Immunogenicity Risk Assessment of Peptide Drugs and their Impurities (using in silico tools)

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Daniela Verthelyi, PhD,  
Christina Howard, PhD

**CUBRC:**
Katie Edwards PhD
“...Differences between the peptide-related impurities in a proposed generic synthetic peptide and those in an RLD of rDNA origin could produce different impurity profiles, which could adversely affect the safety or effectiveness of a proposed generic synthetic peptide product, if uncontrolled...”

Specifically, in lieu of clinical trials. sponsors asked to perform **immunogenicity risk assessment** studies on:

- ... Impurities that are new in the ... generic peptide ... *in excess of 0.1% of the API*....
- ...impurities ... present in both the RLD and generic drug ... if the abundance ...*exceeds that of the RLD*

“...demonstrate ... *that the impurity does not contain sequences that have increased affinity for* ... MHC, known as *T cell epitopes*”
Immunogenicity risk assessment of synthetic peptide drugs and their impurities

Anne S De Groot 1,2, Brian J Roberts 1, Aimee Mattei 1, Sandra Lelias 1, Christine Boyle 1, William D Martin 1
Outline

• Immuno informatics Basics
• Orthogonal Approach to identifying T cell Epitopes in synthetic peptides and impurities
  • In Silico Analysis
  • In Vitro Risk Assessment
• Case Study: Teriparatide
• Prospective Identification of Synthetic Peptide Impurities- The What If Machine
FDA: Novel / Generic Peptides (and their impurities)... should be assessed for Immunogenicity risk.

Biologics developers have been using immunoinformatics tools such as those developed by EpiVax for decades to address... Immunogenicity Risk!


The T cell epitope is linear when bound to the HLA molecule that presents it to the T cell
**immunoinformatics/ in vitro tools illuminate immune responses to peptide drugs**

What does the **Antigen Presenting Cell** present?

What does the **T cell** see?

What effect on immune response?

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**T cell epitope and immunogenicity analysis for peptides and their impurities**
How is In Silico Risk Assessment Done?

EpiMatrix™ T Cell Epitope Prediction

- EpiVax proprietary algorithm: EpiMatrix™
  - Matrix-based algorithm for predicting linear T cell epitopes
- EpiMatrix™ predicts Class II HLA binding potential and "potential" for T cell response
EpiVax tests for binding potential to the most common HLA molecules within each of the “supertypes”* shown to the left.

This allows us to provide results that are representative of >95% of human populations worldwide** without needing to test each haplotype individually.


### Summing Epitopes to Assess Risk

More T cell epitopes = Higher immune response

Total T cell epitope content = Predicted immunogenic potential

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1 + 1 + 1 = Predicted Immunogenic Potential

---

**Immunogenic potential increases with increasing T cell epitope content**

Analysis of each 9mer frame
For probable binding to HLA

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| Total Assessments Performed: 1672 | Deviation from Expectation: -13.95 | Deviation per 1000 AA: -8.34 |
| Adjusted for Regulatory Epitopes | Deviation from Expectation: -34.27 | Deviation per 1000 AA: -20.50 |

EpiMatrix Immunogenicity Score
Treg epitope -adjusted Score

Promiscuous Epitope
Outline

• **Immuno informatics Basics**
  • Orthogonal Approaches for Identifying T cell Epitopes in synthetic peptides and impurities
    • In Silico Analysis
    • In Vitro Risk Assessment
  • Case Study: Teriparatide
  • Prospective Identification of Synthetic Peptide Impurities - The What If Machine
Many peptides are ‘Self Peptides’ e.g. Teriparatide (PTH), GLP-1RA.

The API is usually non immunogenic because “self” and tolerated.

The impurity changes the sequence and can trigger immune response:

- Prediction: in silico
- Test: HLA Binding
- Test: Immune response (in vitro)
Peptide Impurities & Immunogenicity: Impact of Impurities - Duplications

Example Impurity - Duplication of Amino Acid 2:

Scenario 1: **Binder** → **Non-binder**

- HLA Binder
  - 1 2 3 4 5 6 7 8 9
  - P1 P4 P6 P9
- HLA Non-binder
  - 1 2 2 4 5 6 7 8 9
  - P1 P4 P6 P9

Results in a peptide that will no longer bind HLA by shifting subsequent amino acids out of phase

**Low-Risk Impurity***

Scenario 2: **Non-binder** → **Binder**

- HLA Non-binder
  - 1 3 4 5 6 7 8 9
  - P1 P4 P6 P9
- HLA Binder
  - 1 2 2 3 4 5 6 7 9
  - P1 P4 P6 P9

Results in a peptide that will now bind HLA by shifting subsequent amino acids into phase

**Potentially Immunogenic Impurity***

*Based on T cell epitope content alone

EpiVax - confidential
Changes the T cell Receptor Face

Serious Systemic Hypersensitivity:

**Epitope Prediction: Synthesis Side Product**

Potential Neo-T-cell Epitope

Suspicion: Side Products may give rise to novel T-cell epitopes
Not all T cell Epitopes are the Same!
Characterizing Putative Tolerizing T cell Epitopes

Epitopes can be *either* effector or regulatory

Most in silico algorithms cannot differentiate between these two
Depending on whether Treg or Teffector are engaged, immune response may be repressed or activated.
The TCR-interacting face: Epitope
The MHC-binding face: Agretope

JanusMatrix
EpiMatrix

Analyze each peptide and its impurity
For interaction at both faces of the T cell epitope
Impurities that are predicted to be immunogenic in silico have **high EpiMatrix scores** and **low JanusMatrix scores**.

Based on these two parameters, impurities can be divided into **four quadrants**:

- **Epitope Dense**, Highly non-human
- **Epitope Dense**, Highly human
- **Epitope Sparse**, Highly non-human
- **Epitope Sparse**, Highly human
How to read: Immunogenicity Quadrant Plot
Standards: biologics and vaccine antigens

- **Epitope-dense, More common in human proteins**
  - HBV_Surface_Ag
  - Interferon_Alpha

- **Epitope-dense, Less common in human proteins**
  - Interleukin_2
  - EBV-BKRF3
  - Influenza_HA

- **Epitope-sparse, More common in human proteins**
  - Thrombopoietin

- **Epitope-sparse, Less common in human proteins**
  - IgG_Fc
  - Albumin

**Increasing humanness** → (>3 is significant)

**Increasing immunogenic potential** → (0 is random expectation)
Putting it all together:
Application of Quadrant Plots to Generic Drugs / Impurities
Original API may have low risk but impurities move higher

**EpiMatrix** predicts “what the HLA will present” identifies putative T cell epitopes

**JanusMatrix** predicts “what the T cell will see” Human (tolerated) vs. Foreign (immunogenic)

- **Impurity X**
  - High Risk
  - Significant Immunogenic Potential
  - Epitope Dense, Less Common in Human Proteins
  - Teriparatide API

- **Impurity Y**
  - Low Risk
  - Limited Immunogenic Potential
  - Epitope Sparse, More Common in Human Proteins

- **Semaglutide API**
  - Epitope Sparse, Less Common in Human Proteins

- **Most Immunogenic Potential**
  - Epitope Dense, More Common in Human Proteins

- **Least Immunogenic Potential**
  - Epitope Sparse, Less Common in Human Proteins

**EpiMatrix Score (T cell Epitope Content)**
**JanusMatrix Human Homology Score (Humanness)**

- Peptide Therapeutic and impurities
- HLA
- Epitopes
- Antigen Presenting Cell
- Effector T Cell
- Regulatory T Cell
ANDA Peptide Drugs and their Impurities

![Graph showing the immunogenic potential of different peptides and their associations with human proteins.](Image)

- **Most Immunogenic Potential**:
  - Epitope Dense, Less Common in Human Proteins
    - HCV NS3
    - Influenza HA (306-319)
    - Tetanus Toxin (947-967)
    - hCLIP
    - SCT EpiBar
    - Salmon Calcitonin API
  - Epitope Sparse, Less Common in Human Proteins
    - Exenatide
    - Glucagon
    - Lira/Semaglutide
  - Epitope Dense, More Common in Human Proteins
    - Tregitope 167
    - ECV_BHDF1 (171-189)
    - Teriparatide
    - Nesiritide

- **Least Immunogenic Potential**:
  - Epitope Sparse, More Common in Human Proteins

- Legend:
  - Assay Standards
  - Peptide APts
  - Salmon Calcitonin API
  - Salmon Calcitonin EpiBar
  - Salmon Calcitonin Impurities
Do we know the immunogenic risk of all possible impurities?
The “What If Machine”

EpiVax has a “What If” Machine for peptide impurities.

When generic drug impurities are unknown, modifications at each amino acid position in the peptide can be performed in silico, their immunogenicity risk predicted and they can be assigned an impurity risk score.

The “What if Machine”, performs all possible changes to the natural amino acid sequence of the drug substance and measures their impact on the epitope content of the peptide.

This includes: Amino acid modifications, duplications, insertions, deletions and truncations on the epitope content of the peptide drug substance AT EVERY SITE and COMBINATION of sites in the peptide.
Here we will show WhIM analysis examples for several generic peptide drugs (Salmon Calcitonin, Teriparatide, others) and several Novel peptides (that may be of concern).

High-risk impurities identified by the what-if-machine could be identified flagged and communicated to drug manufactures at early stages in the drug development process, saving resources in the effort to ensure the development of safe and effective novel or generic peptide therapeutics.
Teriparatide – Known and Unknown

Teriparatide Impurities

Epitope Content (EMX Score) vs. Humanness (JMX Score)

Most Immunogenic Potential
- Tetanus Toxin (947-967)
- Influenza HA (306-319)
- EBV_BHRF1 (171-189)
- Endo-Leu11
- Des-Gly12
- Des-Leu28
- Des-His26
- Des-Lys13
- Des-His9

Least Immunogenic Potential
- Teriparatide – Known and Unknown
Salmon Calcitonin and Impurities by “WHiM”

Salmon Calcitonin Impurities

Most Immunogenic Potential

Epitope Surface

Epitope Density

Epitope Content (EMX Score)

Humanness (JMX Score)

Epitope Matrix Score

JanusMatrix Score

Assay Standards
Peptide APIs
Salmon Calcitonin API
Salmon Calcitonin EpiBar
Salmon Calcitonin Impurities

Least Immunogenic Potential
WhIM accurately predicted that synthetic teriparatide carries a high risk for generating immunogenic impurities. Due to the presence of a tregitope in the n-terminus, modifications that ablate this feature result in peptide impurities that are significantly more immunogenic when compared to the teriparatide API peptide.
Semaglutide / Liraglutide impurities

Semaglutide / Liraglutide Impurities

Epitope Content (EMX Score) vs. Humanness (JMX Score)
WhIM accurately predicted that synthetic semaglutide has a low risk for generating immunogenic impurities.
Here we will show WhIM analysis examples for several generic peptide drugs (Salmon Calcitonin, Teriparatide, others) and several Novel peptides (that may be of concern).

High-risk impurities identified by the what-if-machine could be identified flagged and communicated to drug manufactures at early stages in the drug development process, saving resources in the effort to ensure the development of safe and effective novel or generic peptide therapeutics.

The algorithm could be used by regulators (to assess novel impurities listed by manufacturers) or by sponsors, who wish to identify impurities that they should be careful to exclude in the synthesis and purification process due to their potential for immunogenicity.

It is recommended that WhIM be used in conjunction with in vitro HLA binding and T cell assays, which serve to validate the predicted immunogenic sequences if they are in fact identified in the drug product, during the course of generic drug development.
Potential synergies for reducing risk:

The **What-if Machine (WhIM)** is an algorithm that, for a given input peptide sequence, **models** (in silico) nearly all **impurities** that may occur during peptide manufacturing and storage.

Use with LC/MS? Combine knowledge of Co-eluting impurities with in silico risk assessment to identify risk.

Prof. Farnsworth contemplates what could be using the What if Machine (in “Futurama”)

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**EpiVax**
Match LCMS with WhIM in ANDA process?
• Immuno informatics Basics
• Orthogonal Approaches to Immunogenicity Risk Assessment
  • Identifying T cell Epitopes in synthetic peptides and impurities
  • In Silico Analysis
  • In Vitro Risk Assessment
• Case Study: Teriparatide
• Prospective Identification of Synthetic Peptide Impurities - The What If Machine
Case Study: Teriparatide

- Teriparatide is a generic drug based off the reference drug product Forteo®

- The teriparatide API peptide is derived from, 34 amino acid of human parathyroid hormone (PTH) (the biologically active region of the hormone)

- PTH is the primary regulator of calcium and phosphate metabolism in the bone and kidney

- The drug is approved by the FDA for the treatment of Osteoporosis in men and women who are at a high risk for bone fracture

- In clinical studies, 2.8% of treated patients develop anti-Teriparatide antibodies after 12 months of treatment
WhiM (What if?) Plot of Impurities for Teriparatide

Teriparatide Impurities

Epitope Content (EMX Score) vs. Humanness (JMX Score)

Most Immunogenic Potential

Least Immunogenic Potential

Assay Standards
Peptide API benchmarks
Teriparatide API
TPT Observed Impurities
TPT Observed Impurities
TPT Impurities tested in vitro

Teriparatide

Salmon Calcitonin
Exenatide
Glucagon
Teduglutide
Tetanus Toxin (825-850)
Tetanus Toxin (947-967)
HCV NS3
Influenza HA (306-319)

EBV_BHRF (171-189)
Lys-Ac26
Des-His14
Des-lys13
Des-Leu28
Des-Leu7
Des-His9
Des-Gly12

Teriparatide

Lira/Semaglutide

HCV NS3
Tetanus Toxin
Teriparatide

Assay Standards
Peptide API benchmarks
Teriparatide API
TPT Observed Impurities
TPT Observed Impurities
TPT Impurities tested in vitro
**In Vitro Class II HLA Binding Assays:** Class II HLA binding assays may be used to measure the relative binding potential of putative epitopes to multiple HLA alleles. EpiVax employs an adapted competition-based HLA binding assay that utilizes highly-purified Class II HLA molecules of “supertype” alleles. Non-linear regression analysis is performed to produce a curve from which an IC50 value is calculated and used to assess binding strength. This assay format is superior in sensitivity and specificity compared to cell-based binding assay formats.

**Naïve Donor T Cell Assay – In Vitro Immunogenicity Protocol (IVIP):** EpiVax has adapted an in vitro assay to test the immunogenicity of novel vaccines and therapeutics with human lymphocytes. This assay utilizes blood from HLA-typed healthy donors in order to closely mimic a natural human immune response.

In this assay naïve PBMCs are cultured with the test article and relevant controls. In parallel, PBMCs from the same donor are cultured without test peptide. After 14 days, the cells are stimulated, as a challenge or first exposure, with the appropriate test article or control. The resulting immunoinflammatory and/or immunosuppressive response is measured via Fluorospot assay.
Review of Teriparatide: In silico

“EpiBar” is highly conserved with prevalent human protein

Teriparatide:

- **EMX Score:** 16.03- elevated
- **JMX Score:** 4.74- potential for tolerance
- **Total Epitope Count:** 19
- 8 hit EpiBar in frame 5
- **Significant** hits for each DRB1 Supertype allele except for DRB1*0901

From this analysis, we expect that Teriparatide will have high epitope content due to the elevated EpiMatrix score, but low immunogenicity due to the high JanusMatrix Score

Potential Treg epitope*

File: FDA_YR2_TERIPARATIDE Sequence: 00_TERIPARATIDE_RLD : 1

**Summarized Results**

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**Scores Adjusted for Traggotope:**

- EpiMatrix Score: 16.03
- EpiMatrix Score (w/o Rank): 16.03

4/7/24
Why is Teriparatide potentially a Treg epitope?
Extensive cross-conservation with self epitopes

Overview of Class II JanusMatrix Results
TERIPARATIDE RLD
Current Database: HUMAN

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Teriparatide “EpiBar” has promiscuous binding
to multiple HLA DR alleles as predicted

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**Summary Results**

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**Scores Adjusted for Tregitope**

- Hydrophobicity: -0.67
- EpiMatrix Score: 16.03
- EpiMatrix Score (w/ flanks): 16.03

**EpiVax**

- HLA Binding
  - 7/8 alleles showed binding affinity.
Teriparatide Impurities RESULTS

Loss of “humanness” increases immunogenicity

When an impurity becomes less human, the immunogenicity increases

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<th>Test Article</th>
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<th>Percent of Responding Donors</th>
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Summary thus far:

- Immuno informatics can help assess Immunogenicity Risk
- Consideration of “human-ness” (Tolerance) is important
- In vitro assays for orthogonal evaluation
  - In Vitro HLA binding
  - In Vitro T cell Assays Assessment
- Not discussed here: Appropriate Controls / Innate Immune Responses / Aggregation
- Approach described here is valid for
  - Novel peptides
  - Host Cell proteins
  - Biologics
• Immuno informatics Basics
• Orthogonal Approaches to Immunogenicity Risk Assessment
  • Identifying T cell Epitopes in synthetic peptides and impurities
  • In Silico Analysis
  • In Vitro Risk Assessment
• Case Study: Teriparatide
• Other Synthetic Peptide Impurities- The What If Machine
Comparison (Control Negative) Bivalirudin

Bivalirudin
(Non Immunogenic)