# Engineering O-glycovariants of etanercept with heightened potency using an O-Glycoengineered CHO cell line platform

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## Abstract

Asparagine linked glycans (N-glycans) on monoclonal antibodies impact drug product safety and efficacy. Several therapeutic proteins also contain serine and/or threonine linked glycans known as O-glycans, but the significance of O-glycans on therapeutic proteins remains elusive. To investigate the impact of O-glycans on therapeutic potency and safety, we successfully generated an O-glycoengineered Chinese hamster ovary (CHO) cell line platform by manipulating critical glycogenes within the O-glycosylation pathways to modulate the expression of O-glycans on therapeutic proteins. As a proof-of-concept study, we utilized this platform technology to generate five different O-glycovariants of etanercept and investigated the impact of O-glycans on the physiochemical and biological properties of etanercept. Our results demonstrate that this CHO cell platform can produce O-glycoengineered therapeutic proteins containing truncated O-glycan structures with and without sialic acid, sialylated Core 1 O-glycans with sialylated Core 3 O-glycans, or sialylated Core 3 O-glycans alone. Moreover, this platform demonstrated to exclusively modulate O-glycans on etanercept as compared to N-glycans. Using the etanercept O-glycovariants, we confirmed that O-glycans on etanercept alters the protein's isoelectric point and one O-glycovariant has heightened potency. Collectively, this work established an O-glycoengineered CHO cell platform that can produce novel engineered proteins with desired O-glycans for higher quality therapeutics.

## Introduction

Protein glycosylation is an enzymatic process that modifies certain residues of proteins by attachment of oligosaccharides. Hundreds of enzymes in the endoplasmic reticulum and Golgi apparatus are known to contribute to the formation of glycans and glycosylation prior to protein sorting to the plasma membrane or extracellular space. Two major glycosylation types are N-linked glycans to asparagine residues (N-glycans), and O-linked glycans to serine, threonine and even tyrosine residues (O-glycans) on proteins traversing through the secretory pathway. N-glycosylation is known to significantly impact drug safety and efficacy of therapeutic proteins. In contrast to N-glycans, the impact of O-glycans of therapeutic proteins on drug safety and efficacy remains unknown.

CHO cells are the most common cell substrate for manufacturing recombinant protein therapeutics or biotherapeutics. Although CHO cells are unique in their ability to produce therapeutic proteins with "human-like" glycans, their ability to produce different O-glycan structures is limited. CHO cells are unable to produce the Core 2, extended core 1, and core 3 complex O-glycans, but exclusively produce mono- and di-sialylated Core 1 [SAα2,3Galβ1,3(±SAα2,6)GalNAc-α] structures. Here we have established clonally-derived O-glycoengineered CHO cell lines through gene-editing and transfection to have distinct O-glycosylation machinery to produce glycoproteins with sialyl Core 3 structures alone, sialyl Core 1 structures with STn antigen or sialyl Core 3 structures, or Tn antigen with and without STn antigen.

Etanercept is a dimeric fusion protein that contains the extracellular ligand binding portion of human p75 tumor necrosis factor receptor 2 linked to the Fc portion of human IgG1 with three N-linked and thirteen O-linked glycosylation sites. Structural, physiochemical, and biological characterization of etanercept and etanercept-ykto, a biosimilar, in combination with data from clinical studies suggest that changes in sialylated O-glycans may impact the safety of etanercept. We used etanercept for our proof-of-principle protein engineering studies and evaluated TNF-α binding, potency, and stability of each O-glycovariant as compared to a reference product. We demonstrate that changes in O-glycans on etanercept can alter the isoelectric point, increase TNF-α binding, and heighten the potency of etanercept without affecting the sensitivity of the therapeutic protein to oxidative stress induced instability.

## Materials and Methods

**Generation of clonally-derived O-glycoengineered CHO cell platform**

To generate stable clones, transfected cells were exposed to antibiotic selection , Fluorescence Activated Cell Sorting (FACS), and one round of limiting dilution cloning at 0.5 cells/well.

**Production and purification of experimental and O-glycovariants of etanercept**

Harvest media underwent sequential chromatography. Etan-Ref was a commercially available therapeutic protein.

**Characterization**

Common techniques were performed to characterize and quantify purity, identity, glycan content, isoelectric point, potency and TNF-α binding..

Statistics and Reagents

**Statistical analyses**

When 1 factor was analyzed, a one-way ANOVA was performed with a Sidak's multiple comparison test to identify statistical differences. When >1 factor was analyzed, a two-way ANOVA was performed with a Tukey's multiple comparison post-hoc test to identify significant differences. A p value of < 0.05 indicated statistical differences.

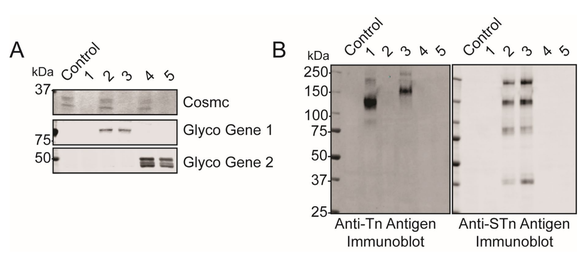



**Figure 1.** Generation of O-glycoengineered CHO cells. A) Representative immunoblot of glycosylation-related (Glyco) genes. B) Immunoblot of truncated O-glycans with antibodies.

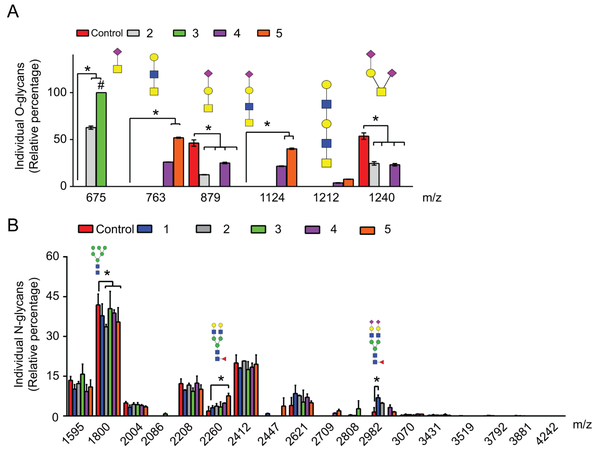



**Figure 2.** Glycosylation characterization of glycoengineered CHO cell line platform. (A) O-glycans and (B) N-glycans identified using CHO cell lysates.

## Results and Discussion

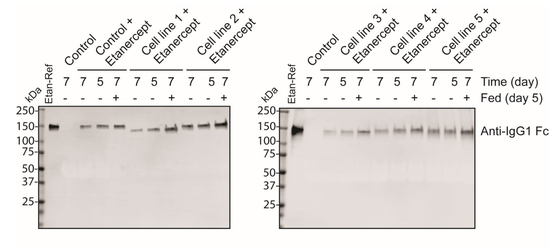



**Figure 3.** Generation of CHO cells expressing Etanercept. Representative immunoblots using an anti-human IgG1 Fc antibody.

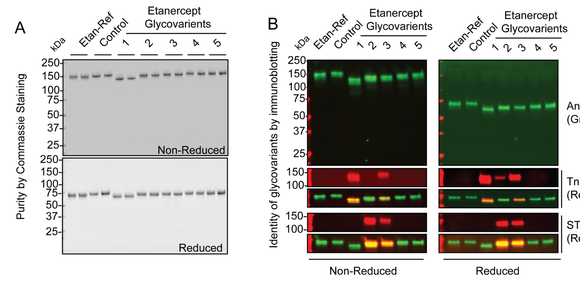



**Figure 4.** Purity and identity assessment of purified O-glycovariants of etanercept. A) Coomassie stained SDS-PAGE. B) Representative immunoblots.

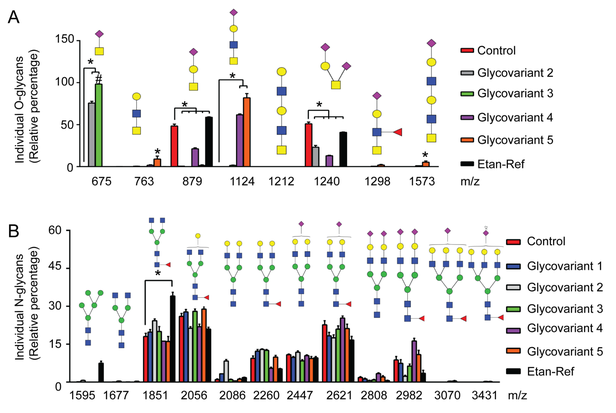



**Figure 5.** Glycosylation characterization of reference material and etanercept O-glycovariants. (A) O-glycans and (B) N-glycans identified using reference material and purified etanercept proteins.

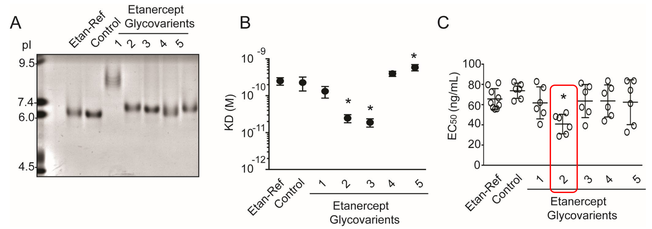



**Figure 6.** Physiochemical and biological characterization of reference material and etanercept O-glycovariants. (A) isoelectric point, (B) TNF-a Binding, and (C) TNF-a Neutralization. The Etan O-glycovariant-2 with STn antigens had heightened potency (red boxed).

## Conclusion

In summary, we established a manufacturing relevant O-glycoengineered CHO cell line platform to produce recombinant proteins. Using this platform, we bioengineered etanercept as a proof of principle study and demonstrated that changes in O-glycans can alter the therapeutic protein's critical quality attributes. Collectively, we propose that this CHO cell platform can be utilized for a variety of different applications such as the production of O-glycan specific ligands for assay development, the production of O-glycovariants of growth factors for cell culture, and the manufacture of therapeutic proteins with designed O-glycan structures. This platform offers a tool to define the quality and safety attributes of O-glycosylation to O-glycosylated therapeutic protein drugs.

## FDA Mission Relevance

O-glycans on therapeutic proteins may alter the safety, quality, and activity of a drug. Using this platform, the impact of O-glycosylation on therapeutic proteins drug safety, efficacy and quality and safety can be determined. This platform is available.

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## Disclaimer Statement

The authors have no interests to declare. This publication reflects the views of the authors and should not be construed to represent FDA's views or policies.

## Publication

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