Low predicted immunogenicity risk associated with FIX variants that can promote coagulation in the absence of FVIII: *in vitro* and *in silico* assessments

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PB0301

INTRODUCTION

- Human factor IX (FIX) variants, such as hFIX-FIAV and hFIX-IDAV, can promote coagulation independently of Factor VIII.[1]
- hFIX variants can ameliorate the bleeding phenotype in hemophilia A mice[1] and could be an attractive approach for development as a gene therapy to treat people with hemophilia A.
- One concern in using coagulation factor mutants is potential immunogenicity.
- hFIX-FIAV and hFIX-IDAV were analyzed to determine the potential for immunogenicity via antigen presentation of epitopes via major-histocompatibility complex (MHC) Class I and II.
- The aim of this research was to determine the risk of immunogenicity of hFIX variants by *in silico* and *in vitro* analyses.

METHODS

STEP 1: *In silico* assessment (ABZENA)

- Wild-type human FIX (wt-hFIX), hFIX-FIAV and hFIX-IDAV amino acid sequences were evaluated for their immunogenic potential.
- Peptides spanning the entire sequence were tested as 9mer peptides in one amino acid increments.
- Algorithms used to screen for potential T cell epitopes by identifying linear motifs of 9-10 amino acids that bind to MHC Class I or II.[2]
  - Class I: Immune Epitope Database (IEDB) peptide library.
  - Class II: iTope™ (MHC Class II binding prediction) and T cell epitope database (TCED™).

Step 2: T cell epitopes assay (PROIMMUNE)

- Ability of each candidate peptide to bind to MHC alleles and stabilize the MHC peptide complex.[3]
- Determination of on and off rate binding properties (strength and stability) for candidate peptides.

RESULTS

Step 1: *In silico* analysis

- **MHC class I**: Four moderate affinity peptides identified in the hFIX-FIAV and hFIX-IDAV variant sequences by in silico analysis underwent Step 2 analysis (**Table 1**).
- **MHC-class II**: No binding peptides were identified in the hFIX-FIAV or hFIX-IDAV variant sequences.
  - No difference in MHC Class II predicted immunogenicity between wt-hFIX and hFIX variants.
  - No further analyses on MHC Class II predicted immunogenicity performed (**Table 1**).

Table 1. Immunogenicity testing

	Step 1 *In silico* analysis		Step 2 T cell epitopes assay
MHC Class I	Four potential moderate affinity non-germline epitopes identified	→	Candidate peptides assessed for binding to various MHC Class I alleles
MHC Class II	Zero non-germline epitopes identified	✱	Step 2 testing not performed

Step 2: T cell epitope analysis

- The quantitative and qualitative binding properties of the MHC class I binding peptides were determined for a selection of the most frequent human leukocyte antigen (HLA) alleles in the general population.
- Three out of the four peptides identified were excluded as potential epitopes (**Table 2**).
  - Peptide 4 showed binding to two MHC Class I alleles A*02:01 and B*35:01
  - Allele frequency in North American Caucasian population:
    - A*02:01: 45.0%
    - B*35:01: 10.7%

Table 2. REVEAL® scores showing the level of incorporation of variant peptides to different MHC class I alleles

Allele	FIX-FIAV Peptide 1 RYNSGK**F**EE	FIX-FIAV FIX-IDAV Peptide 2 QSFNDFTR**I**	FIX-FIAV FIX-IDAV Peptide 3 SFNDFTR**I**V	FIX-FIAV FIX-IDAV Peptide 4 N**A**YNHDIAL	Positive control
A 01:01	0.8	0.5	0.5	0.4	+100
A 02:01	0.6	32.9	0.5	**+132.8**	+100
A 03:01	2.4	**+70.3**	**+77.7**	44.0	+100
A 11:01	1.0	0.9	1.8	1.7	+100
A 24:02	15.2	0.6	0.4	1.3	+100
A 29:02	1.2	1.3	0.6	0.9	+100
B 07:02	0.3	0.3	0.2	16.4	+100
B 08:01	0.3	0.3	0.4	**+59.8**	+100
B 14.02	0.0	0.1	0.0	15.2	+100
B 15.01	0.2	0.8	0.3	11.1	+100
B 27.05	0.6	0.1	0.1	0.0	+100
B 35:01	0.4	0.4	0.1	**+62.8**	+100
B 40: 01	0.2	0.1	0.1	0.0	+100

The modified amino acid in each peptide sequence is underlined. Positive control was a known T-cell epitope peptide with very strong binding. REVEAL score for each MHC-peptide complex calculated by comparison to the binding (on-rate) of the positive control at the latest time point.

- Peptide 4 was determined as posing an extremely low risk for the two alleles due to:
  - Poor stability of the MHC/peptide complexes (A*02:01).
  - Weak binding of the peptide to MHC (B*35:01) (**Table 3**).

Table 3. Binding and on- and off-rate data for Peptide 4

MHC Class I allele	On-rate $T_{½}$ (h)	Off-rate $T_{½}$ (h)	Kinetic score	R score
A*02:01	8.90	0.20	0.02	0.02
Positive control	12.72 ± 2.09	>120	9.43 ± 0.16	9.43
B*35:01	45.90	>120	2.61	1.08
Positive control	375.17 ± 124.83	>120	0.32 ± 0.33	0.32

Kinetic scores are calculated by dividing the off-rate by the on-rate. Higher kinetic scores indicate better epitopes. R scores provide an overall rating for each peptide. The higher the R-score, the better the epitope. R scores ≥45% of the positive control warrant further investigation.

CONCLUSIONS

- hFIX-FIAV and hFIX-IDAV variants are not associated with a significant risk of immunogenicity.
- hFIX-FIAV has been selected for further development.

REFERENCES

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Presented at the 27th International Society on Thrombosis and Haemostasis (ISTH) congress, Melbourne, Australia, July 6-10, 2019