Immunogenicity Risk Assessment of Salmon Calcitonin Peptide Impurities Using In Silico and In Vitro Methods

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PURPOSE

Recent advances in synthetic peptide synthesis have enabled more cost- effective peptide drug manufacturing. Accordingly, peptide drugs initially produced using recombinant DNA (rDNA) technology are now produced synthetically. While peptide synthesis has some advantages over rDNA expression methods, new peptide-related impurities may be generated during synthesis that differ from the active pharmaceutical ingredient (API). Impurity byproducts of the original peptide sequence feature modifications that may alter the immunogenicity risk profile of the drug product. These impurities have become the focus of regulatory review and approval for human use as outlined in the FDA's Center for Drug Evaluation and Research guidance document, "ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin", published in 2021. Here we present a case study that illustrates how in silico and in vitro methods can be applied as orthogonal approaches used to assess the immunogenicity risk of impurities found in synthetic versions of the salmon calcitonin (SCT) drug product.

OBJECTIVE(S)

To apply in silico T cell epitope prediction algorithms and in vitro HLA Binding and T cell assays to investigate the immunogenic risk potential of synthetic peptide impurities in generic Salmon Calcitonin. We propose that a combination of these methods be used to assess the immunogenic risk potential of generic peptide drugs and their impurities submitted through the abbreviated new drug application (ANDA) pathway.

METHOD(S)

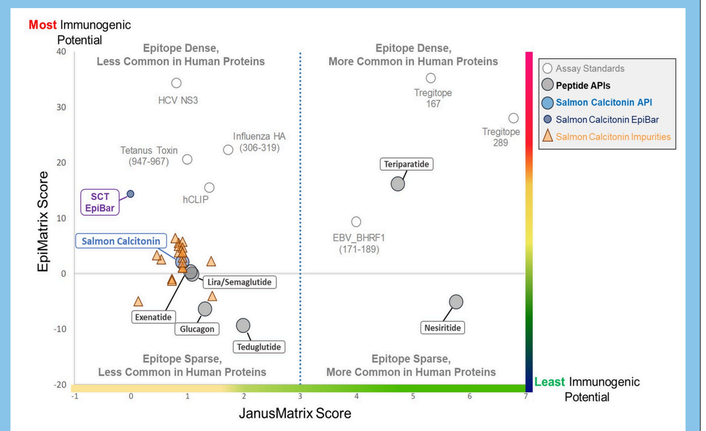
The immunogenicity risk of SCT and several impurities was assessed utilizing three independent (orthogonal) methods: in silico analysis T cell epitope prediction, in vitro class II HLA binding assays, and in vitro naïve T cell assays (De Groot et al., 2023). Using in silico epitope prediction tools, we evaluated the sequences of the salmon calcitonin API peptide and 20 impurities identified through an FDA survey of synthetic peptide impurities found within generic salmon calcitonin. Impurities were ranked for immunogenic risk potential (T cell epitope content and humanness).

Following in silico analysis, a subset of impurity peptides was selected for in vitro assays based on predicted risk. An in vitro HLA binding assay was first employed to assess and compare changes in HLA DRB1 binding affinity between API and impurity sequences with *modifications to HLA-facing residues*. Finally, synthetic salcitonin impurities with changes to *T cell receptor-facing residues* were compared to the SCT drug product for CD4 T cell response in the IVIP naïve CD4+ T cell assay.

In Silico Risk Analysis

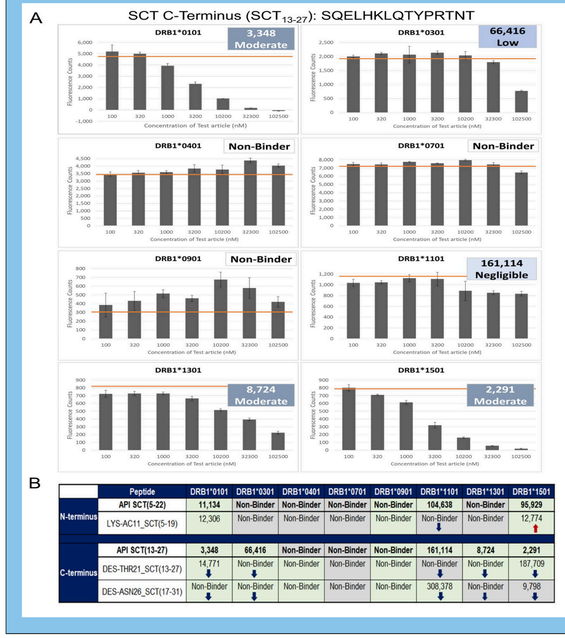
Frame Start	AA Seqeunce	Frame Stop	HLA-DR Supertype Alleles DRB1* 0101	0301	0401	0701	0801	0901	1101	1301	1501	Hits
1	CSNLSTCVL	9										0
2	SNLSTCVLG	10										0
3	NLSTCVLGK	11										0
4	LSTCVLGKL	12										0
5	STCVLGKLS	13										0
6	TCVLGKLSQ	14										0
7	CVLGKLSQE	15										0
8	VLGKLSQEL	16										3
9	LGKLSQELH	17										0
10	GKLSQELHK	18										0
11	KLSQELHKL	19										1
12	LSQELHKLQ	20										1
13	SQELHKLQT	21										0
14	QELHKLQTY	22										0
15	ELHKLQTYP	23										0
16	LHKLQTYPR	24										7
17	HKLQTYPRT	25										0
18	KLQTYPRTN	26										0
19	LQTYPRTNT	27										0
20	QTYPRTNTG	28										0
21	TYPRTNTGS	29										0
22	YPRTNTGSG	30										0
23	PRTNTGSGT	31										0
24	RTNTGSGTP	32										0

Top 10% of random peptides (not significant, near miss) | Top 5% of random peptides (significant hit) | Top 1% of random peptides (significant hit, highly likely)



In silico analysis revealed a 9-mer frame capable of binding nine out of the ten HLADRB1 supertype alleles. This region corresponds to the most foreign region of salmon calcitonin, differing from the native calcitonin by >60.0%. The quadrant plot on the right shows how the predicted immunogenic risk of the SCT API peptide compares to several identified impurities.

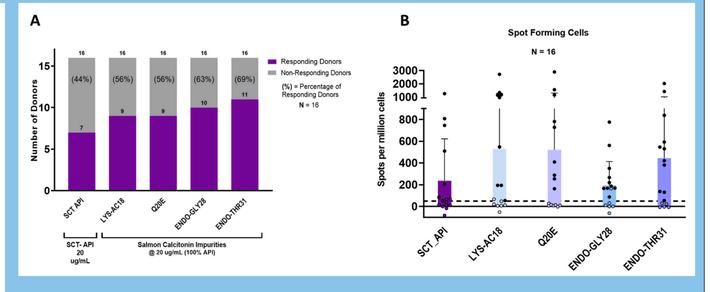
HLA DRB1 Binding Assays



B

	Peptide	DRB1*0101	DRB1*0301	DRB1*0401	DRB1*0701	DRB1*0901	DRB1*1101	DRB1*1301	DRB1*1501
N-terminus	API SCT(5-22)	11,134	Non-Binder	Non-Binder	Non-Binder	Non-Binder	104,638	Non-Binder	95,929
	LYS-AC11_SCT(5-19)	12,306	Non-Binder	Non-Binder	Non-Binder	Non-Binder	Non-Binder ↓	Non-Binder	12,774 ↑
C-terminus	API SCT(13-27)	3,348	66,416	Non-Binder	Non-Binder	Non-Binder	161,114	8,724	2,291
	DES-THR21_SCT(13-27)	14,771 ↓	Non-Binder ↓	Non-Binder	Non-Binder	Non-Binder	Non-Binder ↓	Non-Binder ↓	187,709 ↑
	DES-ASN26_SCT(17-31)	Non-Binder ↓	Non-Binder ↓	Non-Binder	Non-Binder	Non-Binder	308,378 ↓	Non-Binder ↓	9,798 ↓

Naïve CD4+ IVIP T cell Assay



Class II HLA Binding: (Left) *Impurities evaluated for HLA binding potential show that changes in HLA-binding residues can impact binding affinity. The results of the binding assays are in general alignment with the in silico predictions*

CD4+ T cell Assay: (Above) *Each of the selected impurities for the T cell assay generated a response in more donors when compared to SCT (a), however when comparing the intensity of the responses between SCT and the impurities there was no statistical difference. These results align with the in silico predictions and indicate that impurities have the potential to impact the immunogenicity of the drug product.*

CONCLUSION(S)

Collectively, these results indicate that the in vitro class II HLA binding and T cell assays used to compare the immunogenic risk potential of selected synthetic SCT peptide impurities to Salmon Calcitonin were generally aligned with in silico risk assessments of the same sequences. This suggests that this approach of combining orthogonal in silico and in vitro evaluation methods is useful for evaluating the immunogenicity risk of peptide impurities as recommended in the FDA guidance, "ANDAs for Certain Highly Purified Synthetic Drug Products That Refer to Listed Drugs of rDNA Origin".

References:

General Approach:
De Groot, A. S., Roberts, B. J., Mattei, A., Lelias, S., Boyle, C., and Martin, W. D. (2023). **Immunogenicity risk assessment of synthetic peptide drugs and their impurities.** Drug Discov. Today 28, 103714. doi: 10.1016/j.drudis.2023.103714.

Salmon Calcitonin Case Study:
Roberts, BJ, Aimee Mattei, Kristina E. Howard, James L Weaver, Hao Liu, Sandra Lelias, William D Martin, Daniella Verthelyi, Eric Pang, Katie Edwards and Anne S. De Groot. **Assessing the Immunogenicity Risk of Salmon Calcitonin Peptide Impurities Using In Silico and In Vitro Methods**. Manuscript accepted, pending publication

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