

From Discovery to Preclinical: Evaluating Liabilities of a Rare PTM in a Bispecific ADC

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September 18, 2026

Acknowledgments

Analytical Sciences

- Wei Wu
- Hangtian HT Song
- Yunping Huang
- Chris Chumsae
- Li Tao
- Mandy Xie
- Cara D'Amico
- Ismael Jose-Difo
- Thomas Slaney

Up/Downstream Process Development

- Rui Zheng
- Yudi Mejia
- Jacquelyn Schulman
- Dan Huang
- Diana Chereches
- Dane Kazem
- Ashley Bui
- Yizhou Zhou
- Gregory Keil
- Anurag Khetan
- Varsha Vasthare
- Yao Wang (Downstream Lead)
- Christopher Furcht
- Joshua Avant
- Erica Jungwirth
- Andres McRea

Drug Product Development

- Jiawei Gong

Discovery Biology

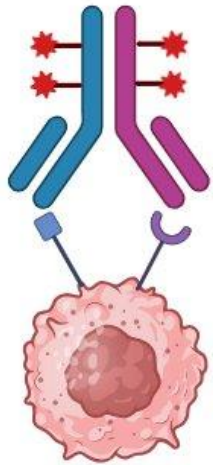
- Daron Forman
- Gabe Mintier
- Angela Chan
- Juhi Juneja
- Holly Schmidt
- Nicole Zaubi
- Christopher Connelly
- Cris Hollander
- Chintan Savla

From Discovery to Development

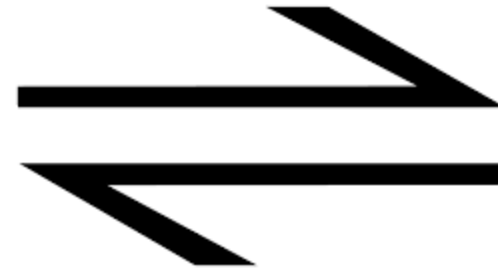
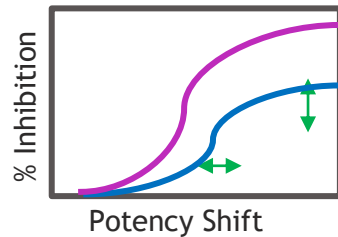
Discovery

Mechanism of Action:

- Co-engaging 2 tumor antigens improves ADC activity
 - Increases internalization and payload delivery
 - Increases total number of available antigens for targeting



Expected Dual-Antigen Binding Drives Activity

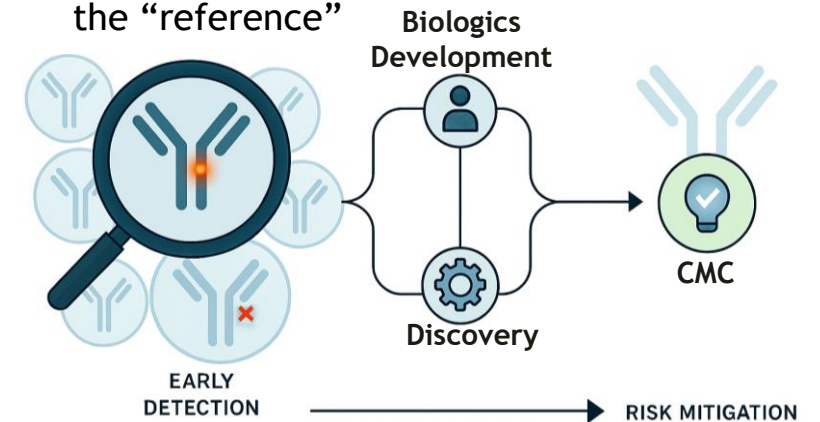


Knowledge exchange is critical for successful identification, risk assessment, and mitigation of molecular liabilities.

Development

Critical Input from Attribute Characterization:

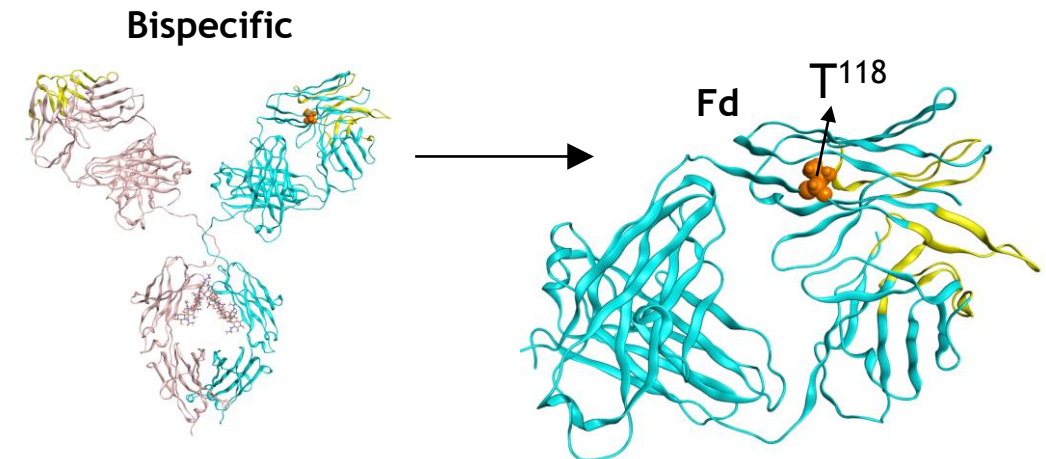
- Identifying molecular liabilities in the form of post-translational modifications in early clones, with Discovery material serving as the “reference”



- The earlier we identify potential liabilities, the earlier we can engage **multiple teams** to investigate the associated risks and support the broader CMC team in making informed decisions

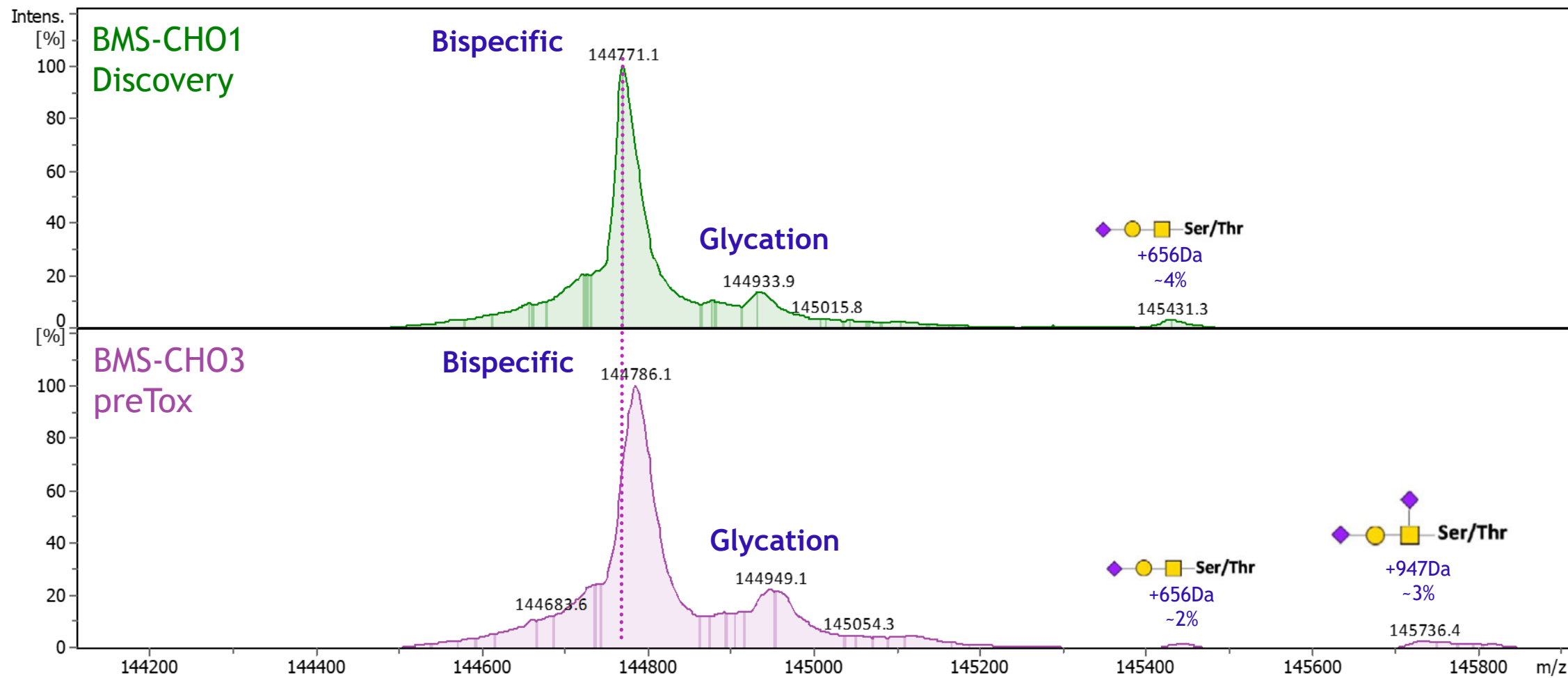
Characterization Background

1. Discovery material was produced using BMS-CHO1 cells.
 - No major chemical modifications detected via peptide mapping
 - Minor O-glycans (~3%) on HC2 T118 near CDR3 region
2. PreTox (preclinical) material produced with BMS-CHO3 consistently showed a mass increase of ~16 Da
3. Since the PTM was identified at the intact MS level, we need to determine:
 - Is it limited to a single amino acid or scattered throughout the mAb?
 - Is it on the LC, HC1, or HC2?
 - Is it incomplete pyroE, oxidation of Met or Trp, hydroxyproline, etc.?



Deglycosylated Intact LC-MS Analysis: Discovery vs. preTox

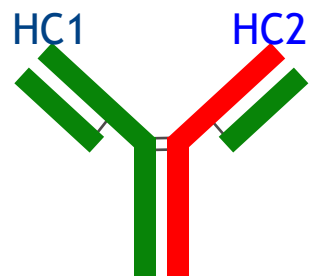
Calc. Mass: 144771Da



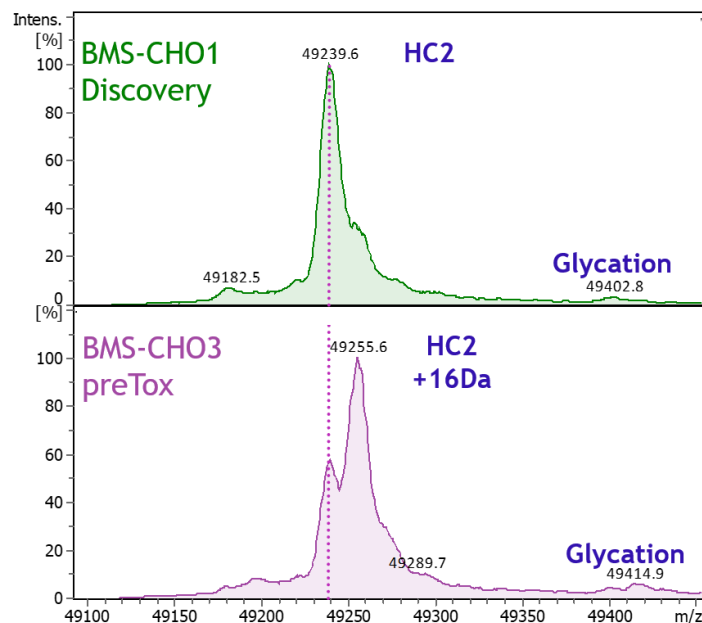
Investigating the +16 Da PTM

Open questions: is this a single-site modification, incomplete pyroE formation, oxidation, or hydroxyproline?

Next: define the impact and determine whether it can be controlled.



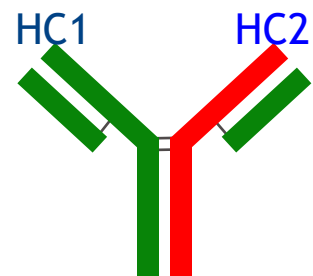
LC-MS analysis of the reduced and de-glycosylated protein indicated that +16 Da PTM is located on the HC2



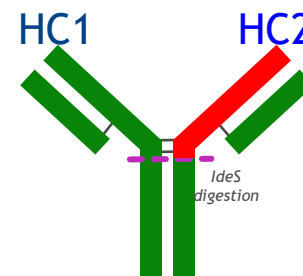
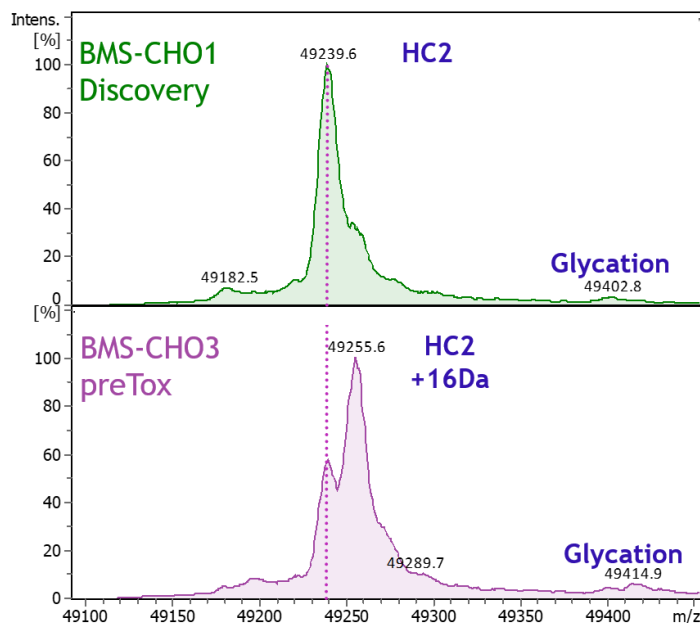
Investigating the +16 Da PTM

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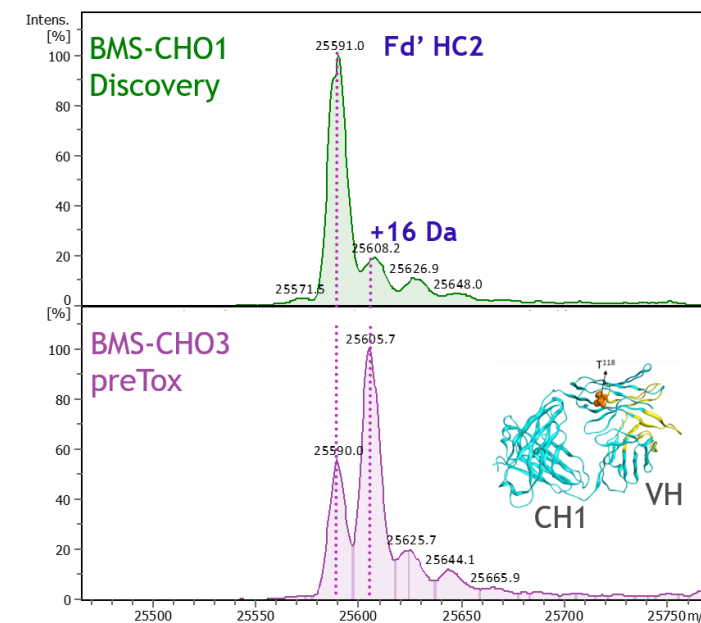
Next: define the impact and determine whether it can be controlled.



LC-MS analysis of the reduced and de-glycosylated protein indicated that +16 Da PTM is located on the HC2



LC-MS analysis of the IdeS treated protein indicated that +16 Da PTM is located on the Fd' of HC2

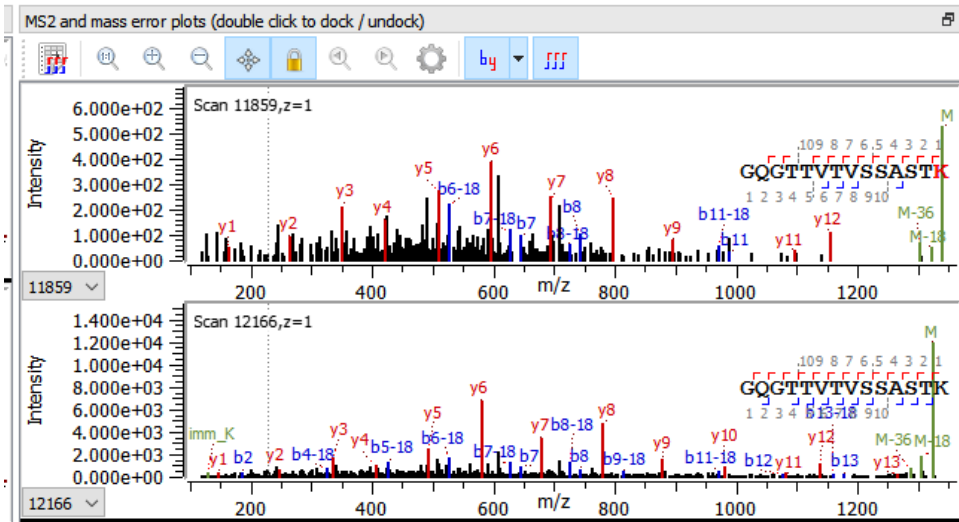


What is Fd'?

IdeS cleaves the antibody below the hinge; reducing the resulting F(ab')₂ then releases the light chain, leaving Fd' – just the VH+CH1 portion of the heavy chain.

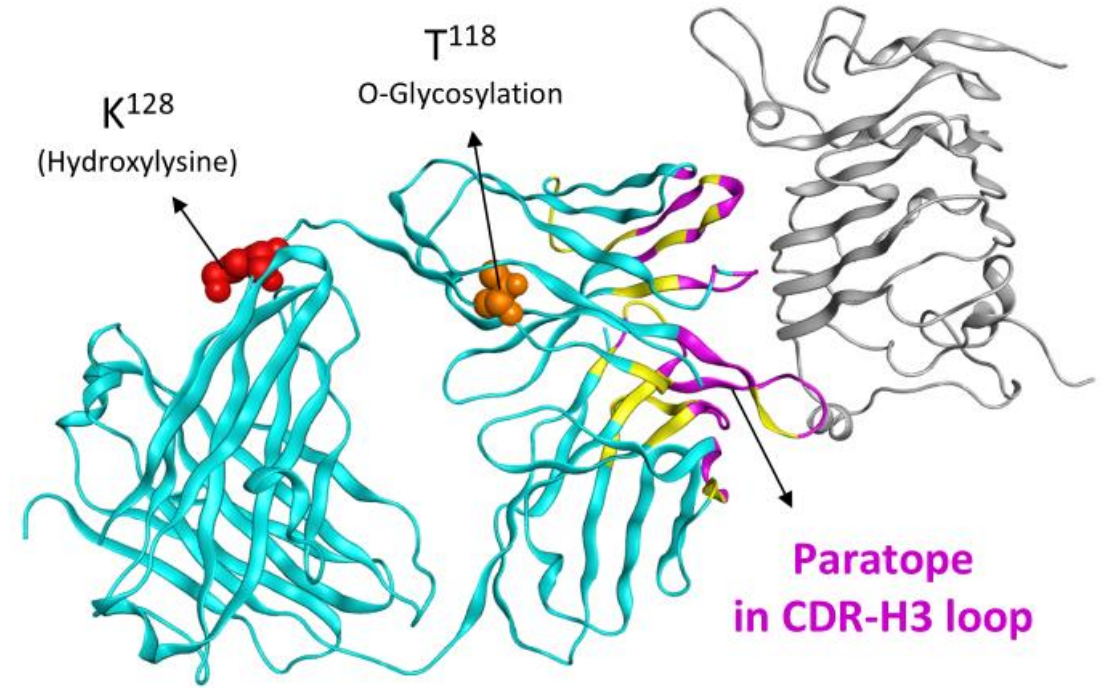
Peptide Mapping Analysis: Identifying the Specific Residue

BMS-CHO3 preTox
HC2 115-135
GQGTTVTVSSASTKGPSVFPL
72%



Hydroxylysine (Hyl) modification was identified at a single site at K₁₂₈

Hyl modification wasn't identified in Discovery material at all.



Modeling based on cryoEM structure of HC2 antibody/antigen complex (Alok Sharma and Naresh Chennamsetty)

Hydroxylysine as a potential Critical Quality Attribute: Impact and Risk Mitigation

Efficacy

Safety

Potency

CHO1 and CHO3 material showed comparable binding (FLISA & SPR)

Immunotoxicity

No cytokine release in human whole blood assay

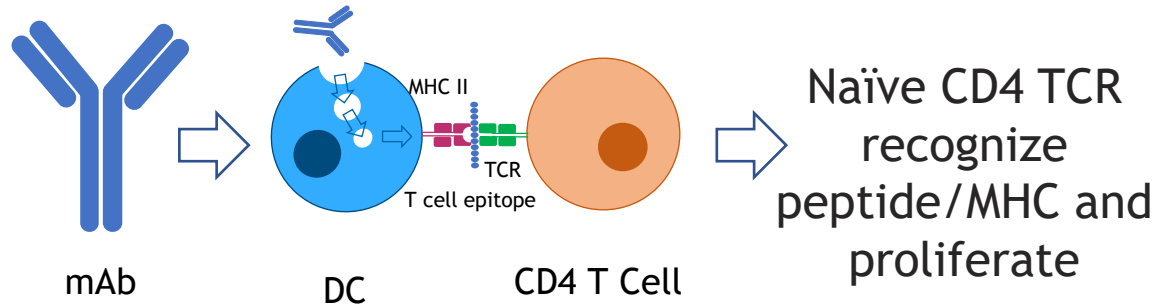
Immunogenicity

CHO3 material showed elevated response (20.8%) resulting in a medium risk assessment. Investigation ongoing

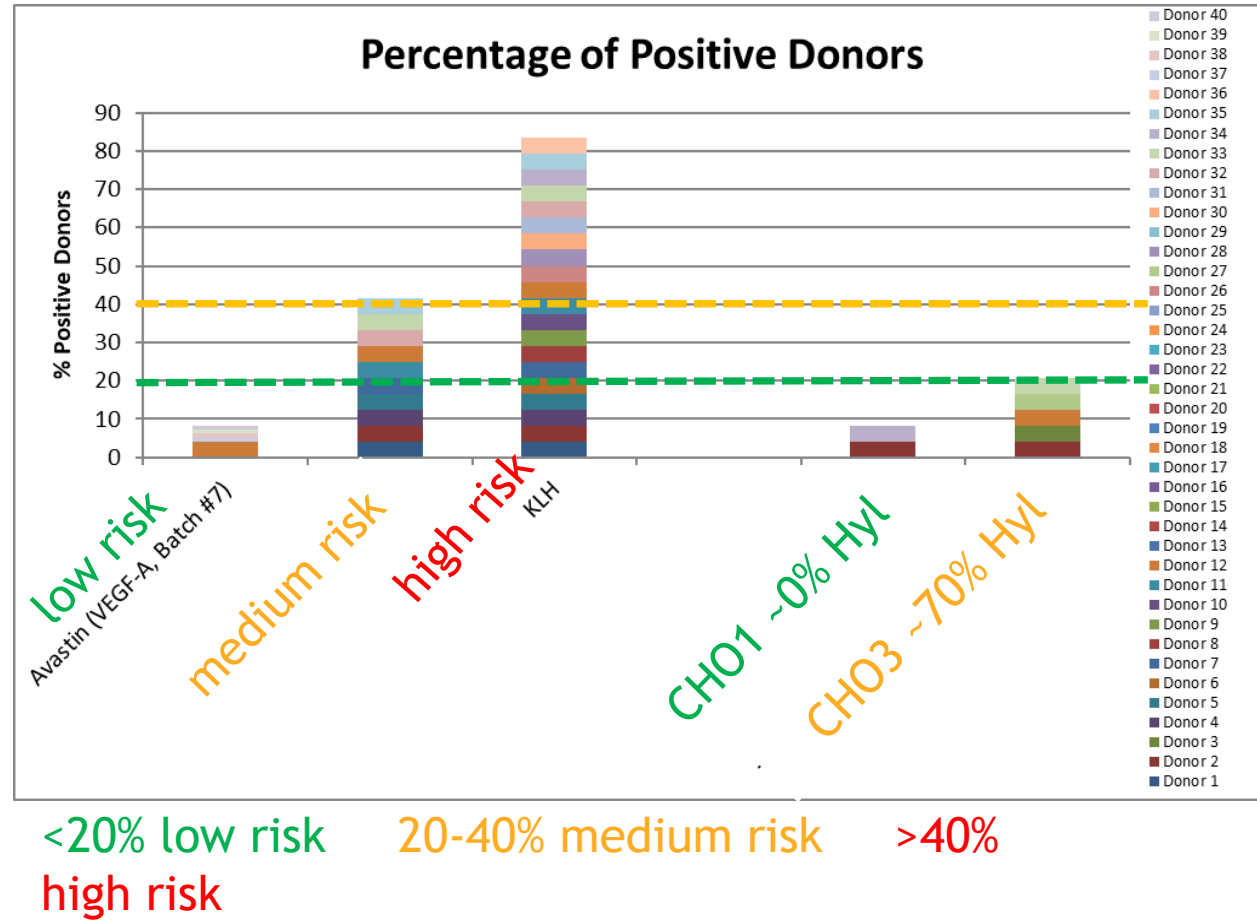
This kind of focused, cross-functional safety evaluation of a single molecular attribute is rare this early in development.

Immunogenicity Risk Assessment of Hyl modification

Antigen processing and peptide presentation



- Hyl modification may alter processing and presentation of peptides from the bispecific
- DC-PBMC (dendritic cells-peripheral blood mononuclear cells) proliferation assay was used to determine if naïve human CD4 T cells recognize peptides that are processed and presented by dendritic cells as foreign



Hydroxylysine Mitigation Overview

Two complementary strategies were pursued to eliminate the ~16 Da hydroxylysine (Hyl) modification identified at ~70% occupancy in BMS-CHO3 preTox material.

Cell Line Development Approach

- Cell line engineering
- Multiplex KO: PLOD1, PLOD2, and PLOD3

Result: Hyl eliminated (~0%) – no impact on cell growth, viability, or productivity

Upstream Approach

- Medium supplement optimization
- Hyl Inhibitors

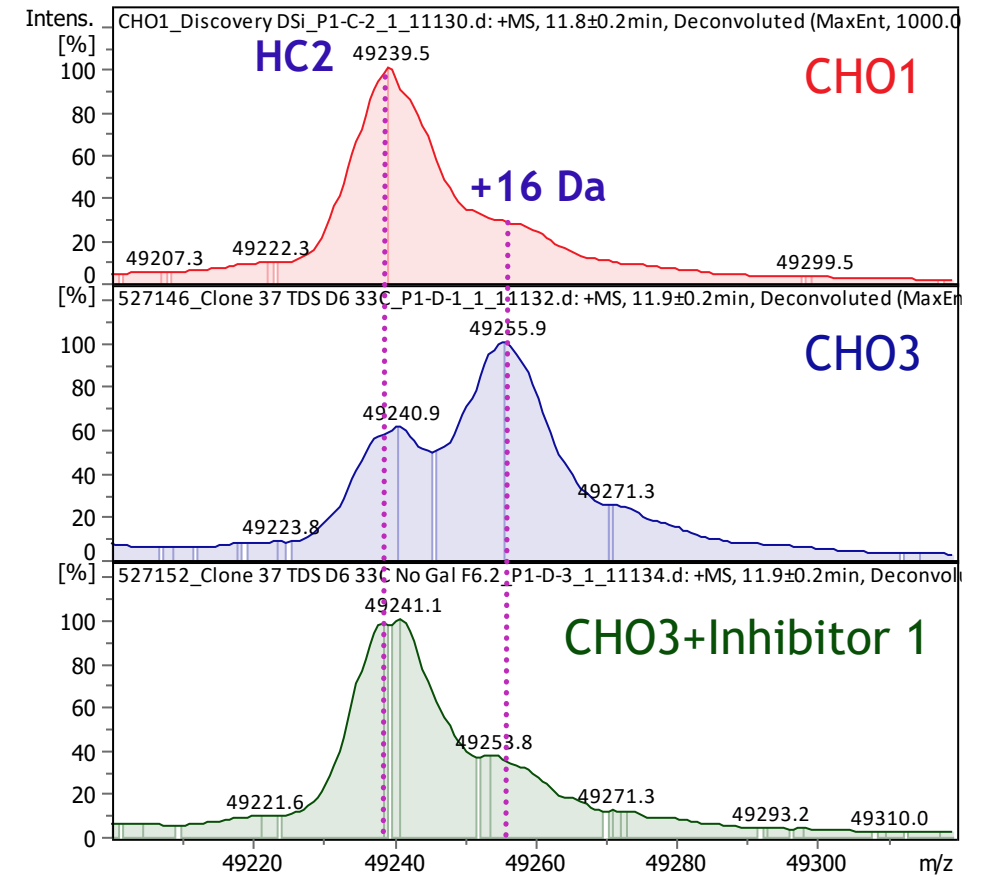
Status: ongoing – fine-tuning reactor day and Inhibitor 1 concentration

Hydroxylysine Mitigation

Shared goal: eliminate Hyl without impacting cell growth or productivity

Working Together with Upstream on Potential Solutions

- From a wide array of reactor conditions (temperature, pH, agitation, dissolved oxygen) and additives (EDTA, Mn, Gal, Inhibitors, and more), Inhibitor 1 was identified as a promising solution.
- The intact MS data shows significant reduction of Hyl levels with Inhibitor 1
- This work is ongoing, and we are fine-tuning the addition of Inhibitor 1 (reactor day, concentration, etc.).



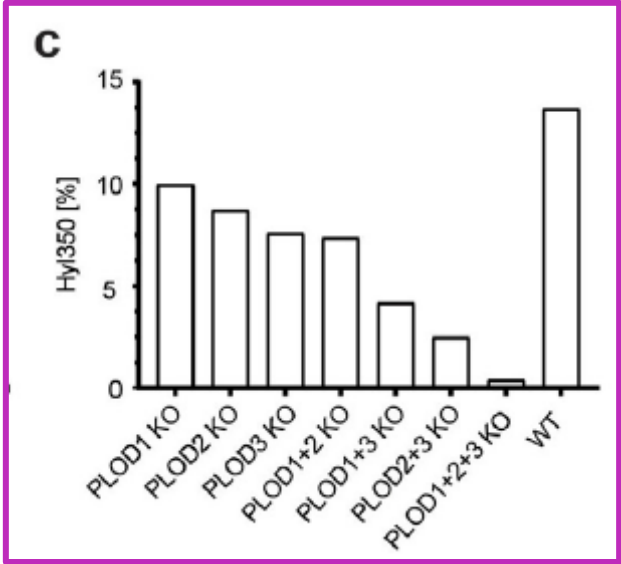
Hyl Identification in the Literature

TABLE 1 Hyl-containing therapeutic peptides and proteins reported.

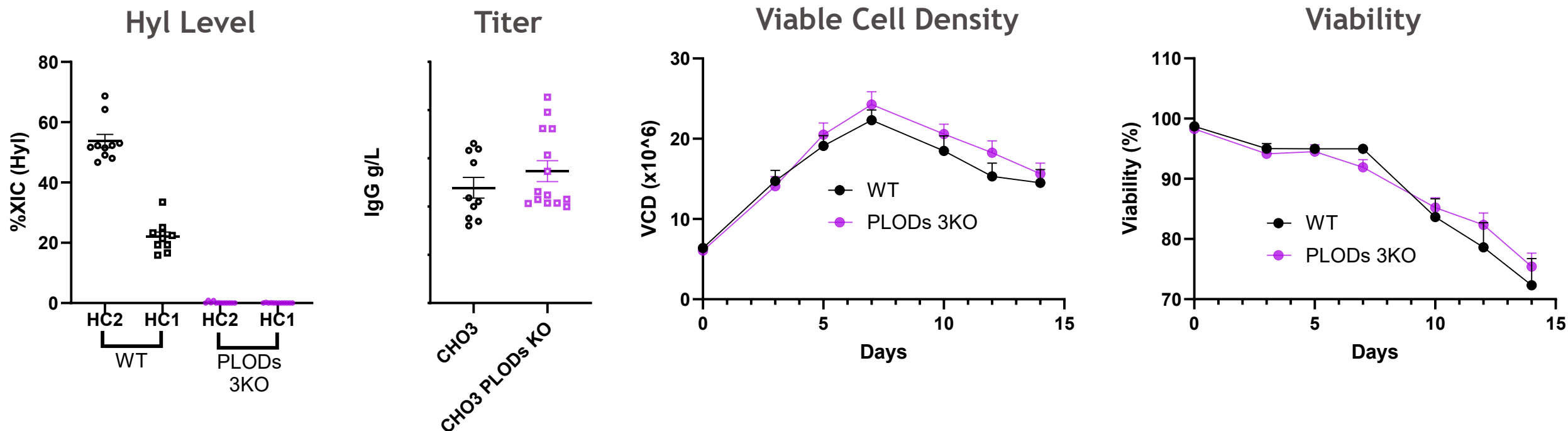
Peptide/protein	Sequence	Level of Hyl	Reference
Recombinant human plasminogen activator (rtPA)	IKGGLFADIASHPWQAAIFAK	Up to 20%	Molony et al. (1995)
Recombinant antibody CD4/rCD4IgG	ILGNEGSFLT K GFSK	Up to 10%	Molony et al. (1995)
De13a, a toxin of marine cone snail	DCPTSCPTTCANGWECC K GYPVCVN K ACSGCTH	Up to 100%	Aguilar et al. (2005)
Somatostatin-28, a peptide hormone from anglerfish	RSVDSTNNLPPRERKAGCKNFYW K GFTSC	Up to 40%	Andrews et al. (1984)
IgG1 mAb	XXXXXXXXXWGQGT L TVSSAST K	Not assessed	Xie et al. (2016)
TCB mAb 1	XXXXXXXXXXXX K GR XXXXXXXXXXXX K DSTYSLSSTLTLS K LTVLSSAST K GPSVFPLAPSSK	12.4%	This work
TCB mAb 2	XXXXXXXXXXXX K GR XXXXXXWGQGV L TVTVSSAST K GPSVFPLAPSSK L K SGTASVVCLLNNFYPR DSTYSLSSTLTLS K ADYEK LTVLSSAST K GPSVFPLAPSSK	2.9%	This work
TCB mAb 3	XXXXXXXXXXXX K GR NQVSLSCAV K LTVLSSAST K GPSVFPLAPSSK	7.0%	This work

The red lysine represents the position of Hyl modification. Please note that “X” represents amino acids of the identified peptides from the mAb CDR regions.

Hyl modification is mediated by lysyl hydroxylases (PLOD1/2/3).



BMS-CHO3 PLODs 3KO Eliminates Hyl with no Impact on Cell Growth or Productivity



PLODs 3KO does not impair cell growth, peak density, viability, or productivity

Summary



Identification

preTox material (BMS- CHO3) showed a consistent ~16 Da intact-mass increase vs. the BMS-CHO1 Discovery reference.



Characterization & Risk

Tiered LC-MS workflow localized hydroxylysine (Hyl) at K128 on HC2 (~70% preTox vs. ~0% Discovery). Binding and cytokine release were comparable, but immunogenicity risk was elevated (20.8%, medium).



Mitigation

Two strategies reduced/eliminated Hyl: Inhibitor 1 supplementation (suppresses lysyl hydroxylase) and a PLOD1/2/3 triple-knockout cell line, with no impact on growth or productivity.

~16 Da

Intact-mass increase

K128 (HC2)

Modified residue, Fd' region

~70% vs ~0%

Hyl occupancy: preTox vs Discovery

20.8%

Immunogenicity signal (medium risk)