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Development of C-terminal α-Synuclein Vaccine for Treatment and Prevention of Parkinson's Disease and Other Synucleinopathies

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INTRODUCTION

- Parkinson's disease (PD) affects 7–10 million people worldwide.[1] PD and other synucleinopathies are characterized by pathological accumulation of α-synuclein (α-syn) in both central and peripheral nervous system neurons, resulting in widespread and progressive motor and non-motor symptoms.[2]
- Currently, there are no approved disease-modifying treatments; however, prasinezumab, a humanized monoclonal antibody targeting the C-terminal region of α-syn, showed signals of efficacy on multiple prespecified secondary and exploratory clinical endpoints in a phase 2 study.[3]
- Prothena is developing active vaccines targeting α-syn epitopes shown in clinical and/or preclinical studies to disrupt synuclein pathology and slow disease progression. These vaccines, which target the underlying pathophysiologic hallmarks of PD, are intended to raise antibody titers against specific α-syn epitopes, resulting in the potential of disease modification, treatment, and/or prevention of PD and other synucleinopathies.
- **We discuss the evolution of a C-terminal tandem peptide-based vaccine and show its superiority over both single-peptide and tandem peptide-based vaccines that combine both C-terminal and mid-region of α-syn.**

MATERIALS AND METHODS

Immunogen Preparation

- Vaccine constructs were prepared as single or tandem peptide vaccines as described in **Figure 1** and **Table 1**.
- Single-peptide vaccines contained a linear peptide, a spacer, and a cysteine for coupling, whereas tandem peptides contained an endopeptidase cleavage site[4] between the peptides, a spacer, and cysteine. Peptides were conjugated to bromoacetyl-CRM197 according to the manufacturer's protocol (FinaBiosolutions) (**Figure 1**).

Figure 1. Design of Vaccine Constructs

SynP, α-syn peptide; XX, endopeptidase cleavage site; CRM197, **a genetically detoxified diphtheria toxin.**

Table 1. Vaccine Construct Designations

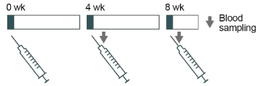
Vaccine	Peptide	Composition
A	Tandem	SC2-XX-SC1-linker CRM
B	Tandem	SC1-XX-SC2-linker CRM
C	Tandem	SC1-XX-SC3-linker CRM
D	Tandem	SC3-XX-SC2-linker CRM
1	Single	SC1-linker CRM
2	Single	SC2-linker CRM
3	Single	SC3-linker CRM

SC1, C-terminal peptide 1; SC2, C-terminal peptide 2; SC3, mid-region peptide; XX, endopeptidase cleavage site.

Study Design

- 5- to 6-week-old Swiss Webster (Taconic) or BDF1 (Jackson) mice were injected every 4 weeks with 25–50 µg of immunogen, and 25 µg QS21 in a 200 µL volume subcutaneously for 3 injections (**Figure 2**). Blood samples were collected 1 week after injections 2 and 3 to evaluate antibody titers. Final serum collections were used for immunochemistry and the *in vitro* B103 cell-based assay.

Figure 2. Treatment and Blood Sampling Schedule



Injections were performed on weeks 0, 4 and 8. Blood collections for serum weeks 5 and 9.

Preparation of Synuclein Aggregates

- Recombinant α-syn was purified as previously described.[5] To generate aggregates, recombinant α-syn (4 mg/mL) was added to Eppendorf low binding tubes containing 1,1,1,3,3,3-hexafluoro-2-propanol to achieve a final concentration of 2.5% v/vv. The solution was shaken at 500 RPM for 5 days at 37ºC and volatiles were allowed to evaporate overnight. Aggregates were collected by centrifugation, resuspended in phosphate-buffered saline (PBS), and sonicated with a probe sonicator (QSonica Q55) prior to use.

Titer Assay

- Antibody titers were determined with an enzyme-linked immunosorbent assay against full-length recombinant α-syn or α-syn aggregates. Plates were coated overnight with 1 µg/mL of either α-syn or α-syn aggregates in PBS and then blocked for 1 hour with 1% bovine serum albumin (BSA) in PBS. Blood samples were diluted in PBS, 0.1% BSA, and 0.1% Tween 20 starting at 1:100 and then serially diluted 1:2. Normal mouse serum was used as a negative control and known positive sera from previous mouse studies were used as a positive control. Plates were washed with tris-buffered saline (TBS)/Tween 20 and incubated with anti-mouse-specific horseradish peroxidase (ThermoFisher Scientific) for 1 hour at room temperature. Plates were washed with TBS/Tween 20, and antibody binding was detected with o-phenylenediamine dihydrochloride substrate (ThermoFisher Scientific) per the manufacturer's instructions, including stopping the reaction with 50 µL H_2SO_4. Plates were read at 490 nm on a Spectromax (Molecular Devices). Antibody titer was defined as the dilution of test sera equal to 4 times the optical density of normal mouse serum at the equivalent dilution.

Immunohistochemistry

- Cryostat sections of fresh frozen PD brain tissue (Banner Sun Health Research Institute) were stained with immune sera at 1:300. Binding of immune sera was detected with a mouse-specific secondary antibody and a DAKO DAB detection kit per the manufacturer's instructions. Staining was processed with an automated Leica Bond Stainer.

Blocking of α-syn Internalization into B103 Cells

Preparation of pHrodo-syn

- α-syn aggregates were labeled using pHrodo Red-SE (ThermoFisher Scientific) per the manufacturer's protocol. Free dye was removed via ultracentrifugation of the aggregated α-syn and repeated rinsing of the resultant pellet. Aggregated α-syn was resuspended in PBS in the original volume and bath sonicated (QSonica) at 10% power for intervals of 10 seconds on and 5 seconds rest for 3 minutes of total sonication time. The degree of labeling was quantified according to manufacturer's instructions and determined to be 2 dye:1 protein.

Preparation of Sera to IgG Cut

- Non-IgG proteins were removed by diluting 50 µL of serum 1:1 with cold saturated ammonium sulfate and incubated at 4ºC for 30 minutes. Precipitate was pelleted by spinning at 1000 x g and resuspended to original volume in PBS and desalted (ThermoFisher Scientific 50 µL Zeba Spin Column).[6]

Internalization of Aggregated α-syn Assay

- Before use, purified pHrodo-labeled α-syn was diluted in growth media to achieve a concentration of 10 µg/mL (a 4x working concentration) prior to the addition of IgG cut samples. IgG cuts were diluted in growth media (4 x working concentration of 1,200 µg/mL; n=3 per group). The 10 µg/mL pHrodo-labeled aggregate working solution was added 1:1 (25 µL/sample) to IgG cuts (25 µL/sample). Antibody/α-syn mixtures were then incubated at room temperature for 30 minutes.
- B103 neuroblastoma cells were washed with warm sterile PBS and lifted with Versene/ethylenediaminetetraacetic acid incubation for 5 minutes at 37ºC in 5% CO_2. Cells were centrifuged at 200 x g for 5 minutes and resuspended to 1,000,000 cells/mL in growth media (50 µL/sample). 50 µL of B103 cells were added to 50 µL antibody/α-syn mixtures in 96-well v-bottom plates. Following a 3-hour incubation at 37ºC in 5% CO_2, cells were washed three times with growth media (200 x g 5-min spins), incubated for 10 minutes at 37ºC in 5% CO_2, washed twice in flow cytometry (FACS) buffer (1% FBS in PBS; no Mg or Ca; 200 x g 5-min spins), and resuspended in 100 µL FACS buffer for flow cytometric analysis of labeled α-syn uptake by B103 cells.

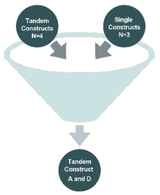
FACS Analysis

- Uptake of α-syn aggregates by B103 rat neuroblastoma cells was evaluated by FACS for pHrodo-labeled fluorescently activated α-syn. α-syn internalization was measured as mean fluorescence intensity (MFI; normalized to 100%) and % blocking determined. Normalization of MFI to 100% involved subtraction of background cellular MFI signal (no pHrodo Tau added) and setting the highest MFI signal to 100% (isotype control).

RESULTS

Prothena took a multi-step screening approach to identify the best peptide vaccine(s) for further development (**Figure 3**).

Figure 3. Screening Funnel



From our initial screening experiments, we showed that:

- All tandem peptide-based vaccines raised titers 7–10 times higher than vaccines with single peptides.
- Serum titers from all vaccinated animals recognized both monomeric and aggregated α-syn, although antibodies raised against tandem peptide vaccines demonstrated a preference for binding α-syn aggregates.
- Among the tandem peptide-based vaccines tested, tandem vaccines A and D passed all screening criteria, whereas vaccine C produced antibodies that did not react with pathological α-syn in PD brains and vaccine B-derived antibodies had high variability in their ability to inhibit α-syn internalization.
- While single-peptide vaccines were inferior to tandem vaccines, we could not determine in this study whether this was due to a higher density of peptide or optimal antigen presentation.

Table 2. Summary of the Characteristics of All Screened Vaccines

Vaccine	Titer	Pathological Synuclein Binding	Inhibition of α-syn Internalization
Single 1	Moderate	Moderate	Poor
Single 2	Moderate	Moderate	Poor
Single 3	Moderate	Negative	Not Tested
Tandem A	High	High	High
Tandem B	High	Moderate	Moderate
Tandem C	High	Negative	Negative
Tandem D	High	High	High

- Tandem vaccines A and D, and single-peptide vaccine 2 were advanced to a larger study.

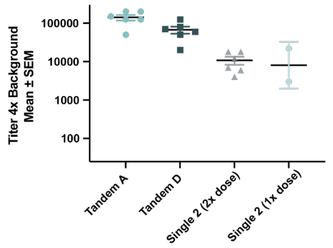
Selection of Lead Tandem Peptide-Based Vaccine A

- Tandem A and D and a 2-fold higher dose of single-peptide vaccine 2 were tested in a larger cohort of a different mouse strain to evaluate inter-species variability. The injection and blood collection schedule were as the first study (**Figure 2**).

Tandem Peptide-Based Vaccine A and D Were Superior to Single-Peptide Vaccine

- The 7- to 10-fold increase in titers between tandem and single-peptide vaccines persisted even after doubling the dose of the single peptide and changing the strain of mice, indicating that peptide density is not the key factor driving the superior immunogenicity of tandem vaccines (**Figure 4**). There was no appreciable difference in titer between the original dose of single peptide and 2x the dose, indicating that the single peptide-based vaccines maximized immunogenicity.

Figure 4. Lower Titer of the Single-Peptide Vaccine to α-syn Is Not Due to Peptide Amount or Mouse Strain

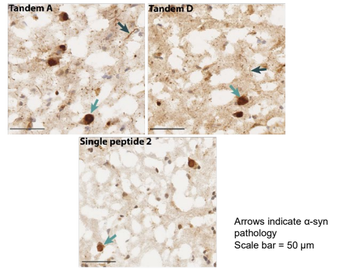


SEM, standard error of the mean.

Staining of Pathological PD Brain Tissue

- Both tandem vaccines generated antibodies that recognized pathological α-syn; however, sera from Tandem A appeared to react to pathological α-syn stronger than sera from Tandem D (**Figure 5**). The single-peptide vaccine did not generate antibodies that recognized the pathology to the same extent as either of the tandems, which may be expected due to the lower titers.

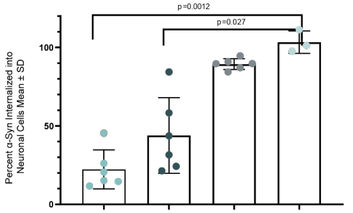
Figure 5. Staining of α-syn in PD Brains



Inhibition of Aggregated α-syn Internalization by IgGs Generated with Tandem Peptide-Based Vaccine

- Tandem A vaccine showed a robust and consistent inhibition of α-syn internalization into B103 cells, whereas Tandem D was more variable and the single-peptide vaccine 2 did not significantly block α-syn internalization (**Figure 6**). Tandem A-treated cells had a 77% reduction (p=0.0012) in α-syn aggregate internalization, whereas Tandem D had a 56% reduction (p=0.027) when compared with non-immune IgG treated (negative-control). Tandem A was nominated as the lead vaccine due to its robust inhibition of internalization of α-syn aggregation with a p value of 0.0012 compared with non-immune mouse IgG using a Kruskal-Wallis multi-comparison test.

Figure 6. Tandem A Robustly Inhibits the Internalization of α-syn into a Neuronal Cell Line



Each point represents the average of 3 replicate points from a single animal. Non-immune IgG was obtained from pools of normal mouse sera

SD, standard deviation.

CONCLUSIONS

- Novel single or tandem vaccines containing short α-syn peptide sequences were developed to optimally target pathogenic, aggregated α-syn, while preventing unwanted off-target reactivity, and to reduce the risk of α-syn-directed T-cell responses.
- We demonstrated that C-terminal/C-terminal tandem peptide-based vaccines were superior to both single-peptide vaccines and C-terminal/mid-region tandem peptide vaccines in all assays investigated: α-syn titers, of pathological α-syn staining in human PD brains, and inhibition of α-syn aggregate internalization into a neuronal cell line.
- **This novel approach of combining synuclein peptides in tandem peptide-based vaccines with an endopeptidase cleavage site may increase specific presentation of the synuclein peptides to the immune system, resulting in higher and more potent titers.**
- **Further development of the tandem peptide vaccine is planned for the potential treatment and prevention of PD and other synucleinopathies.**

AUTHOR DISCLOSURES

All authors are employees of Prothena Biosciences Inc.

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