Prediction and Validation of Immunogenicity and Tolerance to Generic Peptides & Impurities

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ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin*

For each new specified peptide-related impurity that is not more than 0.5 percent of the drug substance, the ANDA applicant should characterize the impurity. Further, the ANDA applicant should provide justification for why such impurity does not affect the safety of the proposed generic synthetic peptide (including with respect to immunogenicity) and why it does not affect its effectiveness. This justification should take into consideration, among other things, the identity and amount of an impurity, the impurity’s impact on the physicochemical and biological

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*ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin, Guidance for Industry, Draft Guidance, October 2017 (Draft — Not for Implementation),
FDA: Generic Peptide Impurities... should be assessed for T cell epitope-related Immunogenicity risk.

OK, so, Develop a comprehensive program for assessing immunogenicity risk building on decades of experience with biologics
T-Cell Dependent Immunogenicity of Protein Therapeutics Pre-clinical Assessment and Mitigation—Updated Consensus and Review 2020

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Founder, CEO/CSO, EpiVax Inc  Associate Scientific Director,

Frances Terry, MPH  
Director of Analysis in Immunoinformatics

William Martin, COO/CIO

EpiVax PANDA team members: Christine Boyle, Holly Clifford, Beth McGonigal, Shah Miah, Sandra Lelias, Chris Talbot, Olivia Morin, Heather Stuart, Mitchell McCallister, Jacob Tiven, and Matt Ardito.
Analyzing Immunogenicity in Peptides/Impurities

- Why do Immunogenicity Screening of Peptides?
- How to do it – In Silico and In Vitro
- Special tools that evaluate “tolerance” and find Treg Epitopes
- FDA Case Studies: Calcitonin and Teriparatide (Impurities)
Start with Peptide Analysis. Change to HLA binding affects T cell activation.

- Impurity can bind and trigger immune response
  - Prediction: in silico
  - Test: HLA Binding
  - Test: Immune response (in vitro)
Peptide Drugs and their Manufacturing-related Impurities

**Truncations:**

**Amino acid deletions:**

**Amino acid insertions:**

**Amino acid duplications:**

**Incorporation of D-stereoisomers:**

**Side chain modifications:**
Peptide Impurities modify HLA binding
E.g. Duplications, on Potential for Immunogenicity

**Example Impurity** - Duplication of Amino Acid 2:

**Scenario 1: Binder → Non-binder**

![HLA Binder](image1)

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

**Scenario 2: Non-binder → Binder**

![HLA Non-binder](image2)

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

**Loss of immune response?**

**New Immunogenic Impurity**
Outline – Analyzing Immunogenicity in Peptide Drugs

• Why do Immunogenicity Screening of Peptides?
• How to do it – In Silico and In Vitro
• Special tools that evaluate “tolerance” and find Treg Epitopes
• Case Studies: Calcitonin and Teriparatide (Impurities)
Four step approach to Peptide Drug Impurities

- **in silico**
  - Epitope Prediction

- **in vitro**
  - HLA Binding
  - T cell Activity

- **Literature**
  - Integrated Review

**In Silico:**
Define potential T cell epitopes in API
Compare with Impurities
How to predict HLA binding of impurities | EpiMatrix
was developed and is curated by EpiVax

• EpiVax proprietary algorithm: EpiMatrix
  – Matrix-based algorithm for predicting linear T cell epitopes
• EpiMatrix™ predicts both class I and **class II HLA** binding potential
Class II HLA DR “Supertypes”

EpiMatrix tests for binding potential to the most common HLA molecules within each of the “supertypes”* shown to the left.

Supertypes comprise families that have similar binding pocket preferences – specific amino acid side chains fit into their HLA binding pockets.


Analyzing sequences for “supertype” HLA DR binding motifs allows us to provide results that are representative of >95% of human populations worldwide** without needing to test each haplotype individually.


### EpiMatrix: Screening a Peptide Sequence for T Cell Epitopes

#### Assessment (Z Score)

- **EpiMatrix Immunogenicity Score**
  - Z Score ≥ 1.64: Hit
  - 1.28 < Z Score ≤ 1.64: Near-Miss
  - Total Number of Hits

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**Summarized Results**

- Maximum Single Z-score: 7.66
- Sum of Significant Z-scores: 22.8
- Count of Significant Z-Scores: 4

**Total Assessments Performed:** 72, Hydrophobicity: 1.36

**EpiMatrix Score:** 49.64

**EpiMatrix Score (w/o flanks):** 49.64

**Total Number of Hits:** 1.28 < Z Score ≤ 1.64 Near-Miss
Protein or Peptide Therapeutic:

1 + 1 + 1 = Response

$T$ cell response depends on:

$T$ cell epitope content + HLA of subject

➢ immunogenicity can be ranked

“Immunogenicity Scale” for proteins
Potential immunogenicity risk

EpiMatrix Predicted Excess/Shortfall in Aggregate
Immunogenicity Relative to a Random Peptide Standard

Protein Therapeutic:

\[
\text{epitope} + \text{epitope} + \text{epitope} = \text{Response}
\]

T cell response depends on:

- \text{T cell epitope content} + HLA of subject
- protein immunogenicity can be ranked

Have been known to be immunogenic
New “PANDA” Peptide Immunogenicity Scale

The first step to assessing Immunogenicity risk of Impurities is to evaluate peptide epitope density on the immunogenicity scale.
### EpiMatrix Cluster Report for GAD65 555-566

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Assessments Performed: 36

Deviation from Expectation: 27.66

Z-score indicates the potential of a 9-mer frame to bind to a given HLA allele; the strength of the score is indicated by the blue shading.

Cluster Regions Outlined: Top 10% Top 5% Top 1%

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**Figure 5. EpiMatrix analysis of a promiscuous epitope.** The GAD65 peptide is an epitope known to be promiscuously immunogenic. It scores extremely high for all 9 alleles in EpiMatrix. Its EpiMatrix Score is 28. Scores higher than 10 are considered to be significant. The band-like EpiBar pattern is characteristic of promiscuous epitopes.
Approach to analyzing unnatural amino acids
Details to be published in manuscripts in preparation

1. Neutral placeholder: Replace unnatural AA(1Nal) with X

AK1NalVAAWLTKAAA

AKXVAAWLTKAAA

2. Replacement Analysis: Replace X with all 20 natural L-amino acids

3. Best Proxy: Choose best-matching natural L-amino acid based on structural and/or chemical properties

AKFVAAWLTKAAA

Most conducive to binding (i.e. worst-case scenario)

AKXVAAWLTKAAA

Neutral Placeholder

AKGVAAWLTKAAA

Least conducive to binding (i.e. best-case scenario)

1-Nal

Bulky Hydrophobic group

Trp

Tyr Ile Leu Trp Val Met Lys Gln His Cys Thr Asn Glu Ser Arg Ala Asp Pro Gly

Risk Estimate Range

Best Risk Estimate

EpiVax - confidential
Outline – Analyzing Immunogenicity in Peptide Drugs

• Why do Immunogenicity Screening of Peptides?
• How to do it – In Silico and In Vitro
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• Case Studies: Calcitonin and Teriparatide (Impurities)
Some generic peptides are not immunogenic
Are they potentially tolerated or tolerogenic?

Epitopes can be *either* effector (inflammatory) or regulatory (tolerogenic).

Most in silico algorithms cannot differentiate between these two. ISPRI uses JanusMatrix.
The T cell epitope has two faces: One that binds HLA, and one that faces up to the T Cell Receptor (TCR).

The T cell epitope is linear when bound to the HLA molecule that presents it to the TCR.
Each MHC ligand has two faces:
1. The MHC-binding face (agretope) and
2. The TCR-interacting face (epitope)

JanusMatrix™ is designed to predict the potential for cross-reactivity between epitope clusters and the human genome, based on conservation of TCR-facing residues in their putative HLA ligands.

Find predicted 9-mer ligands with:
• Identical T cell-facing residues
• Same HLA allele and minimally different MHC-facing residues

Moise L et al. Hum Vaccin Immunother. 2013 Jul;9(7):1577-86
Epitope networks used to provide visual map of epitope cross-conservation to identify tolerogenic (Treg) epitopes

**Cytoscape:** Visual map of epitope cross-conservation

Potentially non-immunogenic due to high cross-conservation with “self”

Potentially immunogenic due to low cross-conservation with “self”
The two-faced T cell epitope
Examining the host-microbe interface with JanusMatrix

H7N9 T-cell epitopes that mimic human sequences are less immunogenic and may induce Treg-mediated tolerance

Better Epitope Discovery, Precision Immune Engineering, and Accelerated Vaccine Design Using Immunoinformatics Tools

T cell epitope redundancy: cross-conservation of the TCR face between pathogens and self and its implications for vaccines and autoimmunity

Integrated assessment of predicted MHC binding and cross-conservation with self reveals patterns of viral camouflage

EpiVax - confidential
Published Treg epitopes in IgG: **Tregitopes**
Also highly conserved by JanusMatrix

- **Tregitopes**
- Discovered by EpiVax
- Highly conserved peptide sequences in Fc and Fab regions of antibodies
- High affinity, promiscuous binders across HLA alleles
- **One mechanism of action of IVIG?**
- Activate antigen-specific regulatory T cells
- Do other self-proteins have tolerogenic epitopes?

Evaluation of Immunogenicity: Consider the potential for tolerance

- Immunogenic peptides have high EpiMatrix scores and low JanusMatrix scores.
- Based on these two parameters, API and their sequence-related impurities can be divided into four quadrants:
Four step approach to Peptide Drug Impurities

- in silico
- in vitro
- HLA Binding
- in vitro
- T cell Activity

In vitro
Independent Validation assays
API and Impurities
In brief, the peptide of interest is incubated with an allele-specific labeled tracer peptide and a soluble HLA supertype monomer are incubated to equilibrium. The following day the binding reaction is halted and the mixture is transferred to assay plates precoated with a pan anti-HLA-DR antibody and incubated overnight. Following this incubation, the plates are developed and peptide binding is indirectly measured by time resolved fluorescence spectroscopy. By using a fixed concentration of the labeled tracer peptide and a range of concentrations for the test peptide, one can generate a multi-point dose ranging curve that enables the calculation of an IC50 value which provides information not only about the ability of the peptide to bind HLA (yes/no) but also about the relative affinity of the peptide to a given HLA-DR supertype. Once can utilize the IC50 values to divide peptides into categories based on their affinity for a given HLA allele, such as high, moderate, low and non-binding. As new technology becomes available and accessible, it will be useful to look at the kinetics of the binding reaction as well.
In Vitro Assay to measure T cell response “IVIP” (Similar assay for Treg response “TTBSA”)

Relative Activity

Naïve T cell Respon

Primary culture (14 Days)

Day 1
- Isolate PBMCs from whole blood filter
- Set up 1” culture
- Prepare HLA typing samples

Day 4
- Half Media Exchange

Day 7
- Half Media Exchange

Day 11
- Half Media Exchange
  - Harvest cells for Fluorospot (IFNg, IL-2)
  - Stimulation #2

Day 14

Day 15

Fluorospot Development
Stimulation (day 1)

• Media (negative control)
• TT 0.5 µg/ml
• TT + peptide (289/FV621 or Teriparatide)  Concentration 10, 20, 40 µg/ml

End points (day 7)

• Proliferation and activation of T eff (CFSE, CD25)
• Characterization markers (CD4 and CD3e)
Full Length Article

Identification of a potent regulatory T cell epitope in factor V that modulates CD4+ and CD8+ memory T cell responses

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Salmon Calcitonin

➢ A Foreign Peptide
  • only 50% sequence homology with human calcitonin
➢ Is slightly immunogenic
  • 40-70% patients develop ADA to SCT\textsuperscript{1,2,3}
➢ Some impurities are potentially immunogenic

Ø The N-terminal human calcitonin-like active site region of SCT contains no epitopes and may be protected by the cyclic nature.

Teriparatide

➢ Fully Human Peptide
➢ Is less immunogenic, may even be tolerogenic
  • Only 3% of patients develop ADA to teriparatide\textsuperscript{4}
  • Teriparatide was shown to increase the number of Tregs by threefold\textsuperscript{5}
  • EpiMatrix & JanusMatrix\textsuperscript{6} identified a promiscuous binding epitope with high degree of human homology to another widely expressed human protein, tubulin.
➢ Some impurities are potentially immunogenic
  • This may explain why some patients (3%) have developed transient antibodies

4. Eli Lilly. FORTEO Package Insert.
Salmon Calcitonin Immunogenicity Analysis
In silico analysis confirms what is known about this drug

EpiMatrix Cluster Detail Report

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Human Calcitonin-like region – no T cell epitopes

Antibody Binding Site

Fully Foreign immunogenic region

Summary of Results

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Scores Adjusted for Tregitope: --

EpiMatrix Score: 4.46
EpiMatrix Score (w/o flanks): 4.46

Total Assessments Performed: 192
Hydrophobicity: -0.54
EpiMatrix Score: 4.46
EpiMatrix Score (w/o flanks): 4.46

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Total Assessments Performed: 192
Hydrophobicity: -0.54
EpiMatrix Score: 4.46
EpiMatrix Score (w/o flanks): 4.46
Salmon Calcitonin: Impurities

Salmon Calcitonin (SCT) API is demonstrated and known to be, immunogenic.

SCT impurities have similar EpiMatrix (moderate) and JanusMatrix (non-human) scores.

Each impurity had similar immunogenicity in silico and ex vivo relative to the API.

- **SCT impurities are immunogenic as predicted, but not significantly more so than the API.**
Teriparatide

- Sold under the name Forteo® (RLD, rDNA).
- Approved for the treatment of Osteoporosis for men and women in the USA.
- Comprised of the N-terminal 34 amino acids of hPTH.
- Primary regulator of Calcium and Phosphate metabolism in the bone and kidney.
EpiMatrix Hits in Frames 4, 5, 7, 8, 11, 21, 23 (Medium and Dark blue shading)

The EpiBar in frame 5 has a high JanusMatrix Human Homology Score suggesting potential for homology-induced tolerance (homology is to the widely expressed human protein tubulin)

**EpiMatrix in Frame 5**

- **EpiMatrix Hits** in Frames 4, 5, 7, 8, 11, 21, 23
- **EpiBar** in Frame 5 = promiscuous binding motif

**EpiMatrix Detail Report**

File: FDA_YR2_TERIPARATIDE Sequence: 00_TERIPARATIDE_RLD : 1

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**Summarized Results**

- **Maximum Single Z-score**
- **Sum of Significant Z-scores**
- **Court of Significant Z-scores**
- **Total Assessments Performed:** 234
- **Hydrophobicity:** -0.67
- **EpiMatrix Score:** 16.03
- **EpiMatrix Score (w/o flanks):** 16.03
- **EpiMatrix Score:** 18.93
- **EpiMatrix Score (w/o flanks):** 18.93
Immunogenic by T cell epitope analysis.

But both in vitro assay and clinical data (3% ADA) . . . much Less Immunogenic than predicted . . . Do

JanusMatrix Analysis:

Conserved with very common intracellular protein, Tubulin.

DO NOT POST – UNPUBLISHED
Teriparatide and Impurities
Immunogenicity vs Humanness

Do Not Post – Publication in Preparation
Teriparatide Summary

• Teriparatide peptide has tolerogenic behavior in vitro, as predicted
  
  • The putative Teriparatide “Tregitope” inhibits CD4+ T cell proliferation and activation in TTBSA in healthy donor PBMCs

• Impurities that retain the same TCR facing sequences are not more immunogenic than Teriparatide (API) in vitro.

• Impurities that remove the TCR cross-conservation but still bind are more immunogenic (publication in preparation)

• Future studies: Does the Teraparatide “Tregitope” tolerize against potential immunogenic impurities when administered together?
Outline – Analyzing Immunogenicity in Peptide Drugs

• Why do Immunogenicity Screening of Peptides?
• How to do it – In Silico and In Vitro
• Special tools that evaluate “tolerance” and find Treg Epitopes
• Case Studies: Calcitonin and Teriparatide (Impurities)
• What if we could prospectively predict and rank all peptide impurities?
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.

The WhIM then calculates the predicted immunogenicity of the impurities and flags the most worrisome for special consideration. Generics manufacturers may be able to use this information to reduce the cost of their ANDA preparation. This list could be used to identify impurities that should be removed.
WHAT IF... (WhIM) Summary of one iteration:
Taspoglutide AA Duplication EXAMPLE (insertion)

**EMX Hits:**
- aa duplication impurities with more EMX than baseline (9) are in red à increase in overall putative T cell epitope content
- aa duplication impurities with less EMX hits than baseline (9) in green à decrease in overall putative T cell epitope content

**JMX HMLGY Score:**
- JanusMatrix Human Homology Score.
- Scores >3 are considered significant for potential homology induced tolerance

**Large number of hits**
High EpiMatrix Score
Low JanusMatrix Score

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FDA Contract with CUBRC and EpiVax
2018-2020 (and 2020-2022…)

Office of Generic Drugs (OGD/FDA) Awards $1M FDA Contract to CUBRC and EpiVax for Demonstration and Validation of Immunogenicity Risk Assessment Methods for Generic Peptide Drugs and Their Impurities

Providence, R.I., October 2, 2018 – EpiVax, Inc. ("EpiVax") and CUBRC, Inc. ("CUBRC") announced today that they have been awarded a two-year $1 million contract from the Food and Drug Administration (FDA) in response to a Broad Agency Announcement (BAA), FDA BAA-17-00123.

The research program will identify best practices and procedures for assessing generic peptides and related impurities for immunogenic potential. "EpiVax has worked hard to be at the forefront of the immunogenicity assessment field. Our proprietary immunoinformatics tools make it possible to perform risk assessments accurately and expeditiously. We look forward to working with FDA scientists to set new standards for immunogenicity risk assessment for generic peptide drug products," stated Annie De Groot, MD, EpiVax’s CEO/CSO.

The FDA recently issued a draft guidance for generic peptide drugs and issued a BAA for immunogenicity risk assessment tools. In response to this BAA, EpiVax scientists will demonstrate and validate risk assessment methods for generic peptides. OGD scientists will also have access to the EpiVax ISPIRI Toolkit for selected peptide drugs and their impurities.

CUBRC will leverage its technical expertise in biomedical research and development along with its experience leading large federal government grants and contracts in collaboration with EpiVax to execute the research. "CUBRC plans to leverage our 34+ year partnership with EpiVax to provide systems integration and program management expertise to advance EpiVax's highly specialized immunoinformatic tools which will help the FDA with evaluation of new generic peptide drugs," stated Katie Edwards, Ph.D., CUBRC’s Prime Technical Program Lead.

EpiVax Advances PANDA Service: Therapeutic Immunogenicity Screening Program

PROVIDENCE, RI, December 29, 2020 /PRNewswire/ -- EpiVax, Inc. (“EpiVax”) today provides an update on the advancement of the company’s peptide therapeutic immunogenicity assessment program.

Peptide Impurity Risk Assessment

The PANDA (Peptide Abbreviated New Drug Application) immunogenicity assessment program was originally developed for screening generic peptide drugs and their impurities following the publication of the ANDA draft guidance by the Food and Drug Administration (FDA). EpiVax performed validation studies of the in silico tools and in vitro validation methods used in the PANDA program in collaboration with CUBRC under contract to the FDA between 2018 and 2020. Work performed by EpiVax under this initial FDA contract is in preparation for publication. Meanwhile, EpiVax announced a new FDA OGD contract in October

“What if” Machine (WhIM)

“EpiVax’s CEO/CSO, Annie De Groot, MD, says, "Having performed protein immunogenicity risk assessment for more than 20 years, we’re thrilled to not only be able to add immunogenicity risk assessment of generic peptides and their impurities to our repertoire of services, but also to see the PANDA approach becoming increasingly useful for assessing the potential immunogenicity of novel peptide therapeutics. The application of our PANDA research aims to help peptide drug developers reduce the risk of failure in the clinical phase of development. We look forward to learning more about the immunogenicity of generic and novel peptide therapeutics and growing our program further in 2021."
Publications cited in this presentation


The EpiVax Team makes this work possible!
T-Cell Dependent Immunogenicity of Protein Therapeutics Pre-clinical Assessment and Mitigation—Updated Consensus and Review 2020

Vibha Jawa¹, Frances Terry², Jochem Gokemeijer³, Shibani Mitra-Kaushik⁴, Brian J. Roberts², Sophie Tourdot⁵ and Anne S. De Groot²,⁶
Clinical Immunology

Volume 224, March 2021, 108661

Full Length Article

Identification of a potent regulatory T cell epitope in factor V that modulates CD4+ and CD8+ memory T cell responses

Anne S. De Groot a, b, c, Amy Rosenberg c, S.M. Shahjahan Miah a, Gail Skowron a, Brian J. Roberts a, Sandra Lélias a, Frances E. Terry a, William D. Martin a
Recent Publications Describing EpiVax Tools

Engineering a bispecific antibody with a common light chain: Identification and optimization of an anti-CD3 epsilon and anti-GPC3 bispecific antibody, ERY974

Hirotake Shiraishi, Atsushi Narita, Mika Kanata-Sakurai, Takahiro Ishiguro, Yuji Sano, Naoki Hironiwa, Takashi Tsuchimi, Hisashi Segawa, Toshiaki Tsunemori, Yoko Ikeda, Yoko Kayahara, Mizuho Noguchi, Tetsuya Wakabayashi, Akibisa Sakamoto, Hiroko Konishi, Tsuchi Kuramochi, Mika Endo, Kunihito Hattori, Junichi Nezu, Tomoyuki Igawa