with A1AT deficiency is life-long intravenous injection of plasma-purified A1AT with a recommended weekly dose of 60 mg/kg of body weight (13). However, with such extended, high-dose therapy, there are some concerns about batch-to-batch heterogeneity of plasma-purified product in terms of glycosylation and quality (13). Moreover, such therapies can be extremely expensive and harbor a risk, however very low, of pathogen transmission (13). These issues underlie the need to identify alternative and cost-effective sources of this protein, ideally produced recombinantly in a cell line suitable for biomanufacturing.

Under normal conditions, A1AT binds irreversibly to neutrophil elastase through its active site where methionine (Met) 351 and 358 residues reside (316). Modification of these Met residues due to exposure to oxidizing agents derived from both endogenous sources, involving activated inflammatory cells, or exogenous sources, such as air pollution and cigarette smoke, results in significant loss of A1AT inhibitory activity (106). Several studies have demonstrated that the sensitivity of A1AT to oxidants contributes to the pathology of pulmonary emphysema in smokers where A1AT is partly inactivated, making it less capable of inhibiting elastase (317, 318).

Thus, an oxidation-resistant variant of A1AT has been previously designed which may provide more durable protection against proteases in vivo (107).

Mature human A1AT is a 52 kDa glycoprotein that consists of a single polypeptide chain with about 14% (w/w) carbohydrates (95). It is *N*-glycosylated at asparagine (Asn) residues 46, 83 and 247 (70). Previous studies have shown that the native human plasma-derived A1AT contains predominantly α-2,6-disialylated bi-antennary structures with little or no core-fucosylation and some α-2,3/6-trisialylated tri-antennary complex-type *N*-glycan structures with and without core-fucose in the molar ratio of 2:1 (98, 319). In addition to bi- and tri-antennary glycans, traces of tetrasialylated tetra-antennary structures were also detected (98).

Although glycosylation is not crucial for the activity of A1AT, it has been demonstrated to greatly affect its in vivo half-life (99). Notably, sialic acid, which is a terminal negatively-charged monosaccharide found on complex *N*-glycans, contributes to extension of A1AT serum half-life (320). While sialylated A1AT has a circulating half-life of 7-9 days in human patients (Campos et al., 2013), asialylation lowers half-life to 5 minutes in blood (320, 321). This can be explained by the presence of asialoglycoprotein receptors (ASGPRs) at the surface of hepatocytes that recognize and bind to asialylated glycoproteins containing terminal galactose (2). Once bound, ASGPRs rapidly internalize asialylated glycoproteins and direct them for degradation (2). In addition to its effect on protein turnover, glycosylation has been also shown to influence immunogenicity of therapeutic proteins in general (322, 323). When expressed in heterologous systems (e.g. production of human therapeutics in insect or plant cells), incorporation of non-human glycans could cause immunogenicity, thereby inducing humoral and/or cell-mediated immune responses (5, 324). Thus, it is valuable to produce A1AT in an expression system capable of making desired human-like post-translational modifications (PTMs) for therapeutic use.

At the present time, there is no recombinant source of A1AT approved for clinical use even though many groups have successfully expressed recombinant A1AT (rA1AT) in various prokaryotic and eukaryotic expression systems. None of these have offered a viable, cost-effective alternative for manufacturing therapeutic A1AT, due in part to the difficulty in producing a human-like glycosylation pattern as well as in achieving productivity levels necessary for its

commercial viability. With these criteria in mind, a cumate-inducible CHO cell line was engineered to produce A1AT with human-like glycosylation by knocking out (KO) two glycosyltransferase genes using CRISPR/Cas9 genome editing. Overexpression of human *ST6Gal1* gene in KO cell line displayed high levels of α-2,6-sialylation. Using the strong inducible CR5 promoter, within 4 weeks post-transfection, we were able to develop stable pools producing over 2.1 g/L and 2.8 g/L of recombinant WT and mutein forms of A1AT with predominant A2G2S2 *N*-glycan structure. By supplementing the culture medium with *N*-acetylmannosamine (ManNAc), the overall sialylation of rA1AT mutein as well as the proportion of A2G2S2 N-glycans were increased, better mimicking native human A1AT glycosylation pattern. The purified recombinant A1AT proteins retained inhibitory activity comparable to Prolastin-C. Finally, substitution of critical Met 351 and 358 residues with valine (Val) rendered A1AT less susceptible to oxidative inactivation while maintaining its anti-elastase activity. Altogether, we present here engineered CHO cells producing high levels of oxidation-resistant A1AT with a human-like *N*-glycan profile. The resulting A1AT mutein could eventually be used as a biobetter for the treatment of A1AT deficiency disorders while offering enhanced resistance to oxidative inactivation, improved biosafety, and cost-effective manufacturing.

Materials and Methods

Plasmids

For gene disruption by CRISPR/Cas9, separate plasmids encoding Streptococcus pyogenes Cas9 (SpCas9) under control of a human elongation factor-1 alpha (EF1α) promoter and single guide RNA (sgRNA) under control of a human U6 promoter were used. SpCas9, containing N- and C-terminal nuclear localization signals, was linked to a GFP reporter via a 2A self-cleaving peptide (Cas9-T2A-GFP). The sgRNA target design for Fut8 and ST3Gal4 was performed using "CRISPy" (325). The chosen sgRNA target sequences are listed in supplemental data Table 3. All plasmids for the production of wild-type (WT) A1AT (A1ATWT), double-mutant A1ATM351V/M358V and human beta-galactoside alpha-2,6-sialyltransferase 1 (ST6Gal1 or hST6) were derived from the previously developed pTT® vector in which expression cassettes for glutamine synthetase (GS) or dihydrofolate reductase (DHFR) genes were inserted supplemental data Table 4 (187, 326, 327).

Prolastin®-C (Grifols, Barcelona, Spain) was generously provided as a gift. The cumate inducible CR5 promoter was as described previously (328). Synthetic promoters 100RPU1 and 100RPU2 (329), human cytomegalovirus (hCMV5) promoter (330), mouse CMV promoter (mCMV), CAG promoter (a fusion promoter comprising a CMV enhancer, the chicken beta-actin promoter and a rabbit β-globin splice acceptor site), Chinese hamster elongation factor-1α (CHEF1) and EF1α-HTLV hybrid promoter (comprising the EF1α core promoter (331) and the R segment and part of the U5 sequence (R-U5') of the Human T-Cell Leukemia Virus (HTLV) Type 1 Long Terminal Repeat (332) were synthesized by GenScript (Piscataway, NJ). pTT®-GFP plasmid (which is devoid of GS expression cassette) was used as a negative control for cell selection. Human A1AT cDNA and hST6 cDNA optimized with Homo sapiens codon-bias were obtained from GeneArt. The double-mutant A1ATM351V/M358V was generated by PCR-based site-directed mutagenesis using pTT81-A1AT plasmid as a template. The forward primers used for substitutions were 5'-CGCTGGCGCCGTGTTTCTGGA-3' (M351V), 5'-AGCCATCCCCGTGTCCATCCC-3' (M358V) and the corresponding reverse primers were 5'-GCTTCGGTGCCCTTCTCG-3' (M351V), 5'-TCCAGAAACATGGCGCCAG-3' (M358V). PCR products were digested with DpnI to degrade the methylated parental DNA. All plasmids were amplified in *Escherichia coli* (DH5α) and purified via ion-exchange chromatography. Finally, the sequences of the newly designed constructs were verified by Sanger DNA sequencing.

Cell Culture and Transfection for Genome Editing

Previously developed cumate-inducible $CHO^{BRI/rcTA}$ cell line (herein named CHO^{wt}) was used for genome editing as well as stable pool selection, and is described in annex 1 (327). Cells were maintained in BalanCD Transfectory CHO media (BCDT, Irvine Scientific, Irvine, CA, USA) supplemented with 4 mM L-glutamine and incubated in shake flasks (Corning, NY, USA) under agitation (120 rpm) at 37°C, 5% CO_2. For routine culture, cells were diluted every 2-3 days to maintain cell densities below 3 x 10^6/mL. On the day of transfection, cells were seeded at a cell density of 3 x 10^6/mL in a 6 well-plate (1.8 mL/well). The DNA:PEI-Max (polyethylenimine-Max; Polysciences) mixture was prepared at a ratio of 1:8 (w:w), with a total of 2 µg DNA per well. The transfected DNA was a mix of plasmids encoding Cas9_T2A_GFP and sgRNAs targeting *Fut8* and

ST3Gal4 at a ratio of 1:1 (w:w). Equal volume of diluted PEI-Max (16 µg per well to transfect) was added to the diluted DNA and incubated for 5 min at room temperature. Polyplexes were then added to the cells (10% v/v final culture volume) and the plate was incubated at 37°C and 5% CO_2 under constant agitation (120 rpm) for 24 h before fluorescence-activated cell sorting (FACS).

Single Cell Sorting of Genome Edited Cells Using FACS and Clonal Expansion

Prior to FACS, cells were resuspended at a cell density of 2 x 10^6/mL in fresh media and filtered through 70 µm cell strainer (VWR International, PA, USA) into a FACS-compatible tube. Single fluorescent-positive (GFP) cells were sorted into 384-well plates (Corning) containing 50 µL BCDT media supplemented with 4 mM L-glutamine and 0.2% [v/v] Anti-Clumping Supplement (Irvine Scientific) on the MoFlo Astrios EQ (Beckman Coulter, Brea, CA) using Summit software. On the day of sorting, the optical alignment and the instrument performance were assessed using Ultra Rainbow fluorescent particles (Spherotech Inc., Lake Forest, IL). With a 200 mW power 488 nm laser as excitation source, the light scattering signals forward scatter (FSC) and side scatter (SSC) allowed the exclusion of debris- and multiple cell-containing droplets. A 513/26 nm bandpass filter was used for GFP detection. For cell sorting, Cas9-GFP positive cells were gated based on the background signal of non-transfected CHO^{wt} cells. The data were analysed using the FlowJo software version (BD Biosciences). Two weeks after cell sorting, clonal colonies were moved to 96-well plate (Corning) and expanded for lectin blot analysis and Sanger DNA sequencing to verify indels.

Lectin-probed Western Blot Analysis

Lectin blotting of purified proteins (0.3-0.5 µg) were performed using biotinylated *Sambucus nigra* agglutinin (SNA), *Erythrina cristagalli* agglutinin (ECL), *Maackia amurensis* lectin II (MALII) or *Aleuria aurantia* lectin (AAL) (Vector Laboratories, Burlingame, CA, USA) as previously described (187).

Generation of Stable CHO Cell Pools

Cells were seeded 48 h before transfection in BCDT media supplemented with 4 mM L-glutamine to reach a density between 1.5 x 10^6- 1.9 x 10^6/mL. On the day of transfection, cells

with viability greater than 98% were centrifuged and resuspended at a cell density of 2 x 10^6/mL in fresh media. In order to compare the cumate-inducible CR5 promoter to various constitutive promoters and select the most effective one for the generation of high-producing pools, PEI-Max was used to transfect CHO^{wt} cells with the expression vectors containing various promoters as described above at a DNA:PEI ratio of 1:5 (w:w), where the final DNA plasmid concentration in cell culture was 1 μg/mL. The day after transfection, cells were resuspended at 0.5 x 10^6/mL in PowerCHO2 chemically defined media (Lonza, Walkersville, MD, USA) supplemented with 50 μM MSX and the cells were passaged in fresh selection media every 2-3 days until the cell viability reached 95%. Cell number and viability was measured using the Cedex AS20 cell counter with 0,2% (w:v) trypan blue (Roche, Laval, Canada).

Similar protocol was used when generating KO cell line expressing hST6 as well as $A1AT^{WT}$ or double-mutant A1AT. KO cells were first transfected in BCDT media with pTT100-hST6 using the same DNA:PEI-Max ratio and final DNA plasmid concentration in cell culture as described above. 24 h post-transfection, cells were resuspended at 0.5 x 10^6/mL in PowerCHO2 media supplemented with 150 nM MTX and 4 mM L-glutamine. When reaching 95% cell viability, this pool was then retransfected with pTT81-$A1AT^{WT}$ or pTT81-$A1AT^{M351V/M358V}$ plasmids and selected with 50 μM MSX.

Flow Cytometry

To confirm the presence of glycoproteins with terminal α-2,6-sialylation at the cell surface, cells were stained with FITC (fluorescein)-SNA lectin (1/2000) for 30 min at 4°C and then washed once with PowerCHO2 media. Cells were then resuspended in 200 μL of PowerCHO2 media supplemented with 4 mM L-glutamine and 5 μg/mL propidium iodide (Promokine, Heidelberg, Germany). After filtering through a 30-40 μm AcroPrep Advance 96-well filter plate (Pall, Port Washington, NY), stained cells were analyzed on a BD LSR Fortessa (BD Biosciences, San Jose, CA) cell analyzer using BD FACSDiva software. The cytometer performance was first assessed using CS&T performance and tracking beads (BD Biosciences, San Jose, CA). FITC and propidium iodide were excited with a 100 mW 488 nm (blue) laser while 530/30 nm and 695/40 nm bandpass filters were employed for the emitted fluorescences detection, respectively. The FSC and SSC light

scattering signals enabled the discrimination of the cells from the debris. Dead cells were excluded based on propidium iodide fluorescence. The SNA positive cells were gated based on negative CHO^{wt} cell signal when stained with the FITC-conjugated SNA lectin (CHO cells lacking α-2,6-sialylation). A minimum of 10 000 live cells were acquired and the FlowJo software (BD) was used for analysis.

Recombinant A1AT Production from Stable Pools

Three days before induction, cells were seeded in BalanCD Growth A chemically defined media (Irvine Scientific, Irvine, CA, USA) supplemented with 150 nM MTX and 50 µM of MSX to reach 3 x 10^6/mL on induction day and incubated at 37 °C and 5% CO_2 under constant agitation (120 rpm). On induction day, expression of the recombinant protein was induced by adding 2 µg/mL of cumate. MSX concentration was adjusted to 125 µM, and F12.7 feed (Irvine Scientific) was added at 7.5% (v:v). Cells were then moved to a 32°C shaking incubator (120 rpm). Every 2-3 days, cultures were fed with 5-10% (v:v) F12.7 feed and additional glucose was added to maintain a minimal concentration of 17 mM. Supernatants were collected between 5-12 dpi or when the cell viability dropped below 70%. Supplementation with 20 mM *N*-acetylmannosamine (ManNAc) was done 24 h post-induction.

Recombinant A1AT Quantification

Protein samples were heat-denatured at 70°C for 10 min under reducing conditions and loaded on a 4-15% Mini-PROTEAN TGX Stain-Free precast gel (BioRad). The migration was done using standard methods. Following completion of electrophoretic separation, the gel was activated by exposing it to UV light for 5 min and the protein bands were visualized with the ChemiDoc MP imager. The total amount of protein was calculated by quantifying the fluorescent signal in a defined area comprising the A1AT band using Image Lab software (BioRad) and background intensity was subsequently subtracted. Then, the TGX Stain-Free gel was stained with Coomassie blue 0.1% (w:v) in 10% acetic acid / 50% ethanol and the protein bands were quantified by densitometric analysis using Image Lab software (BioRad). Linear regression of samples with known concentration of A1AT (3, 1.5, 0.75, 0.375, 0.188 µg) was performed and the slope of the linear regression was used to calculate rA1AT titer in supernatants.

Recombinant A1AT Purification

Recombinant A1AT proteins were purified using Alpha-1 Antitrypsin Select affinity resins (GE Healthcare) according to the manufacturer's instructions. Briefly, filtered supernatants were loaded on a column equilibrated in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (equilibration buffer). The columns were washed once with equilibration buffer and proteins were eluted with 1 M $MgCl_2$ in 20 mM Tris-HCl, pH 7.4. The fractions containing eluted proteins were pooled and elution buffer was exchanged for PBS using NAP-25 columns (GE Healthcare). Purified proteins were quantified by absorbance at 280 nm using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Glycan Analyses by HILIC-UPLC

Glycans were released from the glycoproteins using Rapid PNGase F (New England BioLabs, Whitby, ON, Canada) at 50°C for 10 min, after a 2 min at 80°C denaturation step. The PNGase F and glycoproteins were removed on Discovery glycan SPE 50 mg column (MilliporeSigma, Oakville, ON, Canada), and the glycans were evaporated to dryness under vacuum. They were then labelled with 2-aminobenzamide (2-AB) (Sigma-Aldrich, Oakville, ON, Canada) (333) The excess dye was removed on PD MiniTrap G-10 (Cytiva, Vancouver, BC, Canada). Glycans were analyzed by HILIC with fluorescent detection using an Acquity UPLC wide-pore glycoprotein BEH amide, 300Å, 1.7 µm, 2.1 X 150 mm (Waters Corp., Mississauga, ON, Canada) with the column heated to 60°C and a flow rate of 0.5 mL/min. Glycans were eluted using 100 mM ammonium formate, pH 4.5 (mobile phase A) and 100% acetonitrile (mobile phase B) starting with an initial ratio of 22:78, followed by a gradient to 44:56 over 43.5 min. Peaks were calibrated with a 2-AB labelled dextran ladder standard (Waters Corp.) and compared to GU values in the database Glycobase NIBRT (https://glycobase.nibrt.ie/glycobase/show_nibrt.action). A recombinant broad specificity neuraminidase (MNV-01) was used to identify peaks containing sialic acid.

Monosaccharide Analyses by HPAEC-PAD

Neu5Ac and Neu5Gc were purchased from MilliporeSigma (Oakville, ON, Canada). The monosaccharide standard containing fucose, galactosamine, glucosamine, galactose, glucose and mannose (100 nmol) were obtained from ThermoFisher Scientific (Mississauga, Ontario). Monosaccharide and sialic acid analyses were performed as previously described (334).

LC-MS/MS Analysis

40 µg of each protein was denatured and reduced for 10 min at 80°C in 10 mM DTT, 50 mM ammonium bicarbonate. Reduced cysteines were alkylated with 37.5 mM iodoacetamide for 20 min at room temperature. Excess iodoacetamide was quenched by further addition of DTT. Half of the sample was treated with PNGase F for 4 h at 37°C and all samples were treated with 1:20 (enzyme:protein) with endoproteinase Glu-C (Promega, Madison, WI, USA) overnight at 37°C. The samples were then heated at 95°C for 10 min to deactivate Glu-C. For both samples with and without PNGase F treatment, half of the sample was treated with 1:10 (enzyme:protein) trypsin overnight at 37°C and the other half was treated with 1:10 (enzyme:protein) chymotrypsin overnight at 25°C. The digests were analysed by nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) using an Ultimate 3000 nanoLC coupled to an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific, Sunnyvale, CA). The digests (approx. 1 pmol) The peptides were injected onto an Acclaim PepMax100 C18 µ-pre-column (5 mm x 300 µm inner diameter, Dionex/Thermo Scientific) and resolved on a 1.7 µm BEH130 C18 column (100 x 100 µm inner diameter; Waters Milford, CA) and separated using a 105 min gradient. HCD-MS/MS was performed on multiply charged ions in a data dependant manner. GlycoPIQ, a software developed in-house, was used to search for *N*-linked glycopeptides and to perform relative glycan abundance measurements for each *N*-glycosylation site. The glycopeptide assignments and relative abundance measurements were validated manually.

Elastase Inhibition Assay

The inhibitory activity of the recombinant A1AT and its mutein towards human neutrophil elastase, was measured as described previously with slight modifications (187). Briefly, 9.35 nM

of neutrophil elastase (MilliporeSigma) was first incubated with various amount of purified recombinant A1AT or human-derived commercial protein (0-75 nM) for 15 min at 37°C. Then, 150 µM of the MeOSuc-Ala-Ala-Pro-Val-AMC fluorogenic substrate (MilliporeSigma) was added to measure the kinetics of the reactions on a SpectraMax 340PC spectrophotometer where fluorescence excitation was at 355 nm and emission at 460 nm.

Analysis of Oxidation Resistance

To determine the effect of methionine oxidation on the inhibitory activity of $A1AT^{WT}$ and its mutein against human neutrophil elastase, 4 µM of each purified recombinant protein was incubated with 0-400 mM hydrogen peroxide (Sigma-Aldrich) in PBS buffer at room temperature for 1 hour. After incubation, the treated recombinant proteins were set up for inhibition assay using human neutrophil elastase at equimolar ratio, as described above.

Results

Generation of a Clonal *Fut8/ST3Gal4* Double KO Cell Line and *N*-glycans Characterization

As there is currently no recombinant source of A1AT for therapeutic use, the main aim of this study was to develop CHO cell lines to produce recombinant A1AT, both wild-type and a mutein with improved oxidation resistance, with glycosylation similar to human plasma-derived product. Because the predominant *N*-glycan found on plasma-derived A1AT is A2G2S2 with mostly α-2,6-sialylation, CHO^{wt} cells were first engineered by knocking-out (KO) the *Fut8* and *ST3Gal4* glycosyltransferase genes, which control core α-1,6-fucosylation and α-2,3-sialylation, respectively, using CRISPR/Cas9. Following transfection of CHO^{wt} cells with sgRNAs targeting the *Fut8* and *ST3gal4* genes along with Cas9_T2A_GFP, FACS-based single cell sorting was conducted based on GFP expression. Non-transfected WT cells were used as a negative control to gate between GFP-positive and GFP-negative cells (Figure 17a, top panel). The top 23% of the cell population expressing the highest level of GFP were single-cell deposited in 384 well plates for clonal expansion and further analysis (Figure 17a, bottom panel).

Following expansion of six clones with confirmed indels in targeted genes, only one clone (dKO2) that reached high viable cell density and viability was kept for *N*-glycan characterization.

Following purification of rA1AT expressed by CHO^{wt} cells and the CHO^{dKO2} clone, specific sugar patterns were visualised by lectin blotting. As expected, rA1AT produced by the WT cells contained both core-fucosylation (detected by the AAL lectin) and α-2,3 sialylation (detected by the MALII lectin) (Figure 17b, lane 1). In contrast, the dKO2 clone expressed rA1AT lacking both core fucosylation and α-2,3 sialylation as no signal was detected with AAL lectin and MALII lectin, respectively (Figure 17b, lane 2). It also had significantly higher levels of terminal galactose residues compared to WT cells due to the lack of terminal sialylation on the glycans (shown by the ECL lectin) (Figure 17b, lane 2). The dKO2 clone was then further engineered for the generation of rA1AT with "humanized" *N*-glycosylation as described in the following section.

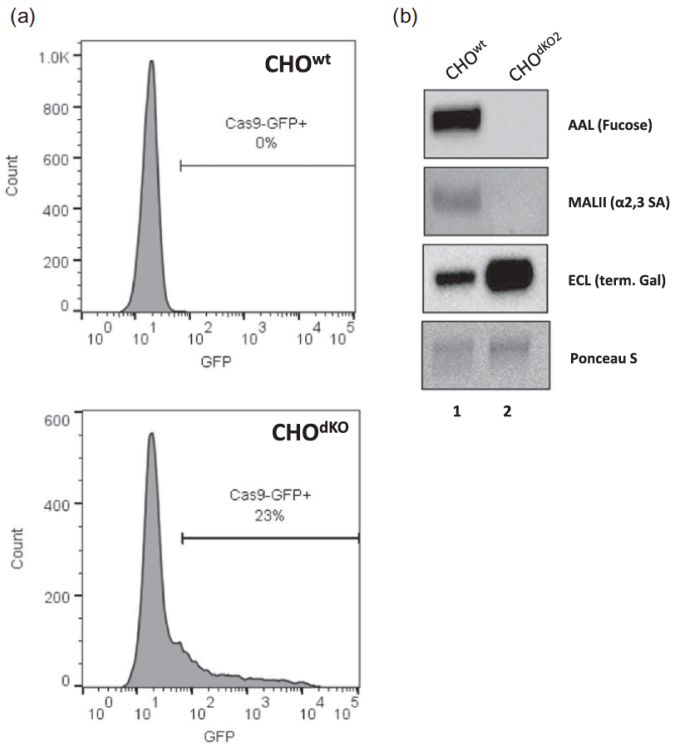

Figure 17. Generation of CHO^{dKO2} double knock-out clonal cell line and its respective *N*-glycan profile. (*a) FACS-based single cell sorting of GFP expressing genome edited cell line (GFP+) with indels in Fut8 and ST3Gal4 glycosyltransferase genes. The most fluorescent GFP+ cells (top 23%) were sorted for clonal expansion to generate homogenous knock-out cell lines. (b) AAL, MALII and ECL lectin blot analysis on rA1AT purified from CHO^{wt} and selected CHO^{dKO2} clone. Ponceau S-stained membrane is used as a protein loading control.*

Introducing h*ST6Gal1* gene in the dKO2 cell line

CHO cells are incapable of producing glycans with α-2,6 sialylation due to lack of ST6Gal1 expression (6). Since α-2,6-sialylated *N*-glycans are the predominant structures found on human plasma-derived A1AT, we aimed to develop a cell line stably expressing ST6Gal1. To this end, the dKO2 clone was transfected with pTT100-hST6 and selected with 150 nM MTX to obtain a stable hST6-expressing pool ($CHO^{dKO2/hST6}$). Cell viability was monitored during MTX selection and the pool reached 95% viability 36 days post-transfection (Figure 18a). After selection, ST6 enzymatic activity was indirectly monitored by detecting α-2,6-sialylation on cell surface glycoproteins by flow cytometry following cell labelling with FITC-SNA lectin, which binds preferentially to sialic acids attached to terminal galactose with a α-2,6-linkage. To determine the proportion of cells with α-2,6-sialylation, FITC-SNA stained CHO^{wt} cells were used to gate between FITC-positive and FITC-negative cells (Figure 18b). As shown in Figure Figure 18b, expression of functional hST6 was confirmed as 90% of the cells within the MTX-selected pool were labeled with the FITC-SNA lectin.

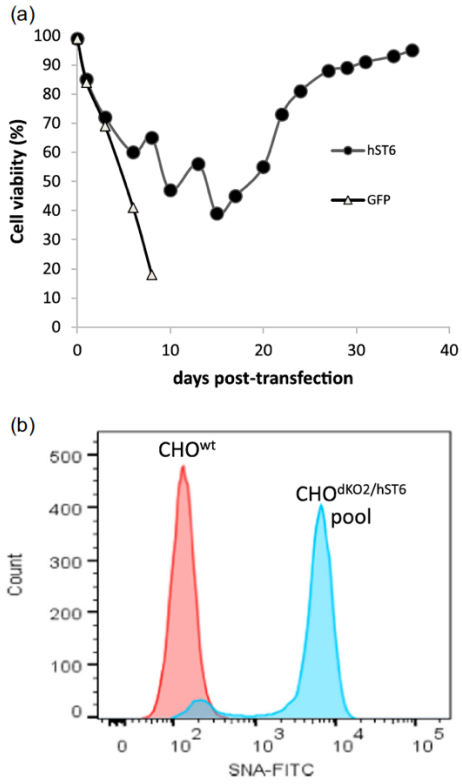

Figure 18. Introduction of α-2,6-sialylation in CHO^{dKO2} cell line. *(a) Cell viability curve of dKO2 cells after transfection with pTT100-hST6 and selection with 150 nM MTX. pTTo-GFPq plasmid was used as a negative control for cell selection. (b) Determination of the α-2,6-sialylation levels on the surface of dKO2 cells expressing hST6 (CHO$^{dKO2/hST6}$) in comparison to CHO^{wt} cells after FITC-SNA lectin staining via flow cytometry. All experiments were carried out twice and shown results represent one set of experiment.*

Selection of the Best Promoter for Highest A1AT Expression from Stable CHO Pool

To maximise volumetric A1AT productivity, we then generated a series of stably-transfected CHO pools with plasmids bearing different promoters including the cumate-inducible CR5 promoter, five commonly used strong constitutive promoters (hCMV5, mCMV, CAG, CHEF1 and EF1α-HTLV), and two recently developed synthetic promoters, 100RPU1 and 100RPU2 (329). The strength of each promoter was compared following stable pool generation from three independent transfections of CHO^{wt} cells with each plasmid construct driving the expression of A1AT (CR5: pTT81; 100RPU1: pTT197; 100RPU2: pTT198; hCMV5: pTT203; mCMV: pTT204; CAG:

pTT205; CHEF1: pTT206; EF1α-HTLV: pTT208). The pools were selected with 50 µM MSX for 20-27 days until >98% cell viability was reached (Figure 19a). After selection, volumetric productivity of each pool was measured at the end of a 5-day fed-batch culture process by SDS-PAGE (stain-free TGX gels imaging and Coomassie blue protein staining) using standards generated by serial dilution of highly purified A1AT protein. The average A1AT titer was highest with the CR5 promoter (1.05 g/L), followed by CHEF1 (433 mg/L), mCMV (400 mg/L), 100RPU2 (296 mg/L), EF1α-HTLV (293 mg/L), CAG (248 mg/L), hCMV5 (180 mg/L) and finally 100RPU1 (158 mg/L) promoters (Figure 19b and c). These results are consistent with previous data comparing the strength of the CR5 promoter to that of CMV5 and EF1-HTLV constitutive promoters in CHO cells (327) and demonstrate that the CR5 inducible promoter can be used to achieve very high-level A1AT expression in CHO cells.

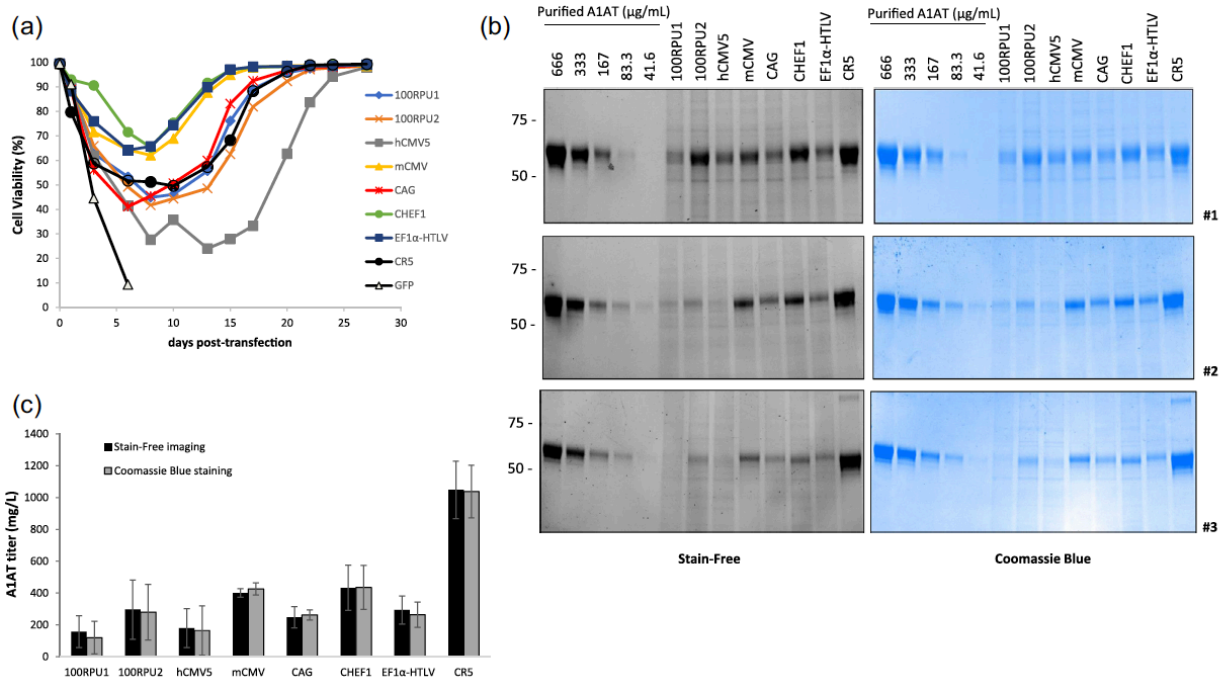

Figure 19. Comparison of cumate-inducible promoter to constitutive promoters for pool generation. *(a) Viability curves of CHO^{wt} cells after transfection with plasmids driving the expression of A1AT through various promoters (CR5, 100RPU1, 100RPU2, hCMV5, mCMV, CAG, CHEF1, EF1α-HTLV) or pTTo-GFPq plasmid (negative control) and selection with 50 µM MSX. (b) A1AT titers in supernatants taken at the end of a 5-day fed-batch culture process from each pool was evaluated by stain-free imaging and Coomassie Blue protein staining using known amounts of purified A1AT protein. (c) Densitometry analysis of the purified protein bands was used to prepare a standard curve and estimate A1AT titers in the supernatants. All experiments were carried out three times and results represent one of the three set of experiment (panel B) or the mean values of the three experiments ± standard deviation (SD; panel C).*

Generation and Production of Human-like Recombinant Wild-type and A1AT Mutein in CHO$^{dKO2/hST6}$ Cells

CHO^{dKO2} cells stably expressing hST6 were then retransfected either with pTT81-A1AT^{WT} or pTT81-A1AT$^{M351V/M358V}$ plasmids and selected with 50 µM MSX for 22 days until cell viability reached >95% (Figure 20a). After recovery, cumate was added to the cultures to induce A1AT expression. Titers measured in supernatants at the end of a 12-day fed-batch culture process

showed that all pools consistently reached similar volumetric productivities with average titers of >2.1 g/L and >2.8 g/L, for A1AT^{WT} and A1AT$^{M351V/M358V}$ pools, respectively (2 independent fed-batch productions) (Figure 20b and c).

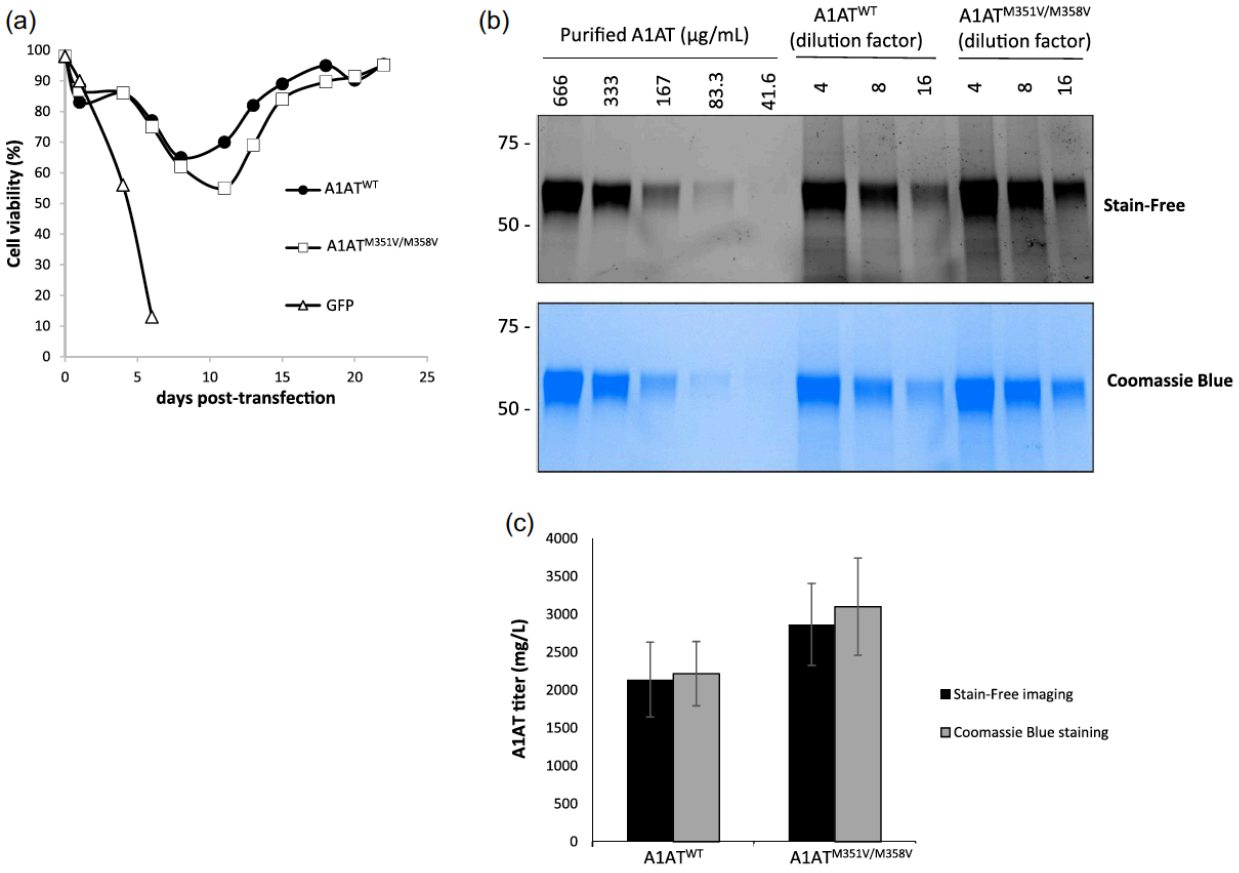

Figure 20. Generation of glycoengineered recombinant wild-type or double-mutant A1AT producing pools. *(a) Viability curves of CHO$^{dKO2/hST6}$ stable pools after retransfection with pTT81-A1AT^{WT} or pTT81-A1AT$^{M351V/M358V}$ and selection with 50 µM MSX. The pTTo-GFPq plasmid was used as a negative control for cell selection. (b) Quantification of A1AT^{WT} and A1AT$^{M351V/M358V}$ in culture supernatants collected at 12 dpi by stain-free imaging as well as Coomassie Blue protein staining using known amounts of purified A1AT protein. (c) Densitometry analysis of the purified protein bands was used to prepare a standard curve and estimate rA1AT titers in the supernatants. All experiments were carried out twice and results represent one set of experiment (panel B) or mean values of two experiments ± SD (panel C).*

Following affinity purification of recombinant A1AT proteins generated by these pools, the respective *N*-glycan compositions were characterized and compared to the commercial plasma-derived therapeutic Prolastin-C using three orthogonal methods: HPAEC-PAD, HILIC-UPLC and nanoLC-MS/MS. HPAEC-PAD analysis of monosaccharides revealed that glucosamine (GlcN), galactose (Gal) and mannose (Man) contents were relatively similar between CHO-produced recombinant proteins and Prolastin-C (Table 2). While traces of fucose (Fuc) were detected in Prolastin-C (0.3 mol Fuc/mol glycoprotein), it remained undetected in rA1ATs, thus confirming the successful KO of the *Fut8* enzyme. Similar to Prolastin-C, sialic acid analysis of each CHO-derived proteins revealed traces of non-human *N*-glycolylneuraminic acid (Neu5Gc) (Table 2).

Table 2. Monosaccharide and sialic acid molecular ratio (mol monosaccharide/mol glycoprotein) after hydrolysis or enzymatic release for rA1ATs produced in glycoengineered CHO cells and plasma-derived Prolastin-C.

Monosaccharide	$A1AT^{WT}$	$A1AT^{M351V/M358V}$	$A1AT^{M351V/M358V}$ + ManNAc	Prolastin-C
Fuc	nd	nd	nd	0.3 (0.0)
GalN	nd	nd	nd	nd
GlcN	13.4 (0.1)	12.3 (0.2)	12.6 (0.2)	14.2 (0.1)
Gal	7.5 (0.1)	6.8 (0.0)	7.0 (0.1)	7.6 (0.0)
Glc	nd	nd	nd	nd
Man	7.3 (0.6)	6.2 (0.0)	6.2 (0.2)	8.2 (0.2)
Neu5Ac	2.9 (0.1)	2.8 (0.2)	4.1 (0.1)	5.0 (0.1)
Neu5Gc	0.01 (0.00)	0.02 (0.00)	0.01 (0.00)	0.003

Note: Results are means of three triplicate reactions performed on the same samples; the standard deviation is indicated in brackets. Nd: none detected

On the other hand, both WT and the mutant version of A1AT were shown to contain considerably lower levels of *N*-acetylneuraminic acid (Neu5Ac) than Prolastin-C. While Prolastin-C exhibited predominantly fully sialylated A2G2S2 (6,6) *N*-glycans (70.6%), the HILIC-UPLC and nanoLC-MS/MS analyses (Figure 21a, b and supplemental data Figure 23) showed that WT and mutein A1AT exhibited significantly lower proportions of this glycan (20% and 22%, respectively). Prolastin-C also contains small amounts of tri-antennary A3G3S3 and A3FG3S3 *N*-glycans (7.3%

each) and trace amounts of tetra-antennary structures (Figure 21a, b and supplemental data Figure 24), which is in agreement with previous observations (98). In contrast, the glycans released from the $CHO^{dKO2/hST6}$-produced WT and mutein A1AT appeared to be more heterogenous but had HILIC profiles very similar to each other (Figure 21a and b). Both rA1ATs had two main peaks with retention times at around 20 min and 24 min, respectively, which were not detected in CHO^{wt}-produced WT A1AT. They also had a fair number of smaller peaks with retention times above 25 min, which are mostly absent in Prolastin-C, suggesting the presence of glycoforms with higher branching patterns. In CHO^{wt}-produced WT A1AT, FA2G2 eluting at 16 min was found as the main *N*-glycan (36%). In comparison, the major peak eluting at 24 min in $CHO^{dKO2/hST6}$-produced WT and mutein A1AT is consistent with A2G2S2 (6,6), as expected, and represented about 20% of the total glycans. However, in contrast to Prolastin-C, we noted that the 24 min peak was not perfectly symmetrical: a significant "shoulder" eluting earlier is observed and is estimated to represent about 8% and 9% of total glycans in WT and mutein A1AT, respectively. We propose that this shoulder peak may represent a mono-sialylated tri-antennary glycan (A3G3S1) since it disappeared after neuraminidase treatment (supplemental data Figure 25). In addition to the main peak, a second major peak also representing about 20% of the profiles with a retention time of 20 min was observed for $CHO^{dKO2/hST6}$-produced rA1ATs. However, while it is not possible to unequivocally assign a structure to this peak, we believe that it corresponds to a mono-sialylated bi-antennary glycan (A2G2S1), an asialylated tri-antennary glycan (A3G3), or a mixture of these.

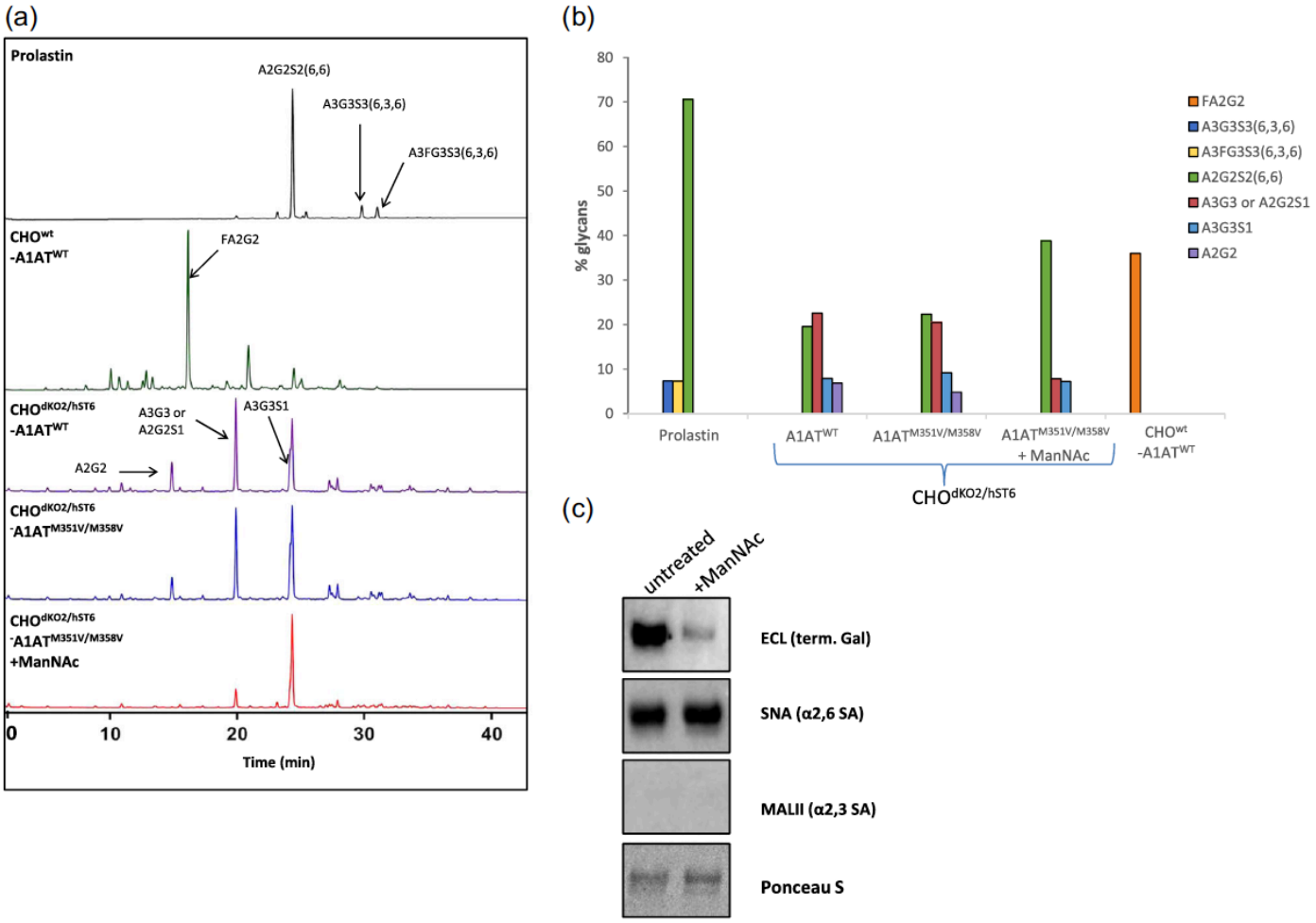

Figure 21. *N*-glycan characterization of purified recombinant WT and double-mutant A1AT from CHO$^{dKO2/hST6}$ pools with or without cell culture media additive in comparison to CHO^{wt}-purified WT A1AT. *(a) HILIC-UPLC analysis of rA1AT purified from each pool and of plasma-derived counterpart, Prolastin-C. (b) Direct comparison of glycan composition between Prolastin-C and rA1AT purified from each pool. (c) Addition of 20 mM ManNAc in cell culture media of double-mutant pool at 1 dpi. ECL, SNA and MALII lectin blot analysis were done on purified ManNAc-treated or untreated A1AT mutein at 12 dpi. Ponceau S-stained membrane is shown for protein loading control.*

Supplementation with *N*-acetylmannosamine to Enhance Sialylation of Recombinant A1AT

In addition to cell engineering strategies, various additives supplemented to the culture medium can enhance the sialic acid biosynthesis pathway and thus increase the sialylation of

glycoproteins during their production. Supplementation of culture medium with ManNAc, a precursor of sialic acid, was previously shown to increase sialylation of recombinant glycoproteins (187, 236, 238). We thus investigated whether the addition of 20 mM ManNAc during production of the mutein pool had a positive effect on A1AT sialylation. Figure 21c shows a significant reduction in ECL lectin (which binds to terminal galactose) binding in ManNAc supplemented culture compared to control culture, indicating that almost all *N*-glycan branches were capped with sialic acid. As expected, no signal was detected with MALII lectin blot (detects α-2,3-sialylation) (Figure 21c). Surprisingly however, no change was observed for the A1AT SNA lectin blot signal (detects α-2,6-sialylation) between control and ManNAc supplemented cultures. We hypothesize that this might be explained if SNA has similar affinities towards mono-, bi- or tri-sialylated glycans. Indeed, HPAEC-PAD analysis revealed that the addition of ManNAc significantly increased the Neu5Ac content of A1AT mutein (4.1 vs 2.8 mol Neu5Ac per mol protein for ManNAc-treated and untreated $A1AT^{M351V/M358V}$ mutein, respectively; Table 2), similar to that of Prolastin-C (5 mol Neu5Ac per mol protein). Furthermore, HILIC-UPLC and nanoLC-MS/MS analysis confirmed almost complete loss of glycans with unsubstituted terminal galactose residues as well as an overall increase (39%) in di-α-2,6-sialylated glycans with ManNAc supplementation (Figure 21a, b and supplemental data Figure 23). Additionally, the "shoulder" peak eluting at 24 min almost disappeared with ManNAc feeding when compared to untreated mutein, suggesting that this glycoform had a higher sialylation level in ManNAc-treated mutein and that additional sialic acid shifted this peak to longer retention times. Together, these results show that addition of ManNAc during production indeed improves α-2,6-sialylation of A1AT mutein, favoring a "human-like" glycosylation pattern.

Anti-elastase Activity of rA1ATs Compared to Commercial A1AT

We then evaluated the inhibitory activity of purified WT and double-mutant rA1ATs against human neutrophil elastase (HNE). A fluorogenic substrate was used to monitor HNE protease activity with and without increasing concentrations of recombinant and human plasma-derived A1ATs. The calculated half maximal inhibitory concentrations (IC_{50}) of $A1AT^{WT}$ and $A1AT^{M351V/M358V}$ (15.4 ± 5.7 nM and 13. 6 ± 3.3 nM, respectively) were found to be not statistically different than that of Prolastin-C (10.5 ± 4.3 nM) ($p= 0.347$; one-way ANOVA) (Figure 22a). These results

confirmed that the anti-elastase activity of both rA1ATs is comparable to that of human-derived commercial protein.

$A1AT^{M351V/M358V}$ is more resistant to oxidation compared to $A1AT^{WT}$

Finally, we investigated if the anti-elastase activity of A1AT mutein was more resistant to oxidative inactivation than $A1AT^{WT}$. Previous studies have demonstrated that the Met^{351} and Met^{358} residues of A1AT are susceptible to oxidation, which results in a loss of anti-elastase activity (107, 110). Accordingly, in the A1AT mutein construct, susceptible Met residues were mutated to valine (M351V/M358V) to generate an oxidation-resistant variant. Figure 22b shows that while $A1AT^{WT}$ drastically lost its ability to inhibit neutrophil elastase under strong oxidizing conditions (>50 mM H_2O_2), the $A1AT^{M351V/M358V}$ construct remained fully active even at the highest H_2O_2 concentration tested.

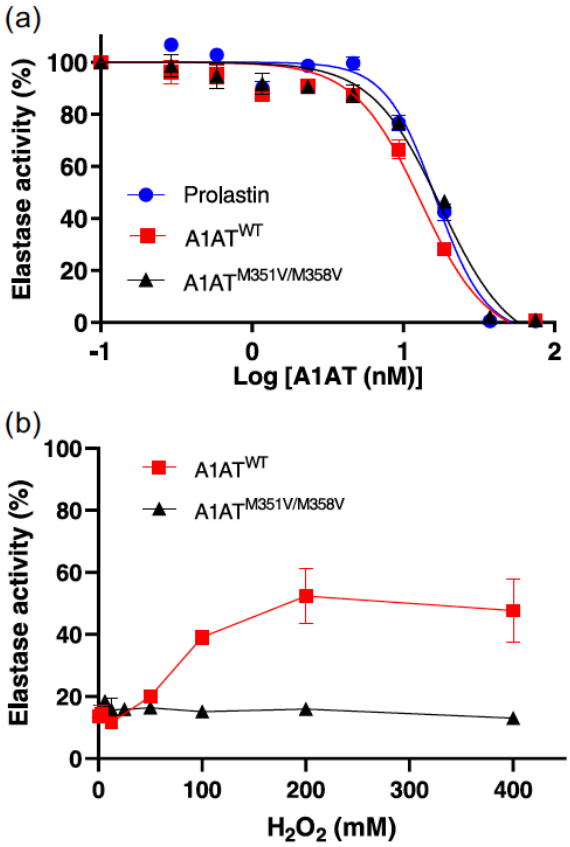

Figure 22. Inhibitory activity of rA1AT proteins against HNE. *(a) Enzymatic activity of HNE was monitored in the absence or in the presence of different concentrations (0-75 nM) of Prolastin-C and rA1AT proteins purified from WT and double-mutant pools. Maximum proteolytic activity of HNE was set to 100%. Experiments were performed four times and shown results represent one of the four set of experiment with error bars indicating SD from duplicates. (b) Effect of methionine oxidation on the anti-elastase activity of WT and double-mutant A1AT upon incubation with 0-400 mM hydrogen peroxide. Error bars represent range of duplicate measurements.*

Discussion

A1AT deficiency is a common hereditary disorder caused by mutations in the *SERPINA1* gene and associated with a high risk for developing emphysema and liver disease (314). Currently, licensed treatment for A1AT deficient-patients is infusions of purified A1AT from pooled human plasma (13). However, this augmentation therapy has drawbacks such as potential risk of contamination with pathogens and lot-to-lot consistency of the plasma-derived product in terms

of glycosylation and quality (13). Moreover, A1AT is susceptible to oxidative inactivation by several oxidants from endogenous and exogenous sources, rendering it less capable of functioning as an antiprotease (106). To address these limitations, we aimed at generating glycoengineered CHO cells capable of producing high levels of recombinant wild-type and oxidation-resistant mutein versions of A1AT, with *N*-glycan patterns resembling those of human plasma-derived protein. Using a previously-described cumate-inducible CHO expression system, and in accordance with previously reported strategies (185, 194), two glycosyltransferase genes (*Fut8 and ST3Gal4*) were first disrupted (dKO2 cells) to allow for expression of A1AT lacking both core-fucosylation and α-2,3-sialylation. Introducing human *ST6Gal1* gene in our dKO2 cells (hST6 pool) then granted the expression of A1AT with α-2,6 sialylation, as previously described by others (196, 199).

A series of strong constitutive promoters were then compared to the cumate-inducible CR5 promoter to drive high levels of A1AT expression in stable CHO pools. Our results showed that volumetric productivity of stable pools generated with the cumate inducible promoter was highest, with an average volumetric titer 2.5- to 6.5-fold greater than the ones generated with constitutive promoters. A possible explanation for this is that the use of an inducible promoter allows for minimising A1AT expression during the pool selection process (done in the "off-mode"), thus reducing cellular stresses/burden caused by recombinant protein overexpression (335-337).

Although a recent study suggested that recombinant A1AT produced in a highly glycoengineered CHO cell line could potentially replace plasma-derived augmentation therapy, the titers obtained from two selected clones were of ~120 mg/L (188). In contrast, we report here unprecedented volumetric productivities of 2.1 g/L for $A1AT^{WT}$ and 2.8 g/L for $A1AT^{M351V/M358V}$ mutein from stable glycoengineered CHO pools. It is unclear if the striking difference in these volumetric productivities are due to a potential negative impact of extensive CRISPR/Cas9 cell engineering, or to the high performance of our expression platform/process. Furthermore, based on cumulated experience with our cumate-inducible CHO platform, it is likely that clonal cell lines isolated from our CHO pools could achieve >2-fold higher volumetric levels of A1AT, making this system even more attractive for manufacturing a commercial product.

Apart from adequate expression levels, appropriate glycosylation is another critical attribute for recombinant proteins intended for therapeutic use. Several studies have reported that the anti-protease activity of A1AT is not influenced by its *N*-glycosylation pattern (186-188). In agreement with these studies, we also found that the inhibitory activity of both rA1ATs towards neutrophil elastase was comparable to that of Prolastin-C. Although glycosylation is not crucial for A1AT protease inhibitory activity, it is worth noting that proper glycosylation may prevent its aggregation and increase its *in vivo* half-life (99). Recombinant A1AT produced in our glycoengineered CHO$^{dKO2/hST6}$ cells in the presence of ManNAc bears predominantly A2G2S2 *N*-glycan with α-2,6-sialylation and very low levels of α-2,3-sialylation in contrast to CHO^{wt}-produced WT A1AT containing asialylated and core-fucosylated *N*-glycans. It is also likely that stable clones isolated from the CHO$^{dKO2/hST6}$ pool may bear significantly higher sialylation levels, as previously demonstrated in another study (188). While a previous study has demonstrated that both mouse and rat ASGPRs are able to recognize glycoproteins with terminal α-2,6- but not α-2,3-linked sialic acid residues (307), the impact of terminal α-2,3 vs α-2,6 sialylation on the serum half-life of A1AT in human is still unclear. Even though CHO-produced recombinant A1AT^{WT} and A1AT$^{M351V/M358V}$ glycans were more heterogenous than those found on Prolastin-C, increased *N*-glycan antennarity may in fact enhance serum half-life, as previously described for erythropoietin (338). Thus, it would be interesting to evaluate in animal models if the increased content in tri- and tetra-antennary sialylated glycans would positively affect rA1AT *in vivo* half-life.

Oxidative inactivation of A1AT is likely contributing to lung damage caused by elastase in individuals exposed to oxidizing agents (339). It is well known that oxidation of Met^{351} and Met^{358} residues located at the reactive site of A1AT results in the loss of its anti-elastase activity (340). To mitigate this, the A1AT$^{M351V/M358V}$ mutein was designed and reported to be significantly less sensitive to oxidation (107). This result was confirmed in our experiments where A1AT$^{M351V/M358V}$ showed much greater resistance to oxidative inactivation, retaining its inhibitory activity under strongly oxidizing conditions, and could be used as a therapy for A1AT deficiency. Indeed, a Phase 1 clinical trial using CHO cell-derived oxidation-resistant recombinant human A1AT-Fc fusion protein (INBRX-101) has recently been initiated (ClinicalTrials.gov Identifier: NCT03815396). However, no details on the glycan profile of this recombinant A1AT-Fc fusion are reported.

In summary, we show that glycoengineered CHO cells combined with the cumate-inducible CR5 promoter may offer a viable expression system to manufacture rA1AT with *N*-glycans resembling those found on native, plasma-derived human A1AT. The recombinant oxidation-resistant mutein offers several potential advantages as a therapeutic for patients with A1AT deficiency; it constitutes a sustainable source of therapeutic protein while decreasing the risk of disease transmission and minimizing batch-to-batch heterogeneity. Moreover, the engineered cell line created here may prove useful for producing other recombinant proteins where fit-for-purpose glycosylation is crucial for their therapeutic efficacy.

Acknowledgments

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Supplemental Data

Table 3. sgRNA genomic target sequences.

Target gene	*Target sequence (5'-3')*
FUT8	*AATGAGCATAATCCAACGCCAGG*
	ATAAAACAATAAGGTCCCCCAGG
ST3GAL4	*GAAACCTTTTCGTACCTATGGGG*
	GCAGTCACGAAAGATCAAGCAGG

Table 4. Plasmids used in this work.

Plasmids	**Promoter**	**Selection marker**	**Recombinant protein**
pTT81-A1AT	CR5	GS/ MSX	human A1AT
pTT81-A1AT M351V/M358V	CR5	GS/ MSX	human A1AT M351V/M358V
pTT100-hST6	CR5	DHFR/ MTX	human ST6Gal1
pTT197-A1AT	100RPU1	GS/ MSX	human A1AT
pTT198-A1AT	100RPU2	GS/ MSX	human A1AT
pTT203-A1AT	hCMV	GS/ MSX	human A1AT
pTT204-A1AT	mCMV	GS/ MSX	human A1AT
pTT205-A1AT	CAG	GS/ MSX	human A1AT
pTT206-A1AT	CHEF1	GS/ MSX	human A1AT
pTT208-A1AT	EF1α-HTLV	GS/ MSX	human A1AT

Site N46	Percentage of Total			
	Prolastin-C	A1AT mutein	ManNAc-treated mutein	WT A1AT
Asialylated	0%	14%	3%	22%
Monosialylated	2%	48%	29%	45%
Disialylated	94%	37%	64%	32%
Trisialylated	5%	1%	4%	1%
Tetrasialylated	0%	0%	0%	0%

Site N83	Percentage of Total			
	Prolastin-C	A1AT mutein	ManNAc-treated mutein	WT A1AT
Asialylated	0%	3%	1%	10%
Monosialylated	1%	53%	20%	59%
Disialylated	51%	36%	58%	26%
Trisialylated	48%	8%	21%	5%
Tetrasialylated	1%	0%	1%	0%

Site N247	Percentage of Total			
	Prolastin-C	A1AT mutein	ManNAc-treated mutein	WT A1AT
Asialylated	0%	7%	2%	5%
Monosialylated	3%	45%	23%	53%
Disialylated	96%	48%	75%	42%
Trisialylated	0%	0%	0%	0%
Tetrasialylated	0%	0%	0%	0%

Figure 23: Sialylation of rA1AT produced by different glyco-engineered CHO pools and Prolastin-C at different sites obtained by nanoLC-MS/MS. *The GlycoPIQ algorithm was used to detect and determine the relative abundance of glycopeptide ions in the nanoLC-MS/MS data.*

Site N46	Percentage of Total			
	Prolastin-C	A1AT mutein	ManNAc-treated mutein	WT A1AT
Biantennary	95%	60%	65%	63%
Triantennary	5%	25%	25%	21%
Tetraantennary	0%	9%	7%	8%
Tetra with LacNAc	0%	1%	1%	1%
High Mannose	0%	2%	1%	2%
Other	0%	3%	2%	5%

Site N83	Percentage of Total			
	Prolastin-C	A1AT mutein	ManNAc-treated mutein	WT A1AT
Biantennary	50%	21%	28%	34%
Triantennary	49%	25%	30%	22%
Tetraantennary	1%	39%	34%	32%
Tetra with LacNAc	0%	14%	8%	12%
High Mannose	0%	0%	1%	1%
Other	0%	0%	0%	0%

Site N247	Percentage of Total			
	Prolastin-C	A1AT mutein	ManNAc-treated mutein	WT A1AT
Biantennary	100%	82%	87%	79%
Triantennary	0%	14%	11%	16%
Tetraantennary	0%	1%	0%	1%
Tetra with LacNAc	0%	0%	0%	0%
High Mannose	0%	1%	1%	2%
Other	0%	1%	1%	2%

Figure 24: Summary of glycan types at the 3 N-glycosylation sites on Prolastin-C and rA1AT as produced by glycoengineered CHO pools and identified by nanoLC-MS/MS and GlycoPIQ.

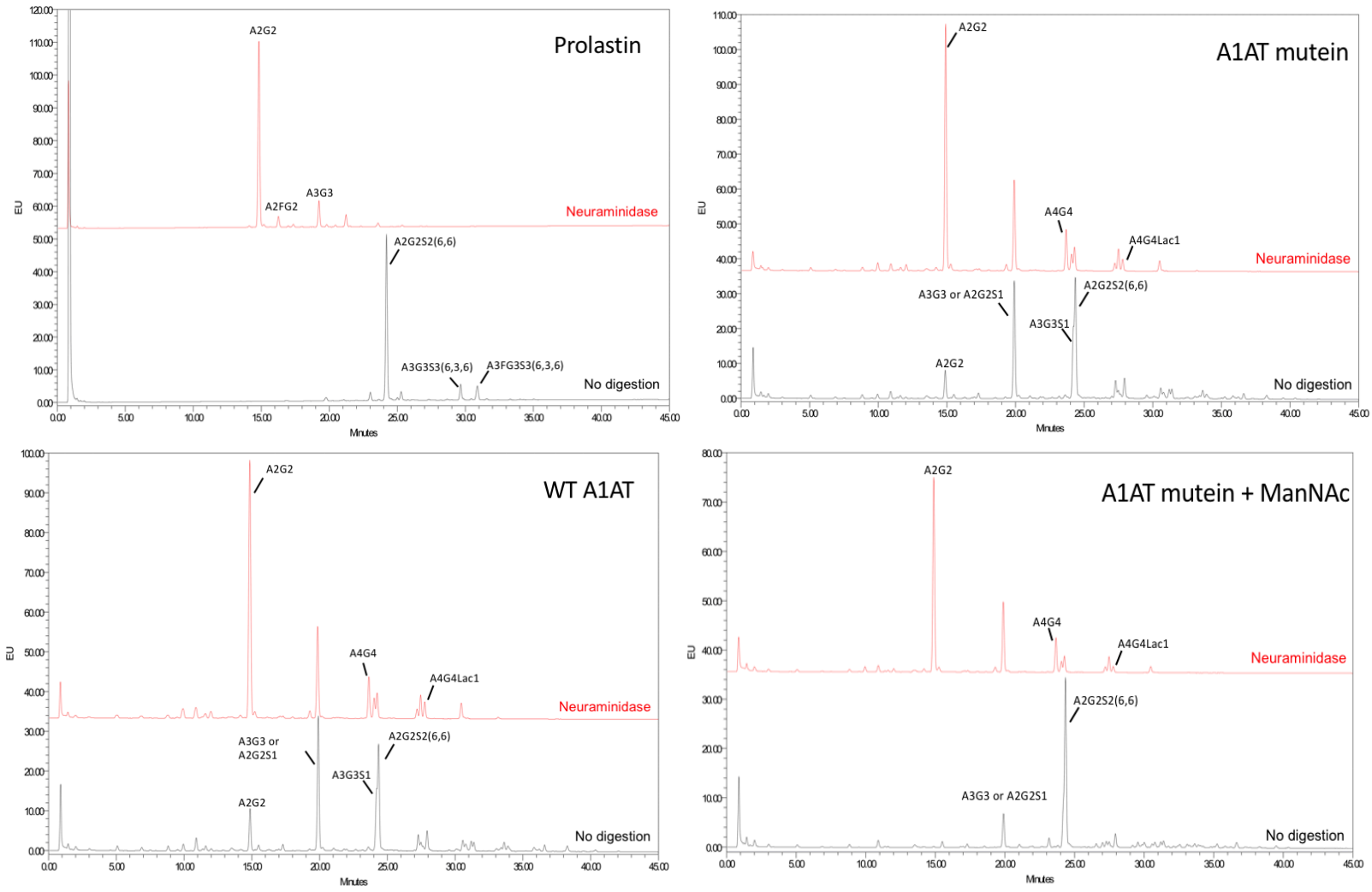

Figure 25: HILIC-UPLC chromatograms of undigested and neuraminidase-treated Prolastin-C and rA1AT samples purified from each pool.

Chapter 4: Impact of A1AT Sialylation on Its Cellular Uptake by Asialoglycoprotein Receptor Orthologs

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Presentation of the Article

This chapter presents the research article entitled " *Impact of A1AT sialylation on its cellular uptake by asialoglycoprotein receptor orthologs ",* which was submitted on May 4th, 2023 in *Scientific Reports Journal.*

In this study, we investigated the impact of different A1AT glycosylation pattern on its interaction with ASGPR orthologs. For this, we first looked at *in vitro* uptake of several glyco-engineered recombinant A1ATs in HepG2 cell line known to express the ASGPR. Because of the poor signal-to-noise ratios obtained with this cell line, we aimed to generate stable CHO pools expressing recombinant human, rat or mouse hepatic lectin 1 (HL-1) and/or HL-2 subunits, and explored their role in ASGPR function with regards to the internalization of glyco-engineered A1ATs. We showed that human HL-1 alone was sufficient to bind and internalize asialylated proteins in contrast to rat and mouse ASGPRs that required both HL-1 and HL-2 subunits to create functional receptors with high affinities. Using FACS, we enriched our CHO pools for cells overexpressing the human, rat and mouse ASGPR on their surface to further increase the signal-to-noise ratio (SNR) of our cell-based internalization assay. Lastly, the glycans of the plasma-derived A1AT clinical product, Prolastin-C, were enzymatically remodelled to compare its uptake

by enriched CHO pools expressing ASGPR orthologs. Our work showed that neither human, rat, nor mouse ASGPR orthologs internalized Prolastin-C when glycans are terminated with α-2,6-Neu5Ac or α-2,8-Neu5Ac-α-2,6-Neu5Ac. On the other hand, the uptake of Prolastin-C containing ~22 % of mono-sialylated bi-antennary glycans with α-2,3-linkage by mouse ASGPR was observed to be statistically higher than that by human and rat ASGPR orthologs.

Abstract

Rapid clearance of asialylated glycoproteins occurs due to the exposure of the penultimate galactose residues, which are recognized by the hepatic asialoglycoprotein receptor (ASGPR), a hetero-oligomer complex composed of two subunits, designated hepatic lectin HL-1 and HL-2. Although it has been proposed that glycans terminating with α-2,6-sialylation, but not those terminating with α-2,3-sialylation, are recognized by mouse and rat ASGPR, little is still known about the impact of sialic acid linkage on glycoprotein interaction with ASGPR orthologs. In this study, we generated stable CHO cell lines expressing human, rat or mouse ASGPR to study their interaction with recombinant A1ATs possessing various glycosylation profiles. While human HL-1 alone accounted for the binding and internalization of asialylated A1AT, both HL-1 and HL-2 subunits were required to form functional and high affinity receptors for the rat and mouse ASGPRs expressed in stable CHO pools. With the aim of improving signal-to-noise ratio of our cell-based uptake assay, we enriched the pools for cells expressing high levels of ASGPRs via fluorescence-activated cell sorting (FACS). Using the clinical plasma-derived A1AT Prolastin-C bearing native or *in-vitro* remodelled glycan structures, our studies revealed no uptake of Prolastin-C when glycans are terminated with α-2,6-Neu5Ac nor α-2,8-Neu5Ac-α-2,6-Neu5Ac by human, rat and mouse ASGPR orthologs. For Prolastin-C bearing biantennary glycans with one branch terminated with α-2,3 sialic acid and the other with terminal galactose, uptake by the mouse ASGPR was statistically higher compared to that of human and rat ASGPR orthologs. Collectively, these findings raise the intriguing possibility that ASGPR orthologs may have slightly different sensitivities towards partially exposed galactose residues.

Key words: Asialoglycoprotein receptor, A1AT, *N*-glycan, sialylation, cellular uptake, CHO cells

Introduction

Asialoglycoprotein receptor (ASGPR) is a well-known hetero-oligomeric membrane-bound C-type lectin receptor expressed primarily in hepatocytes (279, 280). It plays an important role in recognizing and removing asialo-glycoproteins that contain non-reducing terminal galactose (Gal) or N-acetylgalactosamine (GalNAc) from the serum through clathrin-mediated endocytosis, followed by degradation in lysosomes (2, 280, 285). It is highly conserved from rodents to human and is composed of two highly homologous subunits, designated hepatic lectin HL-1 for the major subunit and HL-2 for the minor subunit (290). Both subunits contain a cytosolic N-terminal domain, a single transmembrane segment, a stalk domain, and a Ca2+-dependent C-terminal carbohydrate recognition domain (CRD), which specifically binds to terminal Gal or GalNAc residues on the glycan (284, 291). Although it is generally agreed that the functional ASGPR is a hetero-oligomeric complex consisting of two types of subunits, the exact HL-1/HL-2 subunits stoichiometry in hepatocytes is still not fully understood and the available literature reports some discrepancies (341). While some reported that the ratio of HL-1 to HL-2 is 3:1 (293), others suggested the formation of hetero-tetramers by HL-1 and HL-2 in a 2:2 ratio (291). However, a dominant biological role of the HL-1 was illustrated in the earlier study where cells over-expressing HL-1 could bind asialo-glycoproteins with high affinity in the absence of the HL-2 subunit (294).

Alpha 1-antitrypsin (A1AT) is one of the most abundant plasma-circulating serine protease inhibitor that blocks the activity of a wide spectrum of proteases, particularly neutrophil elastase, in order to protect lungs (313). Human A1AT is a 52 kDa glycoprotein bearing three *N*-glycosylation sites of which 74 % of the glycans are α-2,6-disialylated bi-antennary structures and 14 % are tri-antennary complex-type *N*-glycan structures with a mixture of α-2,3-/α-2,6-linked terminal sialic acid residues (319, 342). Its deficiency is associated with emphysema due to an imbalance between protease/anti-protease activity, which results in degradation of extracellular matrix components of lung tissues caused by unrestrained neutrophil elastase (342). Since mid-1980s, patients with A1AT deficiency have been treated with an augmentation therapy, in which human plasma-purified A1AT is administered to the patients once a week to increase A1AT

concentration in circulation (13, 342). Because of the cost, limited supply and some batch-to-batch heterogeneity of plasma-derived product in terms of glycosylation, many groups have attempted to generate recombinant forms of A1AT in various hosts, yet none is available today as a licensed therapeutic likely because of low volumetric yields and discrepancies in their glycosylation profiles compared to the native version (13). For instance, while A1AT expressed in *E. coli* is non-glycosylated, *Saccharomyces diastaticus* and Chinese hamster ovary (CHO) cells exclusively produce high-mannose and α-2,3-sialylated/core-fucosylated *N*-glycans, respectively, which are nearly absent from circulating human A1AT (13, 143, 186).

Though proper glycosylation is not essential for A1AT activity, presence of sialic acid on the glycoprotein is critical for prolongation of its plasma half-life (99, 320). It was demonstrated that sialylated A1AT undergo a much slower clearance from the circulation in contrast to its asialylated variant, confirming the relevance and clinical importance of proper sialylation (320). The predominant mechanism of asialylated A1AT elimination from the body was also shown to be mediated through the ASGPRs (320). Although some studies have shown that α-2,6-sialylated glycans can also be recognized by mouse and rat ASGPR (290, 307, 308), the impact of α-2,3- vs α-2,6-sialylation on the *in vivo* half-life of A1AT in human is still not clear.

As far as we know, there appear to be no report on how distinct A1AT glycosylation profiles affect its interaction with ASGPR orthologs. To examine this, we first used the HepG2 cells, a human hepatoma cell line known to express the ASGPR receptor (343), to look at *in vitro* uptake of various glyco-engineered recombinant A1Ats. Because of the poor signal-to-noise ratios obtained with this cell line, we sought to generate stable CHO pools expressing recombinant human HL-1 and/or HL-2 subunits, to investigate their role in ASGPR function with regards to the internalization of glyco-engineered A1AT. In addition, we generated stable CHO pools expressing rat or mouse HL-1 and/or HL-2 subunits and demonstrated the requirement of both subunit in mediating the clearance of asialofetuin, a known natural ligand of ASGPR. To improve further the signal-to-noise ratio of our cell-based uptake assay, we enriched our CHO pools for cells overexpressing the human, rat and mouse ASGPR on their surface. Finally, we enzymatically remodeled the glycans of the plasma-derived A1AT clinical product, Prolastin-C, to compare its uptake by enriched CHO pools. Our work shows that comparison of the internalization of α-2,8-

sialylated- and unmodified, α-2,6 sialylated-A1AT by all three ASGPR orthologs revealed no significant difference whereas the uptake of Prolastin-C with ~22% of monosialylated (α-2,3-linkage) biantennary glycans (A2G2S1(3)) by mouse ASGPR was observed to be statistically higher than that by human and rat ASGPR orthologs.

Materials and Methods

Plasmids

Human A1AT and human beta-galactoside alpha-2,6-sialyltransferase 1 (*ST6Gal1* or hST6) complementary DNAs (cDNAs) optimized with *Homo sapiens* codon-bias were obtained from Geneart and cloned into pTT81 and pTT100 vectors, respectively, as described previously (235). Prolastin-C (Grifols) was obtained from the pharmacy. For stable expression of ASGPR in CHO cells, human ASGPR1 (UniProt #P07306) and ASGPR2 (UniProt #P07307.2), rat ASGPR1 (UniProt #P02706) and ASGPR2 (UniProt #P08290) as well as mouse ASGPR1 (UniProt #P34927) and ASGPR2 (UniProt #P24721) cDNA sequences were codon-optimized for expression in CHO cells and synthesized by Genscript. The cDNAs were then cloned into pTT81 vector that possesses an expression cassette for glutamine synthetase (GS) gene (235). All plasmids were amplified in *Escherichia coli* (DH5α) and purified through ion-exchange chromatography. Finally, the sequences of the constructs were verified by Sanger DNA sequencing.

Cell Lines and Protein Expression

Fut8 and ST3Gal4 double knock-out cells (dKO2) as well as dKO2 cells stably expressing human *ST6Gal1* (dKO2/hST6) were derived from $CHO^{BRI/rcTA}$ (herein named CHO^{WT}) cells using CRISPR/Cas9, and were used for stable A1AT expression, as described previously (235). Similarly, CHO^{WT} cells were used for the generation of inducible stable pools expressing human, rat and mouse ASGPRs using a protocol described elsewhere (235).

Protein Purification

All recombinant A1AT proteins produced by glycoengineered CHO cells were purified using A1AT Select resin (GE Healthcare), as described elsewhere (235).

In vitro Sialylation of A1AT

Addition of 2,3-linked Neu5Ac to CHO^{WT}-produced A1AT:

Human ST3Gal4 cDNA with a C-terminal His-tag was transiently produced in FreeStyle-17 media (Gibco) by CHO-3E7 cells as described previously (344). It was purified by immobilized affinity chromatography (IMAC) using a HisTrap excel column (Cytiva), followed by cation-exchange chromatography. For sialylation, the reaction mix included 2 mg of A1AT, 0.2 mg of ST3Gal4, 50 mM MES pH 6.5, 10 mM $MnCl_2$ and 2 mM of CMP-Neu5Ac in a final volume of 1 mL and was incubated at 37°C for 24 h. The reaction progress was followed by isoelectric focussing using a PhastSystem™ (GE HealthCare) and IEF 3-9 PhastGel™ (GE HealthCare, cat. #17054301). The product was purified by anion-exchange chromatography using a Mono Q 5/50 GL column (Cytiva, cat. #17517901) in 20 mM Tris pH 8.0 and a 0-0.5 M NaCl gradient over 16 mL. The fractions containing sialylated A1AT were pooled and the buffer was exchanged to DPBS (Cytiva, cat. #SH30028.03) using a 5 mL HiTrap desalting column (Cytiva, cat. #29048684). The sialylated A1AT was concentrated using an Amicon Ultra-0.5 centrifugal filter (10 kDa MWCO, Millipore, cat. #501024) to 1.85 mg/mL (0.425 mg final recovery).

Addition of 2,3-linked Neu5Ac to asialylated Prolastin-C:

Prolastin C (Grifols) was first asialylated to remove the 2,6-linked Neu5Ac using SialEXO immobilized microspin columns (Genovis, cat. #G1-SM6 -010) according to manufacturer's instructions, except that 2 mg of Prolastin-C was treated with the resin in each column. The asialylation reaction was performed in 50 mM MES pH 6.5 and 100 mM NaCl for 1 h at room temperature. The asialylated Prolastin-C was recovered by spinning the columns for 1 min at 200 x *g*. The resin in the column could be re-used at least 8 times to treat additional 2 mg samples of Prolastin-C. A total of 16 mg of Prolastin-C was treated with 2 SialEXO immobilized microspin columns. The addition of 2,3-linked Neu5Ac to asialylated Prolastin-C (10 mg) was performed using 1 mg of ST3Gal4 in a reaction mix containing 50 mM MES pH 6.5, 10 mM $MnCl_2$ and 2 mM of CMP-Neu5Ac, in a final volume of 2 mL. The reaction mix was incubated at 37°C for 4 h, more ST3Gal4 (0.25 mg) and CMP-Neu5Ac (0.5 mM) were added, and the reaction mix was incubated for a total of 24 h. The reaction was followed by isoelectric focussing as described above. The

ST3Gal4 was removed by capturing it on a 1 mL Ni Sepharose excel column (Cytiva, cat. #17371201) equilibrated in 20 mM MES, 0.5 M NaCl and 5 mM imidazole. Sialylated Prolastin-C was recovered in the flow through and concentrated using an Amicon Ultra-4 centrifugal filter (10 kDa MWCO, Millipore, cat. #801024). The buffer was exchanged to DPBS (Cytiva, cat. #SH30028.03) using a 5 mL HiTrap desalting column (Cytiva, cat. #29048684). The final recovery of Prolastin-C + added 2,3-linked Neu5Ac was 7 mg.

Addition of 2,8-linked Neu5Ac to Prolastin-C

The *Campylobacter* Cst-II α-2,3/8-sialyltransferase was produced with a point mutation (Ile53Ser) as reported previously (345), except that the construct used in this work had a 32 amino acid truncation at the C-terminus (construct CST-81). The reaction mix included 4 mg of Prolastin-C, 0.31 unit of CST-81, 50 mM Tris pH 7.5, 10 mM $MgCl_2$ and 5 mM of CMP-Neu5Ac in a final volume of 2 mL. It was incubated at 37^{o}C for 4 h and the reaction was followed by isoelectric focussing as described above. The product was purified by anion-exchange chromatography using a Mono Q 5/50 GL column (Cytiva, cat. #17517901) in 20 mM Tris pH 8.0 and a 0-1 M NaCl gradient over 20 mL. The fractions containing the Prolastin-C + added 2,8-linked Neu5Ac were concentrated using an Amicon Ultra-4 centrifugal filter (10 kDa MWCO, Millipore, cat. #801024) and the buffer was exchanged to DPBS (Cytiva, cat. #SH30028.03) using a 5 mL HiTrap desalting column (Cytiva, cat. #29048684). The final recovery of Prolastin-C + added 2,8-linked Neu5Ac was 3.1 mg.

Glycan Analysis by Hydrophilic Interaction Liquid Chromatography-Ultra Performance Liquid Chromatography (HILIC-UPLC)

N-glycan analysis of glyco-engineered recombinant A1ATs as well as remodelled Prolastin-C samples was performed as previously described (235).

Protein Labelling

Asialofetuin (ASF) from fetal calf serum Type I (cat# A4781) and fetuin from fetal bovine serum (cat# F2379) were purchased from Sigma. All protein concentrations were adjusted to 2 mg/mL

in DPBS and the conjugation reaction with AlexaFluor-647 (AF647) dye (Invitrogen Molecular Probes) was carried out according to the manufacturer's specifications. Excess dye was removed by a ZebaSpin desalting column (Thermo Fisher Scientific) equilibrated in DPBS. Dye-to-protein ratio was determined by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) at A_{280} and A_{650}.

Internalization Studies

HepG2 cells were seeded in 96-well plates and grown overnight. The next day, cells were washed and incubated with 1 µM of AF647-labelled A1AT glycoforms, ASF and fetuin at 37°C for 3 h. CHO cells stably expressing ASGPRs were induced with cumate 48 h prior to incubation with AF647-labelled A1ATs, ASF and fetuin. Following incubation, the cells were washed thrice with DPBS (with calcium and magnesium) (Wisent, St-Bruno, QC) and then stained with the nuclear dye Hoechst 33342 (Invitrogen Molecular Probes) at room temperature for 15 min. The extracellular background fluorescence was quenched by QSY21 (Invitrogen) and imaging was performed with the ImageXpress Micro XLS (Molecular Devices, San Jose, CA) using a 20X objective, DAPI and Cy5 channels. Images were processed using MetaXpress software (Molecular Devices) and the internalization was measured as integrated fluorescence Intensity (FI)/ cell area.

Western Blotting

ASGPR- transfected cell pellets were resuspended in lysis buffer supplemented with Roche Complete EDTA-free protease inhibitor and incubated on ice for 20 min. The lysate was clarified by centrifugation at 12,000 × *g* for 10 min at 4°C and total protein in supernatant was quantified by Bradford assay (Bio-Rad) before adding Laemmli loading buffer. Western blotting was performed by loading 1.5 µg of protein lysate using rabbit anti-ASGPR1 primary antibody against human HL-1 (Abcam #127896), rabbit anti-ASGPR1 primary antibody against rat and mouse HL-1 (Proteintech #11739-1-AP), mouse anti-ASGPR2-AF647 primary antibody against rat H2 (Santa Cruz #sc-393883), rabbit anti-ASGPR2 primary antibody against human and mouse H2 (Abcam #200196), rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody (Cell Signaling Technology #2118), followed by donkey anti-rabbit-AF647 (Jackson

ImmunoResearch Laboratories #711-605-152), donkey anti-rabbit-AF488 (Jackson ImmunoResearch Laboratories #711-545-152) and goat anti-rabbit HRP Jackson ImmunoResearch Laboratories #111-035-003) secondary antibodies, as described previously (187).

Flow Cytometry

To confirm the presence of ASGPR HL-1 and HL-2 subunits at the cell surface in different pools, cells were first stained with rabbit anti-ASGPR1 primary antibody against human, rat and mouse HL-1 (Abcam #127896), rabbit anti-ASGPR2 primary antibody against human and mouse HL-2 (Abcam #200196), mouse anti-ASGPR2-AF647 primary antibody against rat HL-2 (Santa Cruz #sc-393883), followed by donkey anti-rabbit-AF647 (Jackson ImmunoResearch Laboratories #711-605-152) and donkey anti-rabbit-AF488 (Jackson ImmunoResearch Laboratories #711-545-152) secondary antibodies, as described elsewhere, with the addition of 640 nm laser as excitation source for AF647 (235).

FACS Enrichment

CHO cells stably expressing hHL-1 or rHL-1/rHL-2 or mHL-1/mHL-2 were cultured in PowerCHO2 medium supplemented with 50 µM MSX. Forty-eight hours prior to FACS sorting, cells were induced with 2 µg/ml of cumate and kept at 37°C in a humidified incubator with 5% CO_2. On the day of sorting, cells were first incubated with rabbit anti-ASGPR1 (Abcam #127896) primary antibody against human, rat and mouse HL-1 at 4°C for 30 min. Cells were washed twice with PowerCHO2 medium and incubated with donkey anti-rabbit AF647 (Jackson ImmunoResearch Laboratories) or donkey anti-rabbit-AF488 secondary antibodies at 4°C for 30 min. Cells were then washed twice with PowerCHO2 medium and stained cells sorted on the MoFlo Astrios EQ (Beckman Coulter, Brea, CA). Using 200 mW 488 nm and 640 nm lasers as excitation source, the photomultiplier tubes voltages were adjusted for the following parameters before cell population purification: forward scatter (FSC), side scatter (SSC), 513/26 nm bandpass (BP) filter for FITC detection and 671/30 nm BP filter for AF647. The HL-1 positive cells were gated based on the background signal of non-transfected CHO^{WT} cells. Similar protocol was used when performing the enrichment of HL-2 subunit in enriched rat- and mouse-HL-1 expressing pools.

While anti-ASGPR2-AF647 was used against rat HL-2, cells expressing mouse HL-2 was first incubated with rabbit anti-ASGPR2 primary antibody (Abcam #200196), followed by donkey anti-rabbit-AF488 secondary antibody at 4°C for 30 min.

Confocal Microscopy

CHO^{WT} parental cell line and cumate-induced CHO pools stably expressing human HL-1 were seeded for 24 h onto a doubly coated Fibronectin / Poly-d-lysine 1.5 glass coverslip fused into a 35 mm culture dishes (MatTek) in BalanCD Growth A chemically defined medium (Fujifilm Irvine Scientific) supplemented with 50 µM of MSX (Sigma-Aldrich), 10 % FBS inactivated (VWR) as well as with (CHO-ASGPR) or without (CHO^{WT}) 2 µg/mL of cumate (Ark Pharm Inc). Following adhesion, cells were then treated for 3 h at 37°C, 5% CO_2 with and without various Alexa 647-labeled A1AT glycoforms at 1 µM. After incubation, cells were washed and fixed using 4% paraformaldehyde diluted in DPBS for 30 min at 4°C. Nuclei were stained with Hoechst 33342 (Life Technologies) at 5 µg/mL for 30 min at room temperature. Finally, cells were washed and analysed using the 60X objective of a Fluoview FV10i confocal laser scanning microscope from Olympus. Image J software (NIH) was used for image analysis where cell nucleus and A1AT-Alexa647 signal were represented in blue and in red, respectively.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism v7 software. The level of significance was set at $p < 0.05$ using one-way analysis of variance (ANOVA).

Results

N-glycan Characterization of Glycoengineered A1AT

We expressed A1AT in three distinct CHO cell lines: Wild-type cells (CHO^{WT} or WT), *Fut8 and ST3Gal4* double knock-out cells (dKO2) as well as dKO2 cells stably expressing human *ST6Gal1* (dKO2/hST6) using conditions similar to those described previously (235). Following affinity purification, *N*-glycan composition of the recombinant A1AT generated by these cells was characterized and compared to the commercial human-derived therapeutic A1AT Prolastin-C.

Using HILIC-UPLC analysis, the A2G2S2 (6,6) structure was found to be the predominant (71%) *N*-glycan in Prolastin-C (Figure 26a, b and supplemental data Figure 32) in stark contrast to CHO^{WT}-produced A1AT bearing mainly FA2G2 *N*-glycans (36%). Due to disruption of *Fut8* and *ST3Gal4* genes, A1AT produced by dKO2 cells exhibited predominantly biantennary non-fucosylated and galactosylated A2G2 *N*-glycans, representing 42% of total glycans. Stable expression of the human *ST6Gal1* gene in dKO2 cells significantly reduced the abundance of A2G2 from 42% down to 8% while increasing levels of the A2G2S2 (6,6) *N*-glycans to 20%.

To improve the alpha–2,3-linked sialylation of CHO^{WT}-produced A1AT, *in vitro* enzymatic sialylation (WT+SA) was then performed in the presence of the soluble human beta-galactoside alpha–2,3-sialyltransferase, ST3Gal4. HILIC-UPLC analysis confirmed significant reduction in the proportion of FA2G2 glycans with terminal galactose (36% → 5%) concomitant with an overall increase in the level of FA2G2S2 with α2,3-linked *N*-acetylneuraminic acid (Neu5Ac) up to 39%. Overall, these results illustrate that we were able to generate A1AT with different glycosylation patterns using glycoengineered cells or *in-vitro* glycan remodelling to evaluate their binding and internalization by ASGPRs.

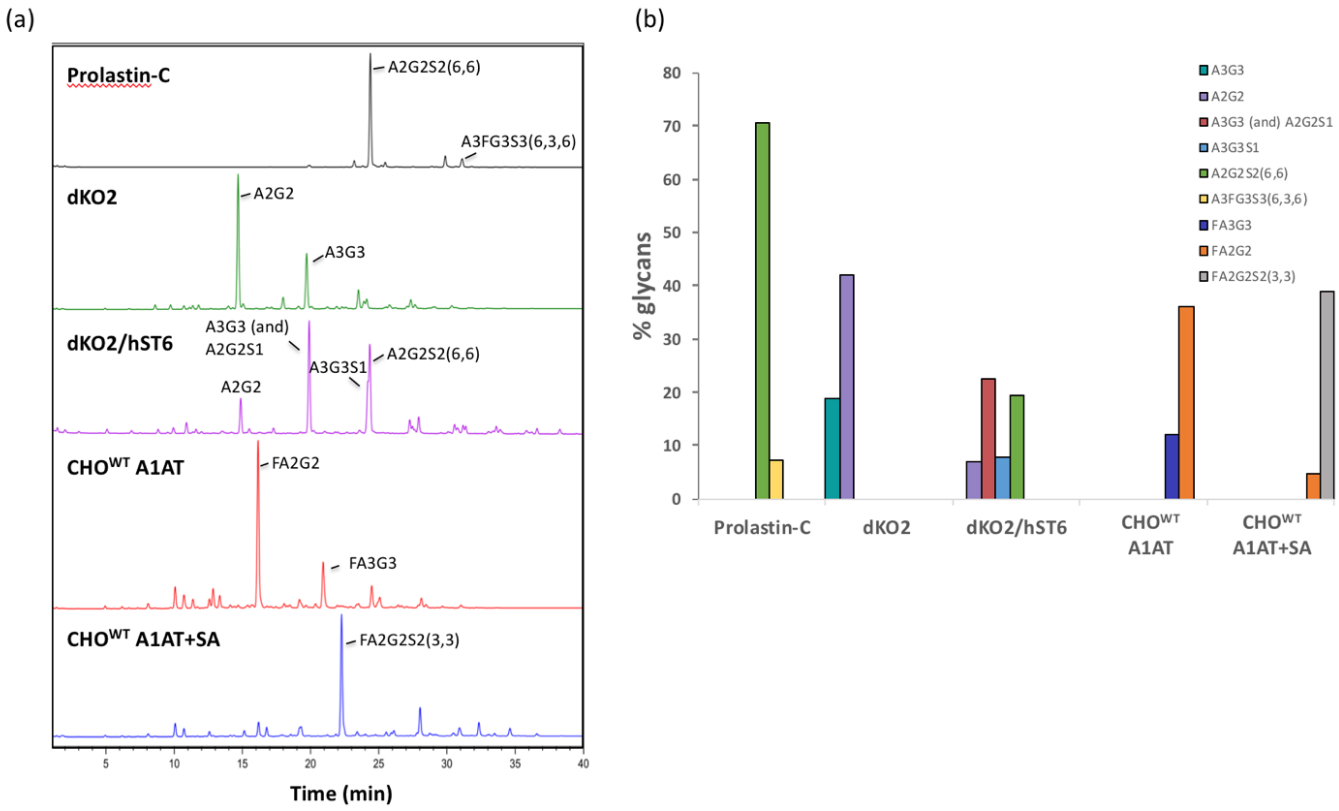

Figure 26. *N*-glycan characterization of recombinant A1AT glycoforms. *(a) HILIC-UPLC analysis of glyco-engineered recombinant A1AT and of plasma-derived Prolastin-C. (b) Glycan content (in percentage) in Prolastin-C and recombinant A1AT glycoforms as determined by integration of HILIC-UPLC profiles shown in panel (a).*

Cellular Uptake of Asialylated A1AT by HepG2 Hepatoma Cell Line

To assess the ability of terminal sialic acid to prevent the glycoproteins from being recognized by the human ASGPR, A1AT glycoforms along with fetuin, which is fully capped with terminal sialic acids and does not exhibit terminal galactose residues (and therefore is not a ligand for ASGPR) (346, 347), and asialofetuin (i.e. asialylated fetuin or ASF, a known natural ligand of the ASGPR) were labeled with the AF647 fluorophore and incubated with human HepG2 cells. These cells are known to express up to 500 000 ASGPRs per cell (343), and are often used to monitor glycoprotein binding to the receptors and internalization (348-350). Following a 3 h incubation at 37°C in order to allow for protein binding and internalization to occur, the remaining extracellular fluorescence was quenched by QSY21, whose absorbance spectrum overlaps with the AF647 fluorophore

emission spectrum, and A1AT uptake was quantified by measuring intracellular fluorescence intensity (FI) using ImageXpress imager (Figure 27a, b). As expected, asialylated A1AT (dKO2-A1AT) and ASF showed a greater uptake by HepG2 cells compared to fetuin due to exposure of terminal Gal on the glycans. On the other hand, dKO2/ST6-A1AT (α-2,6-sialylated) uptake was low and comparable with the plasma-derived therapeutic Prolastin-C. In accordance with its predominance of asialylated *N*-glycans (FA2G2), WT-A1AT exhibited internalization level comparable to dKO2-A1AT and ASF. The uptake was significantly decreased when WT-A1AT glycans were remodelled *in vitro* with terminal α-2,3-sialylation (WT+SA). These observations confirm that sialylation of A1AT efficiently reduce its interaction with ASGPRs that are naturally present at the surface of HepG2 hepatoma cells. However, this HepG2 cell-based assay is limited by its intrinsic high background and low signal-to-noise ratio and would require the generation of an engineered ASGPR-KO HepG2 cell line to control for non-specific binding. Therefore, we aimed at generating a more robust internalization assay using CHO cells overexpressing ASGPRs.

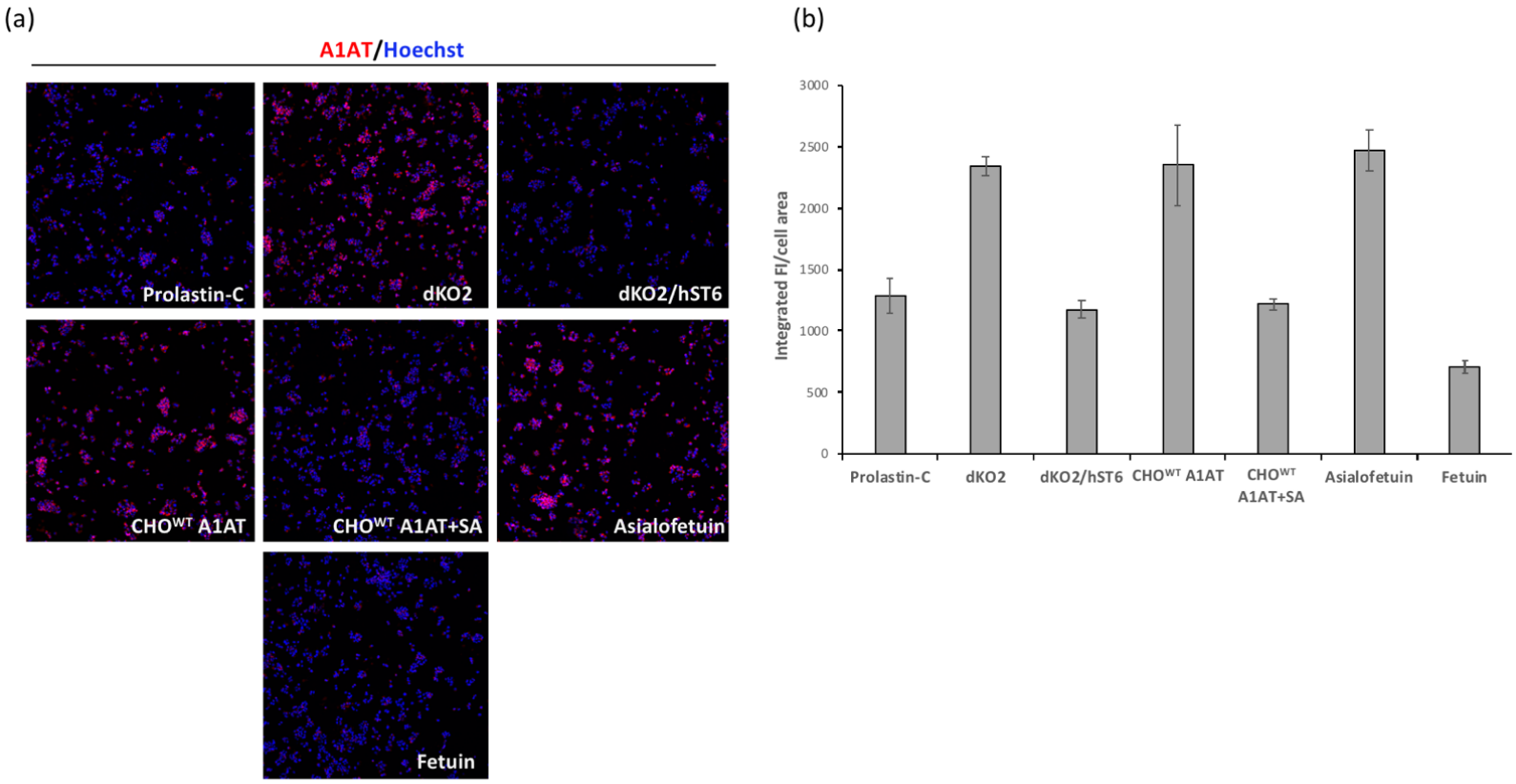

Figure 27. Cellular uptake of A1AT glycoforms by the human HepG2 hepatoma cell line. *(a) Representative processed images of HepG2 cells incubated with AF647-labelled A1AT glycoforms at 37°C for 3 h. ASF and fetuin were used as a positive and negative control, respectively (red = protein; blue = nuclei). (b) Quantification of A1AT glycoforms uptake by measuring integrated cellular fluorescence intensity (FI). All experiments were carried out twice and the data presented are the mean ± standard deviation (SD) of the results obtained in duplicate from one set of experiment.*

Internalization of Glycoproteins by CHO Cells Ectopically Expressing Human ASGPR

While it has previously been suggested that expression of both HL-1 and HL-2 subunits is necessary to generate high-affinity binding sites for asialylated glycoproteins, their relative contribution and optimal stoichiometry is still debated (341). We thus generated CHO pools stably expressing various levels of human HL-1 (hHL-1) and/or HL-2 (hHL-2) (supplemental data Figure 33) in order to monitor their role in asialoglycoprotein (ASGP) binding and internalization. Western blot analysis showed a strong band that belongs to the glycosylated mature forms of

hHL-1 or hHL-2 migrating at ~46 kDa and ~50 kDa, respectively, when each subunit was expressed individually in CHO cells (Figure 28a, top and middle panel, respectively), which is in agreement with the literature (288). As expected, the intensity of these bands was reduced or increased when hHL-1 and hHL-2 were co-expressed at various ratios, indicating proportional expression levels of the target subunits. Consistent with Western blot, flow-cytometry analysis also demonstrated the expression of hHL-1 and hHL-2 at the cell surface in stable pools, and the signal was also proportional to the hHL-1/hHL-2 ratios used (Figure 28b). However, it is worth noting that while anti-ASGPR2 was specific to the hHL-2 subunit, the anti-ASGPR1 antibody used herein, unlike in Western blotting, was also cross-reactive to the hHL-2 subunit (Figure 28b, top panel, HL-2 alone). This could be due to the fact that the anti-ASGPR1 used in this study is polyclonal and that hHL-1 and hHL-2 are 59% identical at the amino acid sequence level.

We next analyzed the uptake of A1AT glycoforms in CHO pools expressing various ratio of hHL-1 and hHL-2 subunits. The ASGPR-expressing CHO pools and non-transfected CHO^{WT} cells were incubated with various AF647-labeled A1AT glycoforms along with AF647-fetuin and AF647-ASF controls at 37°C for 3 h. As shown in Figure 28c, the uptake of asialylated A1AT (dKO2) as well as CHO^{WT}-produced A1AT and ASF were very high in the hHL-1 and HL-1:HL-2 (2:1) expressing CHO pools compared to the CHO^{WT} (representing background signal). The uptake was also significant in the 1:1 and 1:2 pools. Strikingly, cells expressing hHL-2 alone did not show significant uptake when compared to non-transfected CHO^{WT} cells, suggesting that human ASGPR might rely only on the HL-1 subunit to bind and internalize ASGPs as previously suggested (351). These results recapitulated those obtained in HepG2 cells, but with a slightly better signal-to-noise ratio. It is also worth mentioning that some A1AT, fetuin and ASF internalization was detectable in non-transfected CHO^{WT} cells (Figure 28c), demonstrating that these proteins could be internalized to some degree through ASGPR-independent pathway(s) (at least in CHO cells). We therefore concluded that when expressed in CHO cells, hHL-1 is sufficient for the uptake of asialylated glycoproteins.

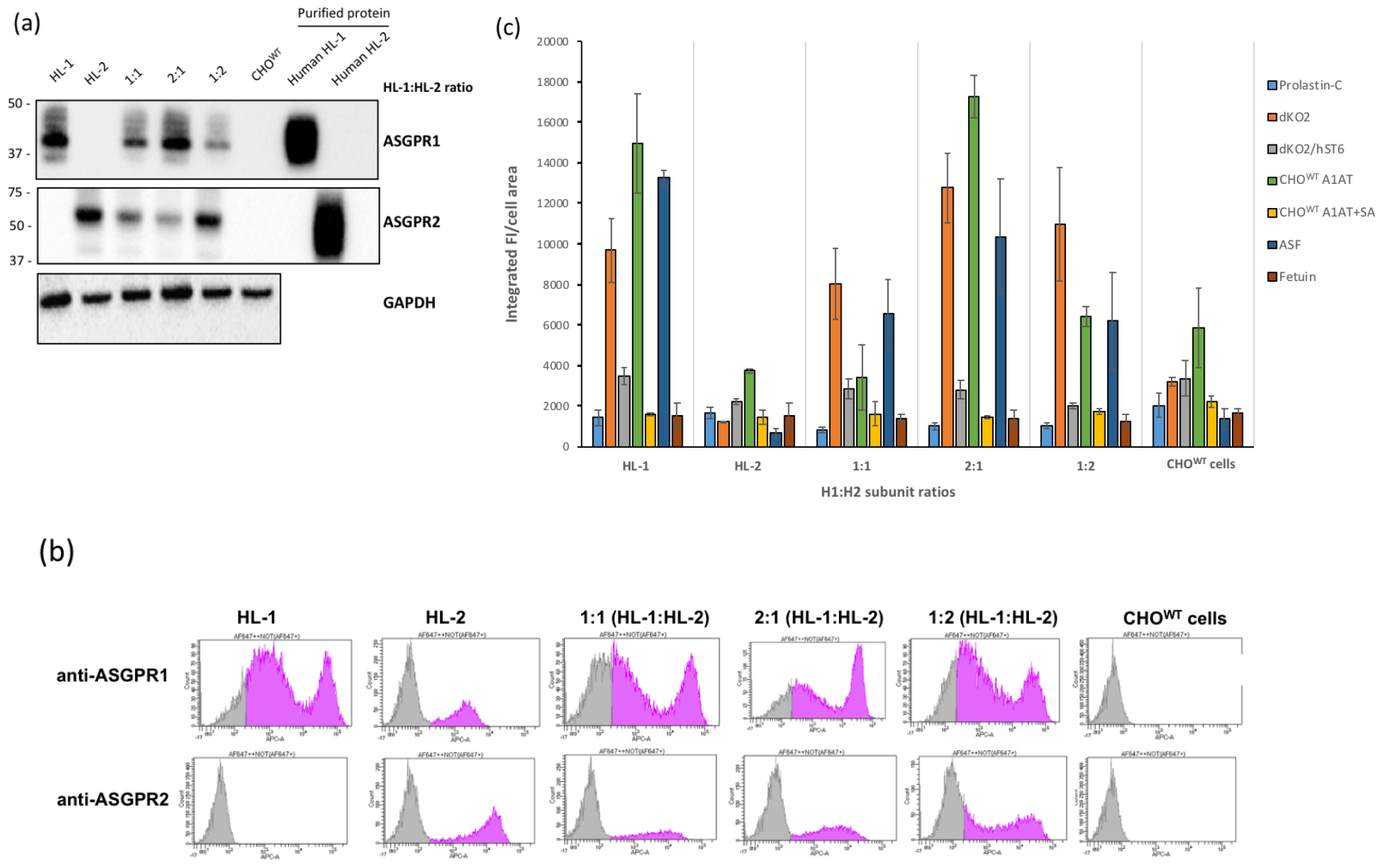

Figure 28. Stable expression of human HL-1/HL-2 in CHO cells and glycoprotein uptake *(a) Western blot of CHO cells extracts confirming the ectopic expression of human HL-1 and HL-2 subunits at various ratios in each pool. GAPDH is used as a protein loading control. (b) Flow-cytometry analysis of CHO cells stably expressing human HL-1 and HL-2 on their cell surface. (c) Integrated FI values of internalized AF647-labelled A1AT glycoforms in different CHO pools as determined by ImageXpress high-content imaging. All experiments were carried out twice and results represent one set of experiment ± SD.*

Internalization of Glycoproteins by CHO Cells Ectopically Expressing Mouse and Rat ASGPRs

We next aimed at evaluating if ASGP uptake by rat or mouse ASGPR orthologs is similar to that of hASGPR. To this end, CHO^{WT} cells were transfected with rat or mouse HL-1 and HL-2

subunits, alone or in combination (at a 1:1 ratio), and the pools were generated following selection with MSX (supplemental data Figure 34). Western blot analysis of cells stably expressing rHL-1 or rHL-2 revealed a single band with an apparent molecular mass of ~41.5 kDa and ~49 kDa, respectively (Figure 29a), while mHL-1 or mHL-2 migrated with an apparent molecular weight of ~42 kDa and ~70 kDa, respectively (Figure 29b), representing the mature glycosylated species (289, 296). Similarly, flow cytometry analysis also confirmed the presence of ASGPR subunits on the cell surface of the rat or mouse ASGPR CHO-expressing pools (supplemental data Figure 35).

To determine if both subunits are necessary for the efficient uptake of asialylated proteins, cells expressing HL-1, HL-2 or both subunits from rat or mouse were incubated with AF647-ASF or AF647-fetuin as a positive and negative control, respectively, for 3h at 37°C. Internalization of fluorescently-labeled glycoproteins was assessed by imaging with ImageXpress. Cells co-expressing both rat or mouse HL-1/HL-2 subunits efficiently internalized ASF compared to CHO^{WT} cells (Fig 29c). There was no difference in the uptake of ASF in cells expressing only rat or mouse HL-1 or HL-2 compared to that observed in CHO^{WT} cells. Our results thus suggest that, in contrast to the human ASGPR, rat and mouse ASGPRs require co-expression of both HL-1 and HL-2 subunits in order to recognize and endocytose asialylated glycoproteins.

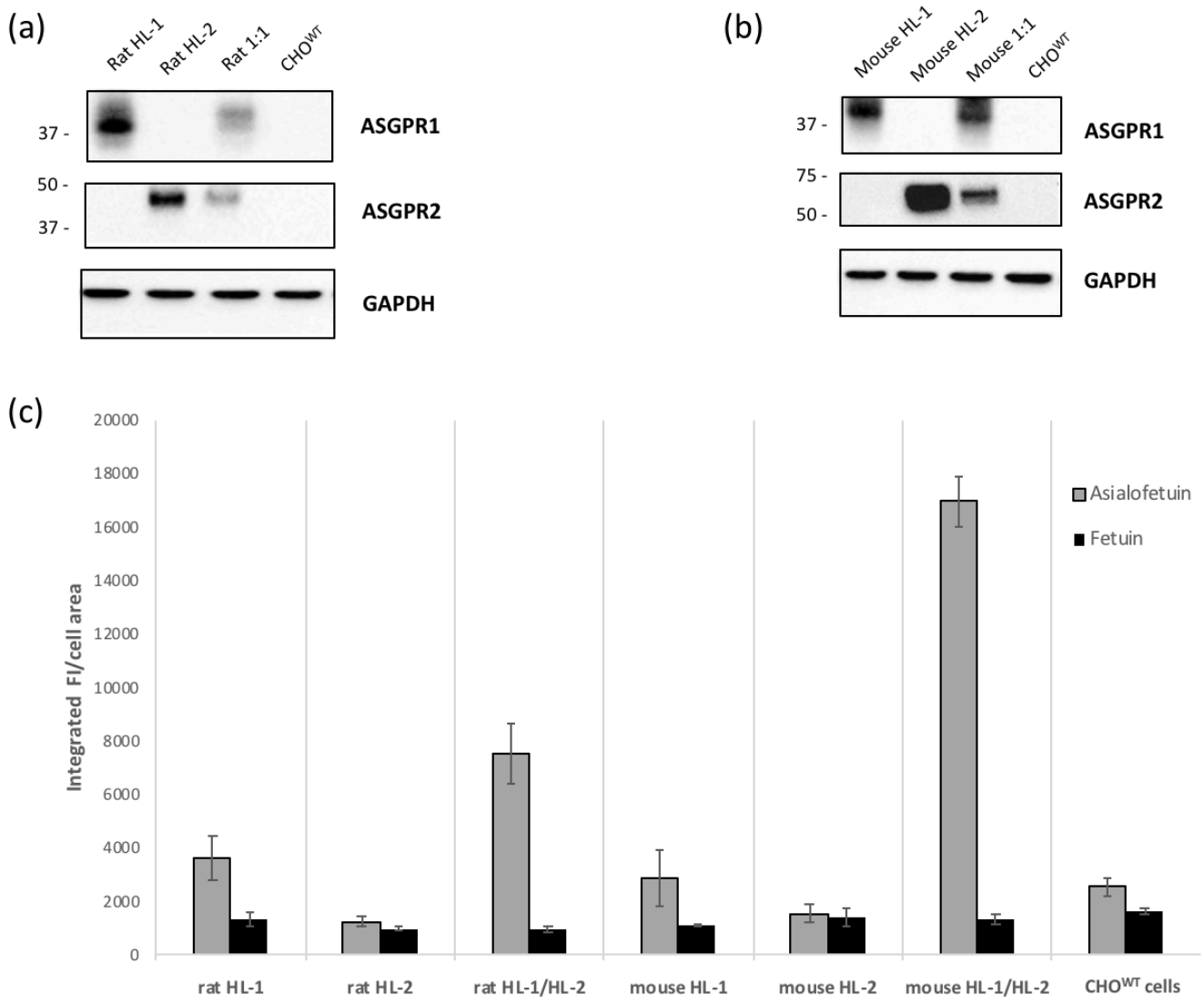

Figure 29. Both HL-1 and HL-2 subunits are required for ASF uptake by rat and mouse ASGPR. Western blot of CHO cells stably expressing *(a) rat ASGPR subunits or (b) mouse ASGPR subunits, alone or in combination. GAPDH is used as a protein loading control. (c) Internalization of ASF and fetuin in rat- and mouse-ASGPR expressing cells as determined by measuring their FI values after incubation at 37°C for 3 h. All experiments were carried out twice and results represent one set of experiment ± SD.*

Increasing Signal-to-noise Ratio Through Enrichment of Human, Rat and Mouse ASGPR-expressing Pools

To isolate cell populations expressing the highest level of ASGPRs on their surface, fluorescence-activated cell sorting (FACS) was carried out following their labeling with antibodies specific to each subunit. For human, rat and mouse pools, cells with the highest HL-1 levels on their surface were enriched upon labeling with rabbit anti-ASGPR1 primary antibody, followed by anti-rabbit-AF488 or anti-rabbit-AF647 secondary antibodies. While the top 15% and 23% positive cells were effectively sorted for human and rat pools, resulting in ~8- and ~3-fold increase in mean fluorescence index (MFI), respectively, we were only able to enrich 1.5% of positive cells for the mouse pool with a ~6-fold increase in MFI compared to non-enriched pools (Figure 30a, top panels). Subsequently, the enriched cell population were analyzed by flow cytometry using AF488- or AF647-labeled antibodies to validate that cell-surface HL-1 expression levels were higher compared to the original unsorted population (Figure 30a, bottom panels). Since both HL-1 and HL-2 subunits are required for the formation of functional rat and mouse ASGPRs, a second sorting exercise was carried out where cells expressing highest cell-surface HL-2 levels were enriched using anti-ASGPR2 specific to rat or mouse. Similar to HL-1 enrichment, while the top 20% of positive cells were enriched for rat with a ~3-fold increase in MFI, we were only able to sort the top 3% positive cells for the mouse HL-2, resulting in a ~5-fold increase in MFI (Figure 30b, top panels). It is likely that, unlike human and rat anti-HL-1/2 antibodies, the antibodies used against mouse HL-1 and HL-2 are not sensitive enough for robust FACS enrichment, thereby resulting in poor cell-surface mHL-1 and mHL-2 labeling compared to their human and rat orthologs, thus limiting the efficacy of ASGPR-expressing cell enrichment. Nevertheless, the resulting enriched rat and mouse ASGPR pools were successfully sorted for cells expressing higher level of ASGPRs at their surface compared to non-enriched pools (Figure 30b, lower panels).

Following enrichment of pools, we performed confocal microscopy to visualize glycoprotein internalization with higher resolution. To this end, AF647-labeled WT-, dKO2- as well as dKO2/ST6-A1AT were incubated with human HL-1 enriched pool along with CHO^{WT} cells at 37°C for 3 h. Following cell fixation, our data clearly show that WT-A1AT and dKO2-A1AT, unlike

dKO2/hST6-A1AT, were readily internalized by the enriched hHL-1 cells in contrast to CHO^{WT} cells, as expected (Figure 30c and 30d).

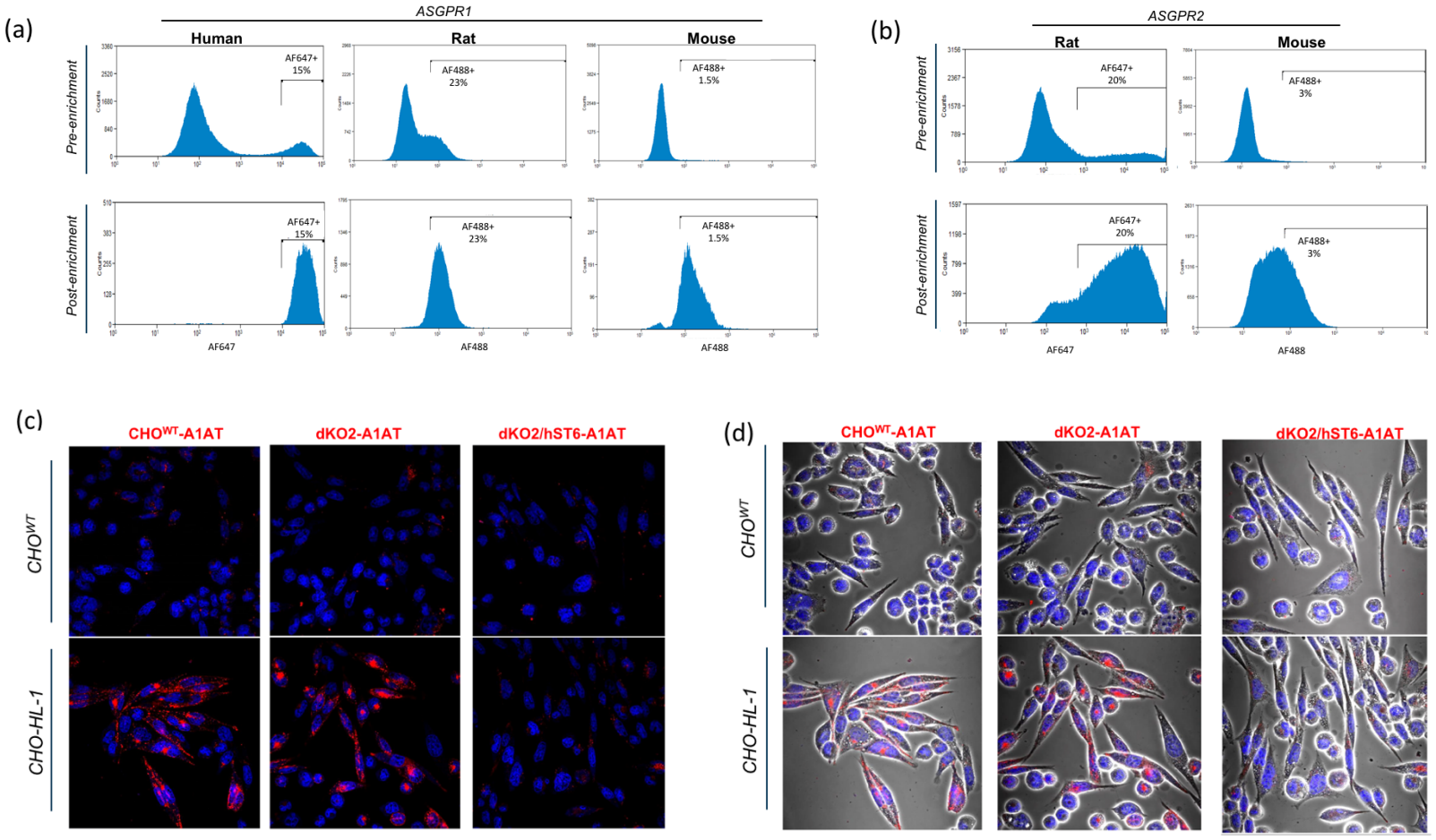

Figure 30. Enrichment of human-, rat- and mouse-ASGPR expressing pools and A1AT glycovariants cellular uptake. *(a) FACS-based enrichment of human-, rat- and mouse-ASGPR pools expressing highest HL-1 subunit levels on their cell surface. (b) Subsequent enrichment of rat and mouse pools for highest levels of HL-2 subunit on their cell surface (c) Fluorescent confocal images of AF647-labeled A1AT (red) internalization by enriched human ASGPR pool (CHO-HL-1) or CHO^{WT}.* Nuclei were stained with Hoechst 33342 (blue). (d) An overlay of the bright-field and fluorescence images showing internalization of A1AT.

Impact of Sialic Acid Linkage on A1AT Interaction with the Human, Rat and Mouse ASGPRs

Although HL-1 and HL-2 subunits share a high percentage of identity in their amino acid sequences across mammalian species, it was reported that ASGPR orthologs may display varying affinities for glycoprotein with different terminal sugar residues (290). To interrogate the ligand specificity of the ASGPRs from human, rat and mouse origin, we performed glycan remodeling of Prolastin-C because of its highly homogeneous *N*-glycan pattern (as opposed to CHO-produced A1AT which shows significant heterogeneity). Replacement of the natural α-2,6-linked Neu5Ac on Prolastin-C by α-2,3-linked Neu5Ac was partially achieved by a two-step asialylation−resialylation process using the immobilized SialEXO microspin columns, followed by incubation with human ST3Gal4. As shown in Figure 31a and b, HILIC-UPLC analysis confirmed almost complete removal of sialic acids from the *N*-linked glycans of Prolastin-C, resulting in Asialo-ProC, where the A2G2 peak was found as the main *N*-glycan (70%). Subsequently, incubation of Asialo-ProC with human ST3Gal4 (ProC-2,3) yielded a main peak corresponding to α–2,3-disialylated bi-antennary glycans, A2G2S2 (3,3), representing about 49% of the total glycans, in addition to a second major peak (22%) corresponding to the A2G2S1 (3) *N*-glycan. This second peak indicates an incomplete sialylation of bi-antennary glycans leading to one branch with a terminal galactose. Finally, Prolastin-C was also incubated with CST-81, a bifunctional α-2,3/2,8-sialyltransferase, in order to add α-2,8 sialic acid termini on the existing α-2,6-sialic acid residues. *N*-glycan analysis of the resulting protein (ProC-2,8) showed that α-2,8-linked Neu5Ac were successfully added to Prolastin-C since all glycan peaks shifted to longer retention times compared to native Prolastin-C sample (Figure 31a). Although it is not possible to determine if all branches have been modified with α-2,8-linked Neu5Ac, it is likely that the major peak, representing 45% of the total glycans with a retention time of 30 min, be consistent with A2G2S2 (6,6) along with one terminal α-2,8-linked Neu5Ac on each branch.

With the remodeled Prolastin-C samples in hand, we verified if ASGPR orthologs display different preferences for the same *N*-glycan termini. Upon fluorescent labelling of remodeled Prolastin-C samples, their degree of labelling was calculated according to manufacturer's instruction and confirmed using Coomassie staining (supplemental data Figure 36 and supplemental data Table 5). Subsequently, labelled proteins were incubated with enriched pools

expressing human, rat or mouse ASGPRs and the Prolastin-C uptake was quantified using the ImageXpress (Figure 31c). Prolastin-C and fetuin, which are capped with sialic acid, were not binding to ASGPR orthologs. As expected, removal of terminal α-2,6-linked Neu5Ac on Prolastin-C (Asialo-ProC) resulted in its efficient internalization by all three ASGPR orthologs, with levels comparable to ASF (except for hHL-1 where internalization is slightly lower than that for ASF). Uptake of Prolastin-C with terminal α-2,8-linked Neu5Ac (ProC-2,8) was also negligible for all three ASGPR orthologs. Interestingly, while α-2,3-sialylated Prolastin-C (ProC-2,3) was not significantly internalized by human and rat ASGPR (levels comparable to CHO^{WT}), the mouse ASGPR showed a significant uptake. Since *in vitro* sialylation of the ProC-2,3 was not complete and the protein still bear ~22% of A2G2S1 glycan, the exposed galactose on one of the antennae may be responsible for this interaction. Because the mouse ASGPR-pool enrichment process was not as efficient as for human and rat ASGPRs, we normalized the data in each pool by determining the ProC-2,3 internalization ratio relative to ASF in order to better compare ASGPR-pools between them. Figure 31d shows that ProC-2,3/ASF internalization ratio for the mouse ASGPR was statistically different, being 3- and 4-fold higher than that of the rat and human ASGPRs, respectively (p=0.0033; one-way ANOVA), suggesting that the mouse ASGPR may have a greater affinity than human and rat orthologs for biantennary structures terminated with one galactose on one branch and one sialic acid on the other.

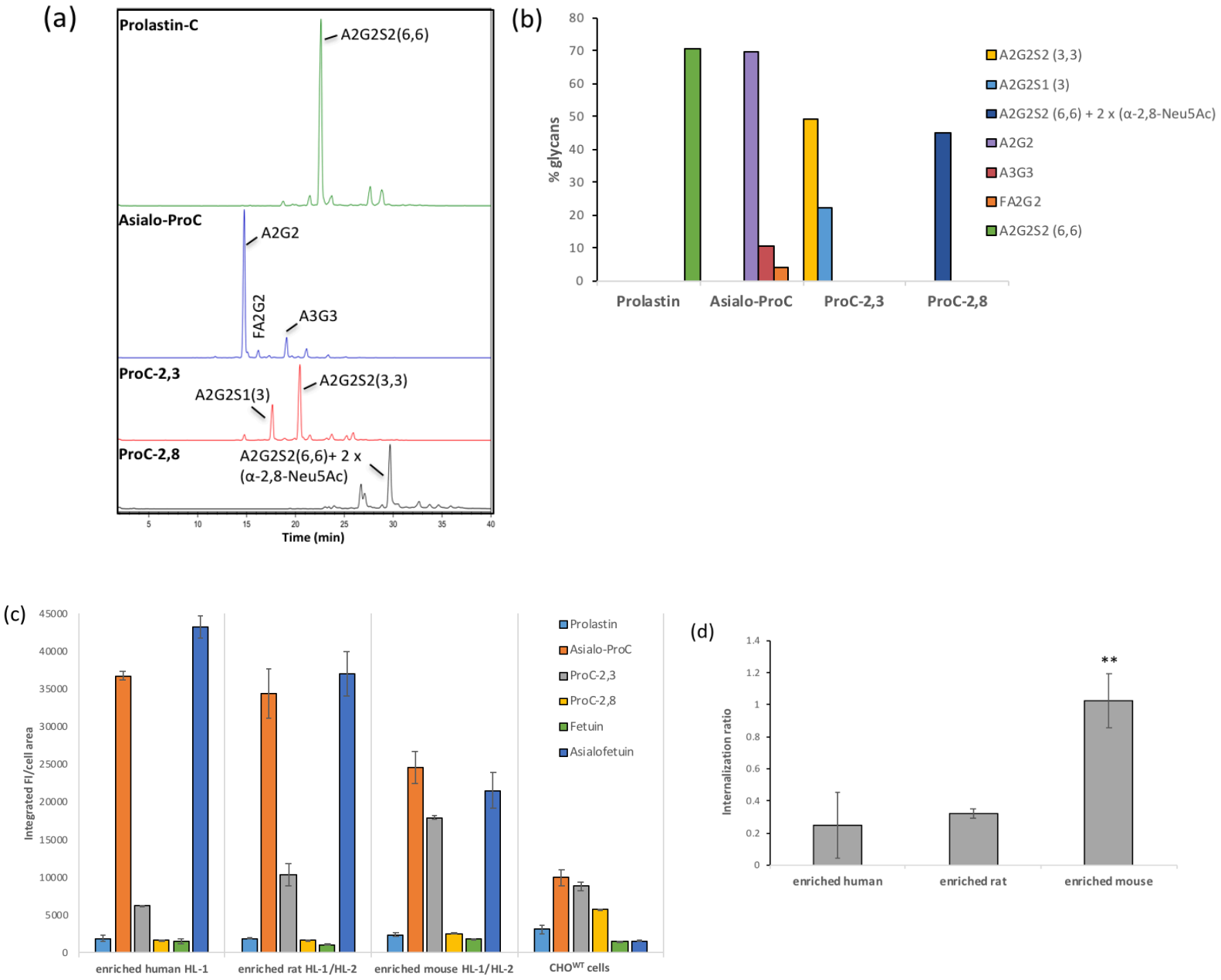

Figure 31. Prolastin-C glycans remodeling and its cellular uptake by ASGPR orthologs. *(a) HILIC-UPLC N-glycan analysis of remodeled Prolastin-C glycans. (b) Composition of N-glycans (in percentage) on each remodeled Prolastin-C as determined by HILIC-UPLC analysis. (c) Protein internalization by ASGPR ortholog pools as measured by quantification of cellular FI. (d) Normalized internalization ratio (relative to ASF) for ProC-2,3 by enriched human-, rat- and mouse-ASGPR expressing pools. Statistical significance was determined using one-way ANOVA, $p < 0.05$. All experiments were carried out four times and results represent one of the four set of experiment ± SD.*

Discussion

The asialoglycoprotein receptor expressed at the surface of hepatocytes was first reported in the early 1970s for its ability to mediate the rapid clearance of asialoglycoproteins (ASGPs) from the blood through binding of exposed terminal galactose sugars (286). Similarly, several studies have demonstrated that removal of sialic acid residues from A1AT results in a shorter plasma half-life compared to the sialylated glycoprotein, and this is accompanied by a concomitant accumulation of the asialoglycoprotein in the liver (320, 352, 353). However, faster ASGPR-dependent *in vivo* clearance of glycoproteins capped with α-2,6-linked compared to α-2,3-linked sialic acids were observed using mice or rat animal models (307, 354, 355). There is a little experimental evidence showing a clear impact of the type of sialic acid linkages on glycoproteins uptake by ASGPRs. To evaluate this impact, we first used a cell-based internalization assay based on the human HepG2 hepatic cell line known to express ASGPRs at its surface (343). Using these cells, and as expected, internalization of fetuin, *in-vitro* sialylated A1AT (WT+SA), dKO2/hST6-A1AT and Prolastin-C was minimal, while that of ASF, WT-A1AT and dKO2-A1AT was maximal. These results were consistent with the sialylation levels of the proteins and demonstrated that A1AT capped with terminal sialic acid in both α-2,3- or α-2,6-linkages is protected from hASGPR-mediated clearance. However, and as reported by another group (350), HepG2 cell-based internalization assay demonstrated poor signal-to-noise ratio between non-sialylated and sialylated proteins (ratio of 2 to 3 max). The high level of background internalization signal, likely responsible for this poor ratio, could potentially be explained by the contribution of intrinsic ASGPR-independent protein binding and internalization mechanism(s). Thus, it was not possible to determine if A1AT capped with α-2,3-linkages are better protected against ASGPR binding compared to A1AT with α-2,6-linkages. With this HepG2 assay, it is also impossible to directly compare interaction of glycoproteins with human ASGPR to that with mouse and rat orthologs, since the use of rat and mouse hepatic cell lines would likely show variable intrinsic ASGPR-independent internalization backgrounds. We thus aimed at developing a new assay based on CHO cells overexpressing recombinant ASGPRs, an assay that would allow us to monitor non-specific binding using naïve CHO cells.

It is generally agreed that both HL-1 and HL-2 subunits are required for a functional ASGPR and several studies have examined the HL-1/HL-2 stoichiometry of the human ASGPR (291, 293). However, a previous study showed that expression of the human HL-1 subunit alone in transiently transfected COS-7 cells resulted in high-affinity binding of ASF (294). In our study, we have confirmed that human HL-1 subunit alone, when overexpressed in CHO cells, is effectively localized at the cell surface, binds asialylated glycoproteins (ASGPs) such as ASF and dKO2-A1AT and internalizes them in the absence of the human HL-2 subunit. In contrast, when hHL-2 was expressed alone, it failed to endocytose ASGPs despite being localized at the cell surface. In addition, co-expression of hHL-2 with the hHL-1 subunit did not enhance the cellular uptake of ASGPs compared to hHL-1 alone. Thus, it appears that, and in agreement with the observations made previously (294), hHL-1 alone is sufficient to mediate binding and internalization of ASGPs. Moreover, compared to HepG2 cell line, CHO cells overexpressing ASGPRs provided higher signal-to-noise ratios and intrinsic ASGPR-independent internalization could be monitored using naïve CHO cells.

We then aimed at developing a similar assay with rat and mouse ASGPR orthologs. When rat and mouse HL-1 or HL-2 subunits were expressed individually in CHO cells, we did not observe any binding to ASF, which agrees with a previous study demonstrating that mice lacking expression of either ASGPR1 or ASGPR2 showed decreased clearance of exogenous ASGPs (296). Accordingly, we demonstrated that expression of both rat and mouse HL-1/HL-2 subunits were required to achieve efficient uptake of asialylated proteins. This indicates that in rat and mouse, HL-2 subunit plays a critical role in the functional assembly of the HL-1/HL-2 ASGPR complex.

Stable CHO pools generated by random integration of plasmid DNA usually show significant heterogeneity because of integration of expression vector at various loci into the host genome. This cause stable CHO pools to contain cell populations expressing proteins at various levels (356). With the aim of further improving the signal-to-noise ratio for our cell-based internalization assay, we performed FACS enrichment of cells harboring the highest levels of human-, rat- or mouse-ASGPRs on their cell-surface. Enrichment of CHO cells expressing highest levels of human HL-1, rat and to a lesser extent mouse HL-1/HL-2 resulted in greater cell-surface density of each receptor subunit as shown by flow cytometry analyses. With the enriched human

pool in hand, we were able to monitor the internalization of ASGPs with much increased signal-to-noise ratio compared to that measured in HepG2 cells, making it easier to distinguish ASGPR-mediated internalization of glycoproteins with various glycosylation patterns from background non-ASGPR mediated internalization.

Some studies suggested that α-2,3 sialylation might prevent clearance of glycoproteins more effectively than α-2,6 sialylation since mouse ASGPR was shown to recognizes the latter in addition to terminal galactose and *N*-acetylgalactosamine residues (307, 308). Other studies have shown that glycoproteins with glycans capped with α-2,3- or α-2,6 sialic acid show similar PK in mice (183) and binding affinities towards hASGPR by SPR (357). Whether the type of sialic acid linkage modulates engagement with the human, rat, and mouse ASGPR orthologs is still not clear. To further address this question, we took advantage of the natural glycan homogeneity of Prolastin-C and of its enzymatically remodeled versions to study internalization through human, rat, and mouse ASGPR orthologs overexpressed in enriched CHO pools. Interestingly, while human and rat ASGPR showed minimal uptake of α-2,3 sialylated ProC (ProC-2,3 uptake was comparable to that of CHO^{WT} cells), ProC-2,3 internalization by mouse ASGPR was found to be higher compared to human and rat ASGPRs. When comparing the internalization ratio between ProC-2,3 and ASF, we have found that it was significantly higher for mouse ASGPR compared to human and rat orthologs (by 3 to 4-fold). Since the sialylation levels of ProC-2,3 was not complete (i.e. it is still contained about 22% of mono-sialylated biantennary glycans), this suggests that mouse ASGPR may have a higher affinity for the exposed terminal galactose present in the A2G2S1(3) glycan compared to human and rat orthologs. Finally, a previous study had demonstrated an improved PK in mice for A1AT with glycans terminated with α-2,8-Neu5Ac-α-2,6-Neu5Ac compared to α-2,6-Neu5Ac alone (50), we have also enzymatically remodeled Pro-C with terminal α-2,8-Neu5Ac residues. Our data showed no differences between the uptake of α-2,6-sialylated Neu5Ac alone versus α-2,8-Neu5Ac-α-2,6-Neu5Ac by ASGPR orthologs, suggesting that the improved PK observed in that study group might be explained by a better protection of A1AT against sialidases or by a reduced protein degradation *in vivo* as a consequence of α-2,8-Neu5Ac addition.

In summary, we have developed a CHO cell-based assay with a much greater signal-to-noise ratio compared to HepG2 cells that allows one to assess recognition and internalization of glycoproteins by ASGPRs. Our results confirmed that the human HL-1 subunit is sufficient for the uptake of asialylated glycoproteins whereas both HL-1 and HL-2 subunits are required for rat and mouse ASGPRs. While neither α-2,8- or α-2,6-sialylated A1AT were ligands of the ASGPRs, mouse ASGPR shows higher internalization of A1AT containing A2G2S1(3) glycan compared to human and rat orthologs. A better understanding of ASGPR affinities for glycan structures present on glycoproteins may provide insights for optimizing pharmacokinetics of recombinantly produced therapeutic proteins, which could be achieved by cell glycoengineering or through other approaches, including the use of culture medium additives or enzyme inhibitors to modulate glycosylation during production.

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Supplemental Data

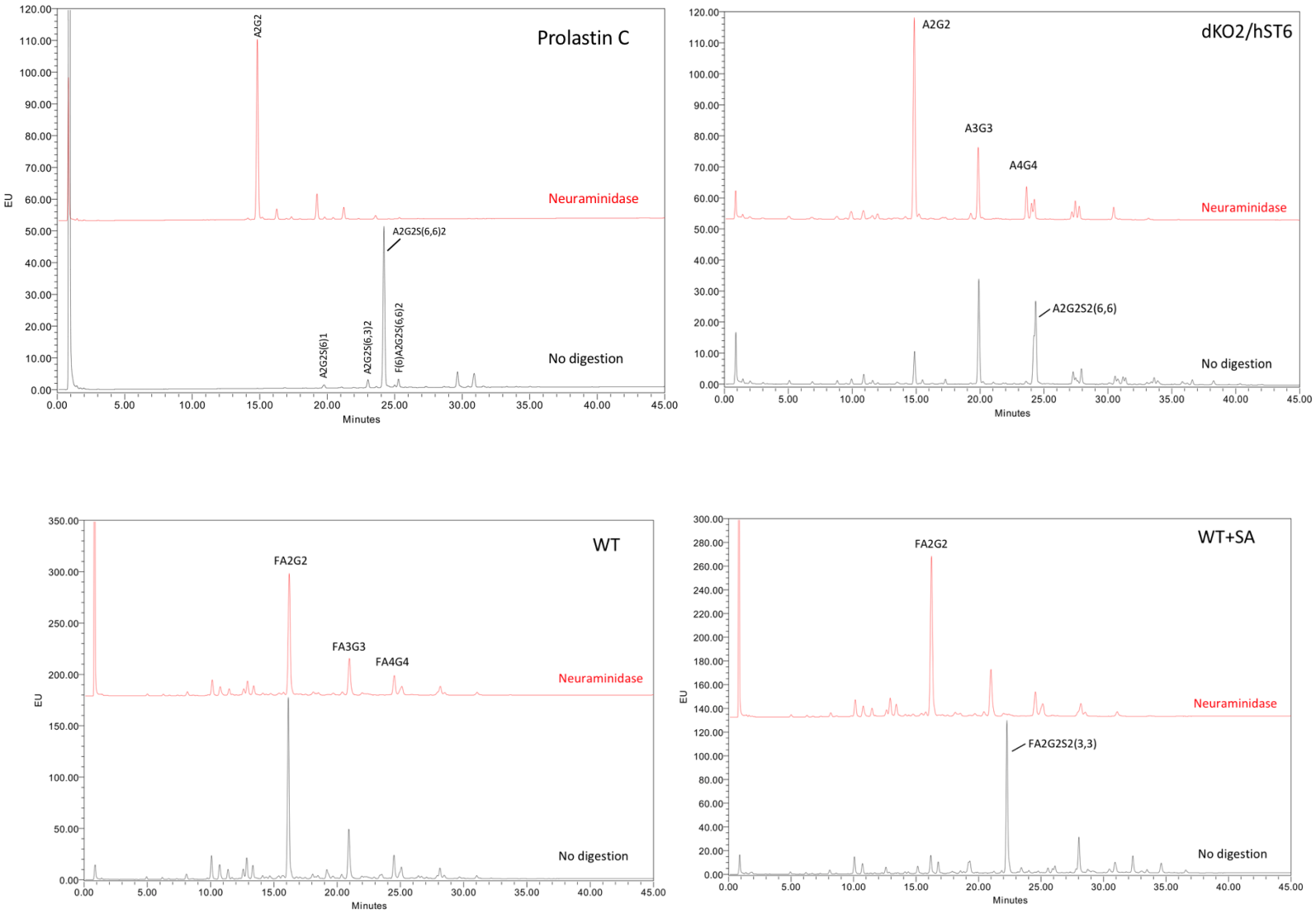

Figure 32: HILIC-UPLC chromatograms of undigested and neuraminidase-treated glycoengineered A1AT proteins and Prolastin-C.

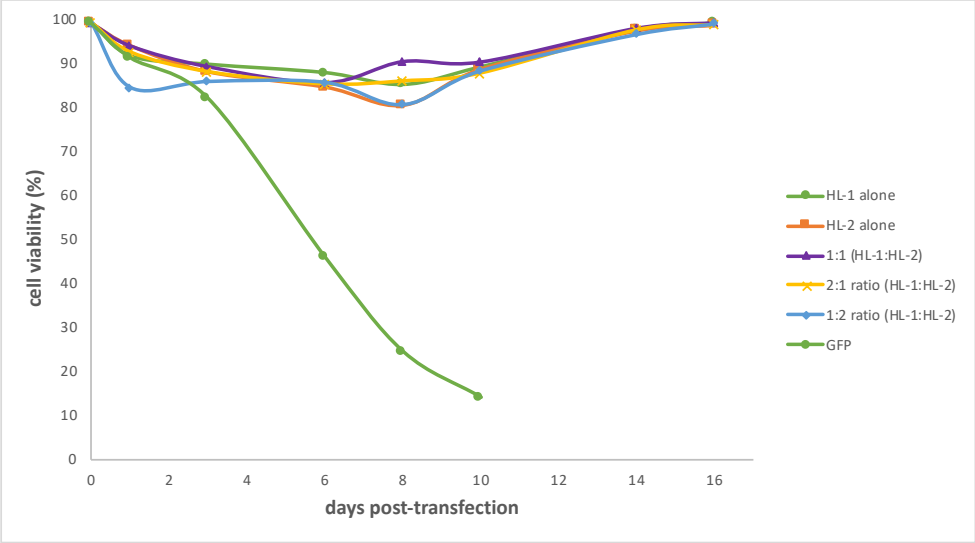

Figure 33: Viability curves of CHO^{WT} transfected with human HL-1 and HL-2 at different ratios.

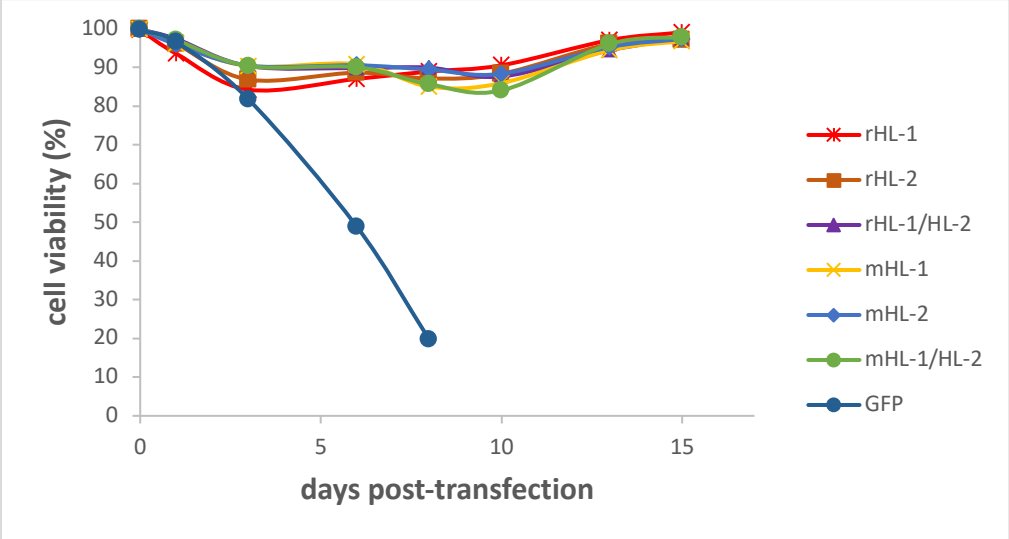

Figure 34: Viability curves of CHO^{WT} transfected with rat or mouse HL-1 and HL-2 at different ratios.

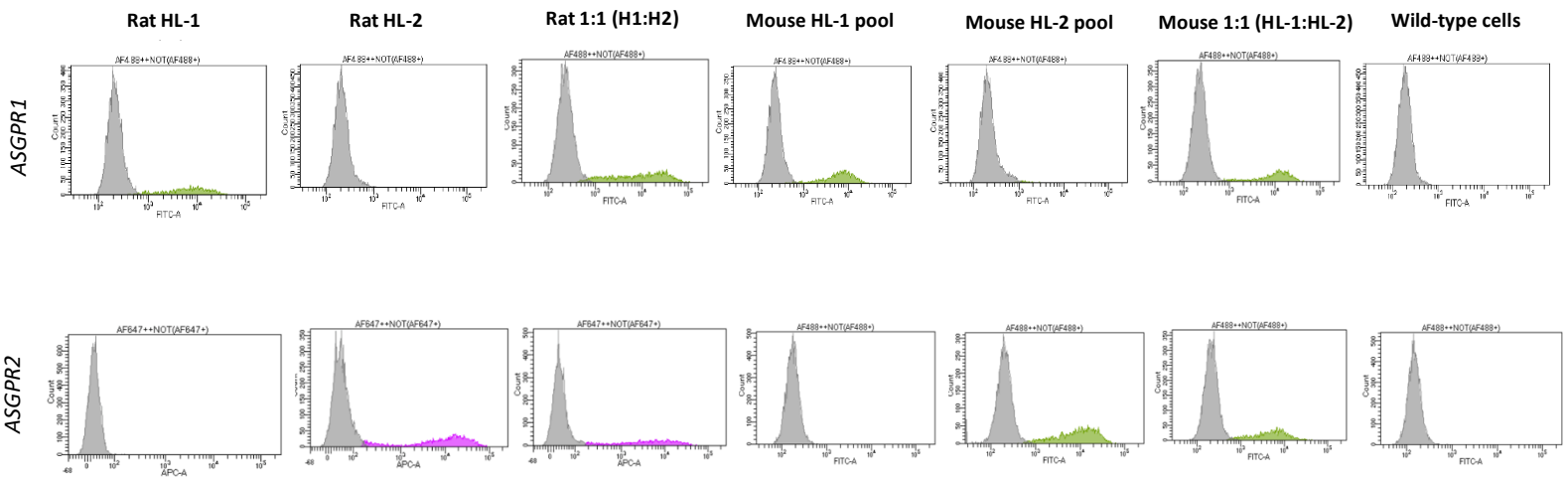

Figure 35: Flow-cytometry analysis of CHO cells stably expressing rat or mouse HL-1 and HL-2 subunits at different ratios on their cell surface.

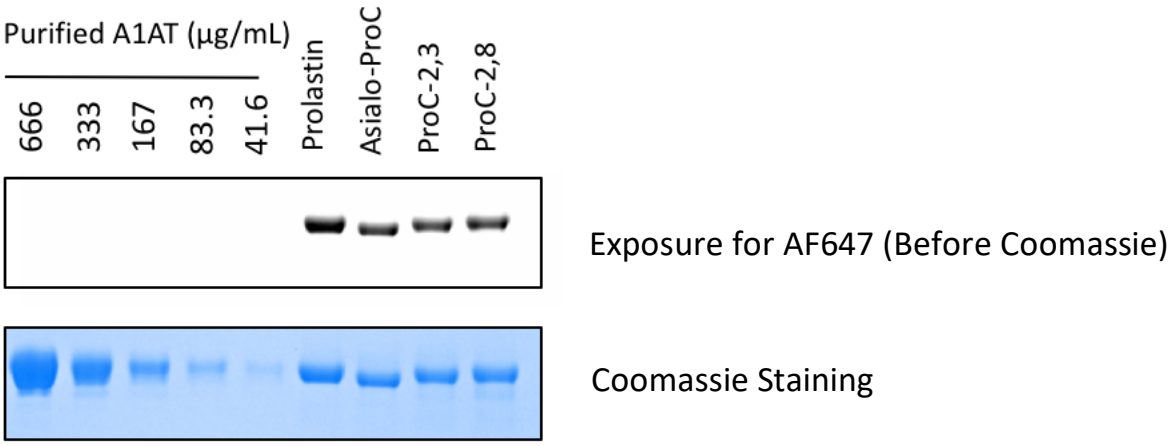

Figure 36: Degree of labelling comparison of remodeled Prolastin samples before Coomassie Staining for AF647 and their quantification by Coomassie Staining

Table 5. Quantification and comparison of AF647-labelled-Prolastin samples using concentration calculation equation according to manufacturer's instruction as well as Coomassie Staining.

Sample Name	DAR	Calculated concentration according to manufacturer's instruction (mg/mL)	Concentration by Coomassie (mg/mL)
Prolastin	3.3	1.9	1.8
Asialo-ProC	3.0	2.3	2.1
ProC-2,3	2.9	2.3	2.2
ProC-2,8	3.3	1.7	1.7

Chapter 5: General Discussion

High-level Production of wild-type and Oxidation-resistant Recombinant alpha-1-antitrypsin In Glycoengineered CHO Cells

A1AT is a serine protease inhibitor which blocks the activity of serum proteases, such as neutrophil elastase, in order to protect lung tissues. It has three *N*-glycosylation sites possessing mostly non-fucosylated, α-2,6-di-sialylated bi-antennary complex type structures (319, 342). In the case of A1AT deficiency (A1ATD), which is a rare disease that is usually underdiagnosed, neutrophil elastase breaks down elastin, reducing lung elasticity, thereby resulting in respiratory complications such as emphysema or COPD (314). To increase awareness for A1ATD, efforts, such as distribution of A1AT test kits free of charge, continuous education of medical staff and dissemination of knowledge regarding A1ATD, should have been made (358). Actually, the only treatment for patients with A1ATD is to receive lifetime injections of plasma purified human A1AT on a weekly basis (13). However, plasma purified A1AT is heterogeneous in terms of its glycosylation state and its quality varies from batch-to-batch. Moreover, such therapies can be extremely expensive and might harbor a risk of pathogen transmittance. There is currently no recombinant source of A1AT because of incorrect glycosylation profile and/or low A1AT expression levels obtained in current recombinant expression systems. Thus far, the production of recombinant A1AT (rA1AT) has been attempted in different cell expression systems with limited success. Despite the availability of various cell lines, CHO cells are the most utilized hosts for the production of recombinant glycoprotein as they can sustain high production rates and can be easily adapted to growth in diverse chemically-defined media (5). Nevertheless, CHO cells do not possess the same glycosylation machinery as human cells, therefore resulting in a different composition of glycans at the surface of recombinant proteins. For instance, CHO cells cannot perform α-2,6-sialylation, which is the predominant capping sugar in human proteins, including A1AT (6). Since glycan composition have been shown to influence immunogenicity, stability, and half-life of therapeutic proteins, it is important to find robust methods that allow tailored and reproducible glycosylation patterns for rA1AT production.

In this context, we aimed at producing high-levels of recombinant wild-type A1AT and oxidation-resistant mutein version with human-like glycosylation pattern in CHO cells for therapeutic uses. For this, we have concentrated our efforts on the generation of CHO cell line that enable the expression of rA1AT with more homogeneous and human-like *N*-glycan structures while allowing the production of the protein at high yields. Ever since its discovery, CRISPR/Cas9 has been greatly preferred as a genome editing tool to modify genomic DNA of any cell line used for recombinant protein production in order to tweak their *N*-glycosylation machinery (212). Therefore, in our study, we used the same technology to disrupt two target genes combined with the overexpression of a human glycosyltransferase and proved the feasibility of tailoring certain *N*-glycan structures via this CRISPR/Cas9 technology to grant the expression of afucosylated A1AT with α-2,6-sialylation.

With the use of an inducible expression system, we demonstrated that the cumate-inducible CR5 promoter is far superior promoter to the strong constitutive promoters for the production of rA1AT in stable pools. We showed that the average volumetric productivity of pools generated with the CR5 promoter being 2.5- to 6.5- fold higher than those generated with the constitutive promoters. This difference could be explained by the variability of the transcriptional efficiency of promoters in CHO cells. Indeed, the CR5 promoter is regulated by the chimeric transactivator rcTA, composed of the cumate binding domain in the reverse configuration fused to the potent viral transactivator VP16, that binds with high affinity to the sequence CuO within the CR5 promoter in the presence of cumate, thereby inducing strong expression of the gene of interest (327), whereas constitutive promoters are regulated by endogenous transcription factors. Moreover, the use of an inducible promoter enables the reduction of A1AT expression during the pool selection process, hence lowering cellular stresses and burden brought on by overexpression of recombinant proteins. Indeed, a previous study showed that the use of an inducible system allowed the isolation of stable clones for the production of monoclonal antibodies that appear to induce cytotoxicity when constitutively overexpressed (335). More recently, Poulain *et al.* also confirmed that the overexpression of the recombinant protein during the pool selection process negatively affects the pool recovery and is associated with an increase in cell death as well as Bip expression, which is a marker for the onset of ER stress (336). Thus, it

is important to separate the growth and production phases to allow cell resources to be more efficiently used in each phase, while improving growth characteristics and enhancing production stability.

Using cumate-inducible CR5 promoter, we here reported unprecedented volumetric productivity of 2.1 g/L for wild-type A1AT and 2.8 g/L for A1AT mutein from stable glycoengineered CHO pools, which were even higher compared to our previously published titer levels obtained in different glycoengineered pools (450-720 mg/L) (5). This result demonstrates that combinations of disrupted target genes for decreased core-fucosylation and α-2,3-sialylation via CRISPR/Cas9, followed by overexpression of α-2,6-sialylation, did not compromise cell growth and productivity unlike reported by another group (188), and proves the high performance of our expression platform/process. Additionally, clonal cell lines derived from our glycoengineered CHO pools may be able to produce >2-fold higher volumetric levels of rA1AT, which would make this system much more appealing for producing a commercial product.

It is known that glycosylation is not required for anti-elastase activity of A1AT (13). However, proper glycosylation has been found to be crucial for its stability, correct protein folding and interaction with other proteins (13, 99). More importantly from a therapeutic point of view is the fact that appropriate glycosylation, i.e. sialylation, enhances the half-life of AAT in the circulation (13). Although rA1AT produced by bacteria can inhibit NE, it is quickly eliminated from the circulation due to lack of glycosylation (115), which renders it inferior to the plasma derived counterpart and impractical as a therapeutic. Therefore, one of the aims of this study was to generate an A1AT molecule with a similar glycoprofile to plasma-derived A1AT. In chapter 3, glycoanalysis of rA1AT produced in our glycoengineered CHO cells revealed that the protein of interest bears predominantly afucosylated, bi-antennary and di-sialylated A2G2S2 *N*-glycans with human-like α-2,6-sialylation and very low levels of α-2,3 sialylation in contrast to unengineered CHO^{WT}-produced A1AT containing predominantly asialylated and core-fucosylated *N*-glycans. Although we were able to generate rA1AT with human-like glycans in our CHO cells, it is important to note that the proportion of this A2G2S2 (6,6) structure (~22 %) is significantly lower than that of commercial plasma-derived therapeutic Prolastin-C (70.6 %). Additionally, glycans released from the glycoengineered CHO cells appeared to be more heterogenous compared to Prolastin-

C, which had more homogenous profile. This is in agreement with the previous studies, where *N*-glycan analysis of the proteins from CHO^{WT} cells resulted in more than 10 annotated N-glycan structures ranging from bi-antennary to tetra-antennary complex-type glycans while native A1AT displayed majorly bi-antennary glycans and a small portion of tri-antennary glycans (186, 188).

With the aim of maximizing the protein sialylation, we tested the effect of cell culture additive on the glycosylation pattern of rA1AT expressed by our glycoengineered CHO cells in addition to cell engineering techniques. Supplementation of a sialic acid precursor ManNAc to the cell culture media during the production increased the overall sialylation of rA1AT mutein as well as the proportion of A2G2S2 *N*-glycans with α-2,6-sialylation to 39 %. Our results clearly show that combining orthogonal approaches that are complementary to cell engineering allowed us to optimize tailored glycosylation profile of rA1AT mutein and favor a "human-like" glycosylation pattern with α-2,6-sialylation during their production, which could potentially enhance its half-life. Indeed, in our previous study, we showed that α2,6-sialylation of A1AT was more stable than α2,3-sialylation throughout the production phase (187). We observed that α-2,3-sialylation of rA1AT decreased over time, exposing the penultimate Gal residues, due to increase in neuraminidase activity towards the end of fed-batch processes as a consequence of cell lysis, whereas the levels of α-2,6-sialylation did not vary. This is most likely caused by the selectivity of CHO neuraminidases for sialic acid residues with α-2,3-linkage. In fact, Warner *et al.* reported that soluble sialidase activity in CHO cell supernatants has a 4-fold preference for α-2,3-sialylated over α-2,6-sialylated glycans (359). As our goal in this project was to produce rA1AT with predominantly α-2,6-sialylated glycans, the low level of neuraminidase activity towards α-2,6-sialic acid in CHO cultures renders our glycoengineered CHO cell platform an attractive option for A1AT manufacturing.

Finally, we demonstrated that the inhibitory activity of both rA1ATs towards neutrophil elastase was comparable to that of Prolastin-C despite the differences in their glycosylation pattern, as expected. Because A1AT is susceptible to oxidant-mediated inactivation by both endogenous (inflammatory cells) and exogenous (smoking, pollution etc..) oxidants, we also showed that substitution of susceptible active site Met residues with Val (M351V/M358V)

rendered A1AT more resistant to oxidative inactivation while retaining its inhibitory activity under strongly oxidizing conditions.

To sum up, we were able to produce high levels of oxidation-resistant rA1AT bearing predominantly A2G2S2 *N*-glycan structure with α-2,6-sialylation, which could be sustainable supply used as a biobetter for the treatment of A1AT deficiency disorders while offering improved biosafety, cost-effective manufacturing, and more stable alternative for augmentation therapy.

Impact of A1AT Sialylation on Its Cellular Uptake by Asialoglycoprotein Receptor Orthologs

Rapid clearance of asialylated glycoproteins is mediated by the hepatic ASGPR, which recognizes the penultimate Gal or GalNAc residues. In addition, previous studies demonstrated that the relative concentration of plasma glycoproteins terminating with sialic acid that is attached to Gal or GalNAc with α-2,6-linkage, and not those with α-2,3-linkage, were also regulated by this receptor using mice or rats animal models (307, 308, 354). Nevertheless, as far as we are aware, the impact of α-2,3- vs α-2,6-sialylation on the *in vivo* half-life of A1AT in human is still not clear.

In this second part of our research project, we wanted to test the impact of the different glycosylation pattern of A1AT on its interaction with the ASGPR orthologs. For this purpose, we first produced α-2,6-sialylated A1AT (dKO2/hST6-A1AT) as well as A1AT lacking terminal sialylation (dKO2-A1AT) in our glycoengineered CHO cell lines that we created in Chapter 3. A1AT was also expressed by CHO^{WT} (WT-A1AT) with the aim of producing α-2,3-sialylated A1AT. Nevertheless, we observed that glycans released from WT-A1AT was mainly asialylated FA2G2 structures (36 %, in Chapter 4). As a result, we performed *in vitro* enzymatic sialylation (WT+SA) using human α-2,3-sialtransferase in order to enhance the α-2,3-linked sialylation of CHO^{WT}-produced A1AT and decrease the proportion of FA2G2 glycans with terminal galactose (36% → 5%).

With the various glycoengineered rA1ATs in hand, we then used HepG2 hepatic cell line, which is known to express the ASGPRs at its surface (343), to evaluate their *in vitro* uptake. Consistent with the sialylation levels of the proteins, our work illustrated that the internalization of WT-A1AT and dKO2-A1AT was maximal, whereas that of WT+SA, dKO2/hST6-A1AT and Prolastin-C was minimal. According to these findings, A1AT is protected from human ASGPR-mediated clearance when it is sialylated with either α-2,3- or α-2,6-linkages. However, it is worthy to note that HepG2 cell-based internalization assay is limited and the results showed a poor signal-to-noise ratio (SNR) between non-sialylated and sialylated proteins, which might be potentially explained by the contribution of intrinsic ASGPR-independent protein binding and internalization mechanisms, causing high background internalization signal. It was therefore impossible to ascertain whether A1AT capped with α-2,3-linkage is more resistant to ASGPR binding than A1AT with α-2,6-linkage.

Because of the poor SNR obtained with HepG2 cell line, our next aim was to develop a novel assay based on CHO cells overexpressing recombinant ASGPRs, which could allow us to evaluate non-specific binding using naïve CHO cells. To achieve this, we first generated stable CHO pools expressing various levels of recombinant human HL-1 (hHL-1) and/or hHL-2 subunits, to investigate their role in ASGPR function with regards to the internalization of glyco-engineered A1AT. In our study, we established that in the absence of the hHL-2 subunit, the hHL-1 subunit alone can efficiently localize at the cell surface, bind asialylated glycoproteins (ASGPs), including asialofetuin (ASF) and dKO2-A1AT, and internalize them. In contrast, although being localized at the cell surface, hHL-2 did not endocytose ASGPs when it was overexpressed alone in CHO cells. Additionally, compared to hHL-1 alone, co-expression of hHL-2 with the hHL-1 subunit did not improve the cellular uptake of ASGPs. Thus, it would seem that hHL-1 alone is sufficient to mediate the binding and internalization of ASGPs, which is consistent with the earlier data showing that overexpression of the hHL-1 subunit alone in COS-7 cells led to high-affinity binding to ASF(294). Additionally, CHO cells overexpressing ASGPRs offered greater signal-to-noise ratios than the HepG2 cell line although it is still worth mentioning that some A1AT internalization was seen in non-transfected CHO^{WT} cells, indicating that these proteins may internalize to some extent through ASGPR-independent pathway(s) in CHO cells.

The next step was to determine if rat or mouse ASGPR orthologs' uptake of ASGP is comparable to that of human ASGPR. Based on our results presented in Chapter 4, we observed no binding to ASF when individual rat (r) or mouse (m) HL-1 or HL-2 subunits were overexpressed alone in CHO cells. This is in good agreement with a previous work showing that mice lacking expression of either ASGPR1 or ASGPR2 on the surface their hepatocytes were not to be able to internalize exogenous ASGPs (296). Similarly, Steirer *et al*. discovered that the level of α-2,6-sialylated glycoproteins was elevated in the plasma of mice lacking the expression of mHL-2 as compared with wild-type mice (308). In our study, we also concluded that effective uptake of ASGPs requires expression of both HL-1/HL-2 subunits in both rat and mouse, and that the HL-2 subunit is essential for the functional assembly of the HL-1/HL-2 ASGPR complex in rats and mice unlike in human.

Given that transgene integration in the host cell genome is a random phenomenon, and transcriptional variation could occur as a result of integration site context, stable CHO pools are typically made of a very heterogenous cell populations in terms of growth rates and protein expression levels (356). With the aim of further improving the SNR, we performed FACS enrichment to isolate cell populations expressing the highest level of human-, rat- or mouse-ASGPRs on their cell-surface. Our results illustrated that while enrichment of CHO cells expressing highest levels of human HL-1, and rat HL-1/HL-2 led to higher cell-surface density of each receptor subunit, that of mouse HL-1/HL-2 did not result in as much increase in the number of these subunits on the cell surface as was observed with the human and rat subunits. We suggested that the effectiveness of ASGPR-expressing cell enrichment was likely constrained by the fact that, unlike human and rat anti-HL-1/2 antibodies, the antibodies used against mouse HL-1 and HL-2 are not sensitive enough for robust FACS enrichment, leading to poor cell-surface mHL-1 and mHL-2 labelling in comparison to their human and rat orthologs. In spite of this, the resulting enriched mouse ASGPR pool was successfully sorted for cells expressing higher level of ASGPRs at their surface in comparison to non-enriched pools.

Despite sharing a high percentage of identical amino acids in their sequence, Park and Baenziger reported that ASGPR orthologs display different preferences for the same *N*-glycan termini (290). To interrogate this and the impact of distinct A1AT sialylation on its interaction with

ASGPR, in the last part of our research work, we performed glycan remodeling of Prolastin-C due to its highly homogenous *N*-glycan pattern in contrast to CHO-produced A1AT, which exhibits significant heterogeneity in its glycosylation pattern. This very homogeneous and well-studied *N*-glycan pattern of Prolastin-C allowed us to install different sialic acid linkages on the glycosylation sites of A1AT and to track all steps of remodelling. In contrast to previous studies demonstrating faster ASGPR-dependent *in vivo* clearance of proteins capped with α-2,6-linked instead of α-2,3-linked sialic acids (307, 308, 354), we did not observe any *in vitro* binding of native Prolastin-C bearing α-2,6-linked sialic acids to ASGPR orthologs (Chapter 4). Likewise, two other studies observed no differences in their PK using mice and their binding affinities towards hASGPR by SPR when glycans are capped with with α-2,3- or α-2,6 sialic acid. Additionally, the uptake of Prolastin-C with terminal α-2,8-Neu5Ac-α-2,6-Neu5Ac (ProC-2,8) was also negligible and comparable to that of α-2,6-Neu5Ac alone for all three ASGPR orthologs, although Lindhout *et al*. reported an improved PK in mice for α-2,8-Neu5Ac-α-2,6-Neu5Ac-linked A1AT compared to native version (50). It is important to note that biologics frequently exhibit complex and species-specific pharmacokinetic profiles due to their distinct physiochemical features (360), which might explain the differences we observed between our *in vitro* study and the others. Interestingly, for α-2,3 sialylated Prolastin-C (ProC-2,3), we found that its uptake by the mouse ASGPR was statistically higher compared to that of human and rat ASGPR orthologs. Since the sialylation levels of ProC-2,3 was not complete, and it contains ~22 % of mono-sialylated bi-antennary glycans with one branch terminated with α-2,3 sialic acid and the other with terminal Gal (A2G2S1(3)), our findings suggest that mouse ASGPR may have a higher affinity for the exposed terminal Gal residue found in that A2G2S1(3) glycan relative to human and rat orthologs.

In conclusion, we have developed a CHO cell-based assay with a significantly higher SNR than HepG2 cells, which could be used to evaluate the recognition and internalization of glycoproteins with distinct glycan structures by ASGPRs. We showed that ASGPR orthologs may have slightly different sensitivities towards partially exposed galactose residues. A better comprehension of ASGPR affinities for glycan structures found on glycoproteins may provide valuable insights for enhancing pharmacokinetics of recombinantly produced therapeutic proteins, where altered glycan profile can potentially shield the therapeutic protein from

recognition and clearance. This knowledge can guide the engineering of glycosylation patterns to enhance the therapeutic potential, efficacy, and safety of these proteins.

Chapter 6: Conclusion and Future Directions

A1AT is one of the most abundant plasma proteins that inhibits serine proteases, including NE. Its deficiency is a genetic disorder affecting more than 3.4 million individuals worldwide. Currently, there is no recombinant source of A1AT available for use as a drug due to the great difficulty in producing it with natural glycosylation pattern and the high quantities needed. As a consequence, the first objective of this thesis was to develop an oxidation-resistant recombinant version of A1AT with human glycosylation pattern expressed in glycoengineered CHO cells for therapeutic use. By knocking-out two glycosylation-gene targets using CRISPR/Cas9 technology, followed by an overexpression of human α-2,6-sialyltransferase, we have achieved to generate a glycoengineered cumate-inducible CHO cell line that was capable of producing over 2.1 g/L and 2.8 g/L of active recombinant WT and mutein forms of A1AT with an enhanced resistance to oxidative inactivation, respectively, bearing predominant human-like A2G2S2 (6,6) *N*-glycan structure.

CRISPR/Cas9 is a powerful genome-editing tool with a wide range of research and potential therapeutic applications. However, the use of CRISPR/Cas9 requires a comprehensive knowledge of their off-target effects to minimize the risk of deleterious outcomes (361). While disrupting two target genes by CRISPR/Cas9 combined with the overexpression of a human glycosyltransferase enabled us to produce of rA1AT with human-like *N*-glycan structures, we did not explore if our protocol gave rise to undesired off-target DNA modifications in the CHO genome. In order to validate the target specificity of CRISPR/Cas9 technique, several different methods such as deep sequencing, web-based prediction tools, and chromatin immunoprecipitation sequencing (ChIP-seq) could be used to assess these off-target effects (362).

All the stable production of rA1ATs described in Chapter 3 were performed in volumes not exceeding 30 mL of culture medium in fed-batch mode. Nevertheless, to generate a sufficient quantity of product for preclinical studies and ultimately for commercial production, scaling up in

CHO cell-based biopharmaceutical production is a fundamental component of process development in the biotechnology industry. For mammalian cell culture system, however, process scale-up from the laboratory to production scale can be challenging due to various factors, involving their shear sensitivity, oxygen transfer, bulk liquid mixing, dissolved carbon dioxide (dCO_2) removal, pH and temperature control etc. Therefore, it would be valuable to test the productivity of the glycoengineered pool expressing rA1ATs using larger volumes and to optimize conditions accordingly in order to increase recombinant protein titer (363).

Although α-2,3 sialylation was suggested to prevent clearance of glycoproteins more effectively than α-2,6 sialylation in the literature (307, 308), little is known about the impact of α-2,3- vs α 2,6-sialylation on the *in vivo* half-life of A1AT. Therefore, the second objective of this thesis is to assess the effect of different A1AT sialylation on its cellular uptake by ASGPR orthologs.

By developing a CHO cell-based assay with a much greater SNR, we showed that HL-1 alone was sufficient for the uptake of asialylated glycoproteins by human ASGPR, whereas, rat and mouse ASGPRs required both HL-1 and HL-2 subunits in order to form functional and high affinity receptors. In our studies, we further demonstrated that co-expression of human hHL-2 with the hHL-1 subunit did not enhance the cellular uptake of ASGPs compared to hHL-1 alone (Chapter 4), however, we did not investigate if co-expression of HL-1 and HL-2 for rat or mouse ASGPR at different ratios (other than 1:1) would result in more receptor complexes being formed, thereby increasing the receptor's binding capacity. Therefore, it would be interesting to explore if co-expressing both rat and mouse subunits at different ratios could enhance receptor function and improve the uptake of ASGPs.

With the use of Prolastin-C bearing native or *in-vitro* remodelled glycan structures, our studies revealed no uptake of Prolastin-C when glycans are terminated with α-2,6-Neu5Ac nor α-2,8-Neu5Ac-α-2,6-Neu5Ac by human, rat, and mouse ASGPR orthologs. For Prolastin-C containing ~22% of mono-sialylated bi-antennary glycans with α-2,3-linkage (A2G2S1(3)) in addition to main A2G2S2 (3,3) structure (49%), uptake by the mouse ASGPR was statistically higher compared to that of human and rat ASGPR orthologs. According to our results, we did not detect the advantage of α-2,3 over α-2,6-linkage for preventing the uptake of Prolastin-C by ASGPR orthologs. However,

we observed that mouse ASGPR may have a higher affinity for the exposed terminal Gal residue found in the mono-sialylated bi-antennary A2G2S1(3) glycan relative to human and rat orthologs. It would be also informative if future studies investigate the correlation between the increased number of branches with exposed Gal residues of A1AT and the degree of binding to ASGPR orthologs.

In conclusion, the oxidation-resistant, α-2,6-sialylated rA1AT described in this research project could represent a viable biobetter drug, which would ultimately serve in augmentation therapy for patients with A1ATD while offering a safe as well as more stable alternative for augmentation therapy and ensuring A1AT homogeneity between batches compared to that of clinically used. However, it should be noted that unlike biosimilars, which follow a shortened regulatory path, biobetters are considered as an investigational new drug requiring pre-clinical and clinical studies to prove their safety and efficacy in all the indications for which they want approval. In addition, the engineered cell line created in this project will be a promising host for production of other recombinant proteins where fit-for-purpose glycosylation pattern is crucial for their therapeutic success. Finally, a better understanding of ASGPR affinities for glycan structures present on glycoproteins may provide insights for optimizing pharmacokinetics of recombinantly produced therapeutic proteins, including A1AT. Future studies should examine the safety and efficacy of the rA1AT described herein *in vivo* via pharmacokinetic assay.

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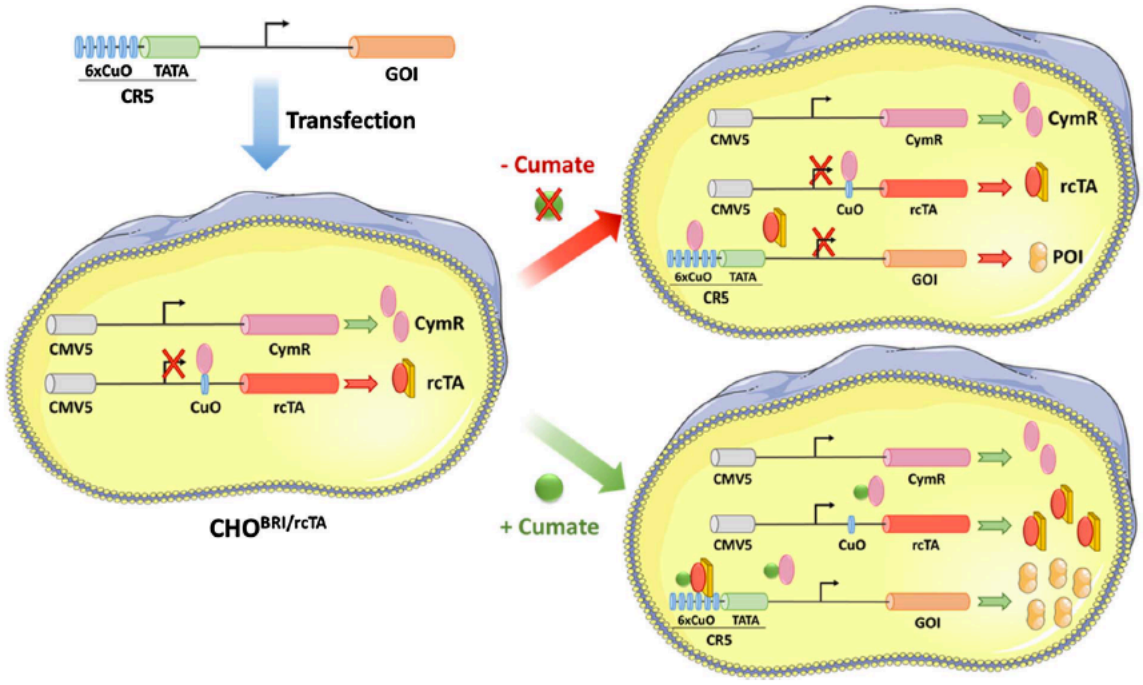
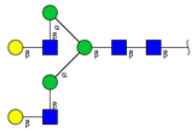
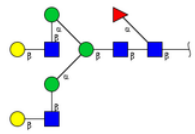
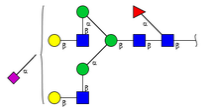
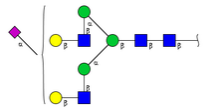
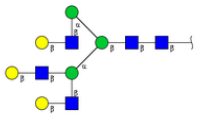
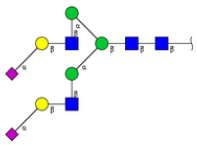
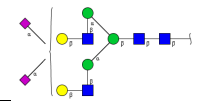
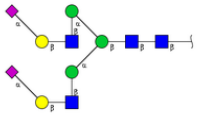
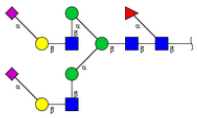
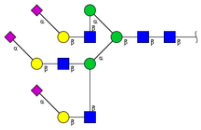
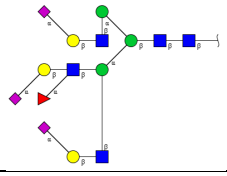
Annex 1: The cumate-inducible CHO$^{BRI/rcTA}$ expression system



The CHO$^{BRI/rcTA}$ cell line stably expresses both the cymene repressor (CymR), which is able to bind specifically to the CuO operator sequence in the absence of cumate, and the cumate reverse transactivator (rcTA). Expression of the rcTA is controlled by the CMV5-CuO promoter and the binding of the CymR (constitutively expressed) to the CuO site prevents rcTA expression. For recombinant protein production, plasmid encoding the gene of interest (GOI) is controlled by the CR5 promoter that contains six repeats of the CuO sequence placed upstream of the cytomegalovirus promoter TATA box. Upon transfection of cells with this inducible plasmid, addition of cumate, causes a conformational change of the CymR where it dissociation from the CuO sequence, thus allowing rcTA expression. Subsequently, binding of cumate to the rcTA enables its binding of the CuO operators present in the CR5 promoter, and thus inducing the expression of GOI (Figure adapted from Poulain *et al.*, 2017 (327))

Annex 2: N-linked glycan structures nomenclature

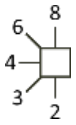
Structure	Name
	A2G2
	F(6)A2G2
	A2G2S(3)1
	A2G2S(6)1
	A3G3
	A2G2S(3,3)2
	A2G2S(6,3)2
	A2G2S(6,6)2
	F(6)A2G2S(6,6)2
	A3G3S(6,6,6)3

Structure	Name
	A3FG3S(6,3,6)3

Monosaccharide symbol

- Galactose
- Mannose
- *N*-Acetylglucosamine
- *N*-Acetylneuraminic acid
- Fucose

Linkage position



Annex 3: Articles as a second author or co-author

Lalonde, M. E., Koyuturk, I., Brochu, D., Jabbour, J., Gilbert, M., & Durocher, Y. (2020). Production of α2,6-sialylated and non-fucosylated recombinant alpha-1-antitrypsin in CHO cells. *Journal of biotechnology, 307*, 87–97. https://doi.org/10.1016/j.jbiotec.2019.10.021

Forest-Nault, C., Koyuturk, I., Gaudreault, J., Pelletier, A., L'Abbé, D., Cass, B., Bisson, L., Burlacu, A., Delafosse, L., Stuible, M., Henry, O., De Crescenzo, G., & Durocher, Y. (2022). Impact of the temperature on the interactions between common variants of the SARS-CoV-2 receptor binding domain and the human ACE2. *Scientific reports, 12*(1), 11520. https://doi.org/10.1038/s41598-022-15215-5