Beyond Pegylation: Biobetters to Enhance the Efficacy and Safety of Protein Therapeutics with a Focus on Diminishing Immunogenicity

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• Non-human and some human proteins are inherently immunogenic to humans. Studies to identify immunogenic “hot spots” may allow engineering to reduce immunogenicity

• Product degradation in vivo limits clinical performance via loss of activity and enhanced immunogenicity. Therefore, degradation reactions of therapeutic proteins should be evaluated in the in vivo context in which the therapeutics function

• If evidence that degradative processes in vivo impact immunogenicity and potency, consider engineering proteins to reduce susceptibility to such modifications.
Ability to Detect and Monitor Types and Levels of Protein Degradation

- During Manufacture: in process testing and controls
- In Storage: stability studies
- In Vivo: need to apply advanced technologies to understand the fate of therapeutic proteins in vivo; should be a high priority
  - In inflammatory environments: diabetes, autoimmune disease, infection, cancer, aging
  - In wound healing, tissue injury and regeneration
  - Tissue specific environments
Integrity of Therapeutic Proteins in in Vivo Environments Presents Challenges
(from Wald D et al 2012)
Chemical Degradation Pathways Associated with Immunogenicity and Autoimmunity: Profound Influence of In Vivo Environment

- Aggregation
- Oxidation/Aldehyde mediated modifications
- Truncation
- Deamidation
- Citrullination (Deimination)
- Others
Mechanisms by which Chemical Degradation of Proteins Induce Immune Responses

• Generate novel epitopes:
  – Direct modification of therapeutic protein
  – Indirect: modification alters antigen processing and presentation
    • alteration of sites, extent, and rapidity of protease activity;
    • generation of novel or previously cryptic peptides presented by HLA to T cells;

• Enhanced HLA binding of modified product peptides:
  – deamidated and citrullinated proteins have enhanced binding to specific class II HLA molecules

• Generate Aggregates
  – Alteration of charge, hydrophobicity etc
Immunogenicity Risk Assessment

- Immune response is to degraded protein only; no cross-reactivity on native therapeutic protein
  - Potential loss of efficacy if degraded protein still has activity
  - Safety: may cause hypersensitivity/inflammatory responses
  - Fosters *Epitope Spread*

- Immune response cross reacts to native therapeutic protein
  - Loss of product efficacy
  - Safety: hypersensitivity/inflammatory responses
  - Safety: cross-reactivity on endogenous protein counterpart
Proteins Behave Differently in Vivo
(Demeule et al 2009 Analytical Bochem)

Omal:IgE 1:1 PBS

Omal: IgE 1:1 Serum

Sedimentation coefficient ($S_{20, W}$)

$\begin{array}{c|c|c|c|c|c|c|c|c|c|c} S & 0.00 & 0.02 & 0.04 & 0.06 & 0.08 & 0.10 & 0.12 & 0.14 & 0.16 & 0.18 \\ \hline \text{c(s)} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ \end{array}$

$\begin{array}{c|c|c|c|c|c|c|c|c|c|c} 7.1 & 13.3 & 17.7 & 21.5 & \text{c(s)} & 0 & 0 & 0 & 0 & 0 & 0 \\ \end{array}$

$\begin{array}{c|c|c|c|c|c|c|c|c|c|c} 4.3 & 8.7 & 12 & 15 & 16 & 18 & 20 & 22 & 24 & 26 & 28 & 30 & 32 \\ \hline \text{c(s)} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ \end{array}$
Local Environment Exerts Profound Influence on Protein Aggregation

(Demeule et al 2006)
• “For new protein drugs, an open question will be the potential aggregation after in vivo injection, since the body can present a destabilizing environment to the protein, leading to aggregation. Further research could be focused on the analysis of therapeutic protein aggregates formed in vivo”
  – Demeule, Gurny and Arvinte 2006
Protein Alteration by Oxidation/Aldehyde Formation

- **During Manufacture**
  - In cells undergoing stress or cell death processes: upregulation of modifying enzymes, ROS

- **In Storage**
  - Container closures: leached metal ion (eg tungsten) catalyzed oxidation
  - Preservative mediated oxidation

- **In Vivo**
  - In Inflammatory environments: enzyme catalyzed and non-enzymatic chemical changes; generation of reactive oxidative species/aldehydes
Inflammatory Environment: Neutrophil Defenses and Diabetes Aldehyde-Tag Proteins

• Reducing sugars and lipid peroxidation in activated neutrophils release aldehydes that tag lysine residues of target proteins
  – Impairment of protein function
  – Crosslinkage of proteins to generate aggregates
  – Enhanced uptake into macrophages by scavenger receptor for aldehyde-tagged proteins
  – Enhanced immunogenicity
Self-Tolerance is Broken by Aldehyde Modifications of a Self Protein
(Fearon D et al 2000)

![Graph showing IgG anti-RCC levels over time with different treatments.

- Orange circles: glycolaldehyde + Cytochrome C
- Blue circles: glycoaldehyde+Inhibitor+Cytochrome C
- Red circles: Cytochrome C alone

Days: 0, 7, 14, 21, 28, 35, 42

IgG anti-RCC levels:
- 100,000
- 10,000
- 1,000
- 100

The graph shows a significant increase in IgG anti-RCC levels starting from day 21 and peaking around day 28, followed by a slight decrease around day 35 and stabilization around day 42.
Detection of Aldehyde Modified Protein by GC/MS in Human Inflammatory Tissue
(Hazen SL et al JBC 1997)
Degradation Pathways Associated with Immunogenicity and Autoimmunity

- Oxidation/Aldehyde Modifications
- *Deamidation*
- Citrullination (Deimination)
Deamidation and Formation of Isoaspartate
(Reissner and Aswad Cell Mol Life Sci 2003)

Introduces negative charge
Change in mass
Change in hydrophobicity
Protein Deamidation Potentially Linked to Immunogenicity via Several Mechanisms

• IsoAsp residues induce aggregation
• IsoAsp residues alter antigen presentation:
  – β-peptide linkage connecting isoasp residue and its C-terminus neighbor are not recognized by most proteases and peptidases; altered antigen processing
• IsoAsp residues prevalent in infected, stressed, apoptotic and aged cells:
  – presence of deamidated protein in milieu with innate immune adjuvants (vaccine)
Environmental and Structural Factors in Rate of Deamidation
(Wakankar A and RT Borchardt JPharm Sci 2006)

• Environmental factors in vivo promoting deamidation: can’t alter
  – pH: base catalyzed;
  – Temperature: directly correlated with rate;

• Structural: potential for engineering
  – Primary Sequence: deamidation rate greatly influenced by aa residue C terminal to Asn; steric bulk and flexibility of residue important;
  – Secondary structure: significantly lowers target residue susceptibility to deamidation when located in organized secondary structure; conformational restriction;
  – Tertiary structure: attenuated deamidation rate due to overall reduction in conformational mobility;
Therapeutic Proteins at High Risk of Deamidation Mediated Sequelae

- Extended in vivo half-life: prolonged exposure to high temperature and favorable pH eg mAbs
- Proteins targeted to inflammatory sites or tissue repair
- Primary sequences containing asparagine and glutamine/proline “hot spots”: alter for peptide/protein therapeutics where possible?
- Proteins with minimal secondary/tertiary structure: engineer with protective structural elements?
Deamidated snRNP Breaks Self Tolerance
(Mamula MJ et al JBC 1999)

T cell Proliferation
Stimulating Antigen
- Isoasp snRNP
- asp snRNP
- native snRNP

Counts per minute

Antibody Response
- Isoasp snRNP antisera
- asp snRNP antisera

O.D. (405nm)

Immunizing Antigen

Test Antigen
Extensive and Rapid Deamidation of a MAb in Vivo: Argument for Protein Engineering?
(Huang L et al Analyt. Chem 2005)

Extracted Ion Chromatogram of Asn55 Containing Peptide from MAb

Rate of Asn55 Deamidation in Vivo

- 312 hr: 67.9%
- 120 hr: 45.5%
- 24 hr: 28.7%
- 0.25 hr: 25.5%
Post Translational Modifications/Degradation Pathways Associated with Immunogenicity and Autoimmunity

- Oxidation
- Deamidation
- Citrullination (Deimination)
Deimination Catalyzed by Peptidyl Arginine Deiminase

Uysal H et al Imm Rev 2010

L-arginine (+ charged)

Peptidyl arginine deiminase (PAD)

Ca^{2+}

L-citrulline (neutral)

\[ \text{L-arginine} \rightarrow \text{L-citrulline} + \text{NH}_4^+ \]
Chemical Degradation Associated with Autoimmunity

• Arginine deimination/Citrullination:
  – Catalyzed by peptidyl arginine deiminase
  – Requires supraphysiologic concentrations of Ca++
  – Associated with numerous normal physiologic processes
    • Keratins and keratin associated protein filaggrin citrullinated during terminal differentiation of keratinocytes: extensive cross linking-cornification
  – Associated with inflammatory environments
  – Highly associated with autoimmune diseases
    • Antibodies in RA joint fluid to citrullinated proteins: fibrinogen/fibrin, vimentin, type II collagen, and α-enolase.
    • Citrullination of MBP: produces more open conformation; heightened susceptibility to proteases, decreased compaction
Citrullinated Proteins Preferentially Expressed in Inflammatory Tissues: Link to Autoimmunity
(Makrygiannakis D et al Ann Rheum Dis 2006)
Citrullinated Self-Proteins Break Self Tolerance to Native Self Protein in RA

Wegner N et al  Imm Rev 2009
To Biobetter or not?

- Assessment of therapeutic proteins in vivo particularly in specialized environments and inflammatory sites reveals that relatively rapid and profound alterations in structure and function may take place and may diminish efficacy and enhance immunogenicity.

- Such considerations should prompt further study and consideration for biobetters with protein engineering to prevent such modifications.
Mitigation Strategies for Immunogenicity

• Engineer proteins to be less immunogenic
  – Remove T/B cell epitopes in inherently immunogenic proteins
  – Alter propensity to aggregate, deamidate, oxidize etc
  – Pegylation, other means to shield epitopes and extend PK

• Engineer the patient’s immune response
  – Immune suppression
  – Immune tolerance induction
  – Appropriate in some, but not all, clinical scenarios
Pegylation may not be Enough
(Lipsky PE et al 2014)

*Mean value at week 17 adjusted for a single incorrect patient value.
Recombinant immunotoxin for cancer treatment with low immunogenicity by identification and silencing of human T-cell epitopes

Ronit Mazor,, Jaime A. Eberlea, Xiaobo Hua, Aaron N. Vassalla, Masanori Ondaa, Richard Beersa, Elizabeth C. Leea Robert J. Kreitmana, Byungkook Leea, David Baker, Chris King, Raffit Hassana, Itai Benharb, and Ira Pastan

Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892;
Recombinant Immunotoxin to Mesothelin
(Mazor et al 2014)
T Cell Epitope Heat Map of a Recombinant 2 Domain Immunotoxin

(Mazor et al 2014)

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Strategy: Remove Immunogenic Domain not Critical for Activity, Introduce Mutations in T Cell “Hotspots” in Activity Critical Domain

(Mazor et al 2014)
Introduction of Alanine Substitutions for T Cell Epitopes in Domain III
(Mazor et al 2014)
Engineered RIT with Domain 2 Deletion and 6 Point Mutations in Domain 3 has Dramatically Reduced T Cell Responses

(Mazor et al 2014)
Marked Reduction in Patient Antibody Binding to Deimmunized RIT

(Mazor et al 2014)
• Can a biobetter be too good?
Biobetter Monotherapy: Monogenic Disease

No amelioration of disease

Glucocerebrosidase
Gaucher

Biobetter gcerebrdase

Full amelioration of disease

AEs-Widespread apoptosis

Biobetter gcerebrdase

No amelioration of disease

α-glucosidase
Pompe

Biobetter α-glucosidase

Full amelioration of disease

AEs-Inability to store glycogen

Biobetter α-glucosidase
Biobetter in Polypharmacy for Polygenic Disease

No amelioration of disease → Clinical remission DAS$_{28}$ → SAEs + No enhancement of efficacy

↑

TNF antagonists + DMARDs

↑

Biobetter TNF antagonists + DMARDs
Acknowledgements

- Steven Kozlowski
- Daniela Verthelyi