Bioorganic Chemistry xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

O-GlcNAcylation of truncated NAC segment alters peptide-dependent effects on α-synuclein aggregation

Philip Ryan[a,b,c], Ming-ming Xu[d], Andrew K. Davey[a,b,c], Michael Kassiou[e], George D. Mellick[d,*], Santosh Rudrawar[a,b,c,*]

[a] School of Pharmacy and Pharmacology, Griffith University, Gold Coast, QLD 4222, Australia
[b] Menzies Health Institute Queensland, Griffith University, Gold Coast, QLD 4222, Australia
[c] Quality Use of Medicines Network, Griffith University, Gold Coast, QLD 4222, Australia
[d] Griffith Institute for Drug Discovery, Griffith University, Nathan, QLD 4111, Australia
[e] School of Chemistry, The University of Sydney, NSW 2006, Australia

ARTICLE INFO

Keywords:
α-Synuclein aggregation
Parkinson's disease
O-GlcNAcylation
Neurodegenerative diseases

ABSTRACT

Numerous post-translational modifications (PTMs) of the Parkinson's disease (PD) associated α-synuclein (α-syn) protein have been recognised to play critical roles in disease aetiology. Indeed, dysregulated phosphorylation and proteolysis are thought to modulate α-syn aggregation and disease progression. Among the PTMs, enzymatic glycosylation with *N*-acetylglucosamine (GlcNAc) onto the protein's hydroxylated amino acid residues is reported to deliver protective effects against its pathogenic processing. This modification has been reported to alter its pathogenic self-assembly. As such, manipulation of the protein's *O*-GlcNAcylation status has been proposed to offer a PD therapeutic route. However, targeting upstream cellular processes can lead to mechanism-based toxicity as the enzymes governing *O*-GlcNAc cycling modify thousands of acceptor substrates. Small glycopeptides that couple the protective effects of *O*-GlcNAc with the selectivity of recognition sequences may prove useful tools to modulate protein aggregation. Here we discuss efforts to probe the effects of various *O*-GlcNAc modified peptides on wild-type α-synuclein aggregation.

1. Introduction

There is no cure for the neurodegeneration that results in Parkinson's disease (PD). Specifically, understanding what causes PD and the mechanisms leading to the neurodegenerative process is being pursued to advance disease modifying therapies and clinically relevant diagnostics. The main pathological hallmarks of PD arise from the misprocessing, misfolding and self-assembly of a 140 amino-acid protein, α-synuclein (α-syn). α-Syn is highly abundant in the brain and, when not degraded, forms beta pleated sheets and fibrillar aggregates which eventually form insoluble Lewy bodies and Lewy neurites. These aggregates have been used to characterize PD at autopsy [1].

The predominant α-syn isoform is a 140 amino-acid sequence consisting of three distinct domains: an *N*-terminal repeat domain spanning residues 1–60; a central, hydrophobic region spanning residues 61–95; and the proline-rich, highly acidic *C*-terminal region that spans residues 95–140. The central domain hosts the non-amyloid-β component (NAC), a stretch of residues implicated in the pathogenic self-recognition interactions that lead to pathological aggregation. Exactly how far upstream, along the pathogenic cascade, the self-assembly of α-syn sits is still undetermined. It is also unclear whether other specific molecular events are required to trigger the process. The pathogenic process in PD is associated with α-syn self-assembly, although the exact mechanism responsible for inducing neurotoxicity is poorly defined. It is suggested to be either characteristic of α-syn oligomers, or somehow triggered by supraphysiological levels of α-syn monomers [2–4]. Aggregation prone species may also be able to propagate between neurons [5,6], initiating secondary nucleation events with endogenous α-syn upon arrival, accelerating disease spread and progress [7].

In addition to PD, there are an array of neurodegenerative diseases (NDs) characterized by the misfolding and self-assembly of neurotoxic proteins. These proteins include amyloid-β and tau associated with Alzheimer's disease (AD); Huntingtin in Huntingtons disease (HD); and various prion-related disorders. Chemical agents that can bind these proteins in their monomeric or oligomeric forms selectively and with high-affinity may be useful as early-stage diagnostic tools if detected *ex vivo*. Compounds able to effectively inhibit the aggregation process are speculated to be useful as therapeutic tools. Small peptides are able to

* Corresponding authors at: School of Pharmacy and Pharmacology, Griffith University, Gold Coast, QLD 4222, Australia.
E-mail addresses: g.mellick@griffith.edu.au (G.D. Mellick), s.rudrawar@griffith.edu.au (S. Rudrawar).

https://doi.org/10.1016/j.bioorg.2019.103389
Received 13 August 2019; Received in revised form 15 October 2019; Accepted 21 October 2019

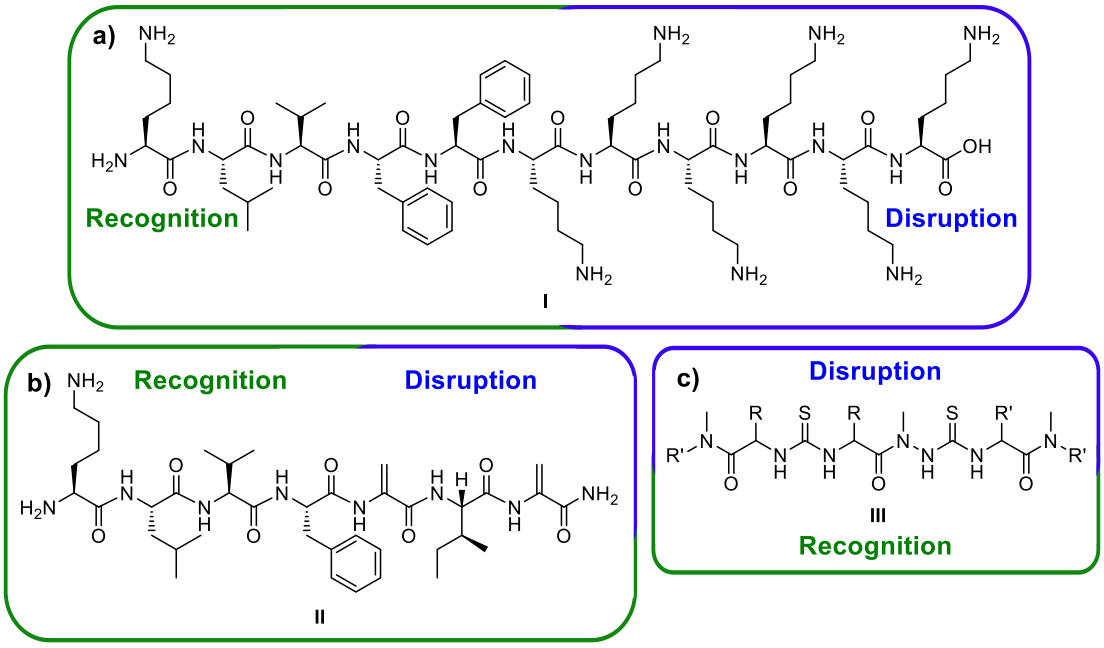


Fig. 1. Literature examples of peptidomimetic inhibitors of Aβ aggregation. (a) The sequence KLVFFKKKKK (**I**) possesses a chain of cationic kosmotropic lysine units as disrupting domain [12]; (b) Compound **II** bears dehydroalanine (ΔAla), a residue which induces specific structural conformations [10]; (c) a novel bis(thiourea) hydrazide pseudopeptide scaffold (**III**) consists of a blocking face resulting from *H*-bond-incompetent sulfur atoms and *N*-methylamides, and a complementarity face resulting from the increased acidity of the thiourea protons [11].

bind amyloidogenic proteins selectively, competitively and, when appropriately functionalised, are able to disrupt amyloid growth [8]. Murphy and co-workers reported a 'hybrid peptide' (**I**, Fig. 1a) which possesses a high-affinity for binding Aβ, supplied by the well-known Aβ-selective CHD (KLVFF) recognition sequence, coupled to a disruptive lysine hexamer sequence that interferes with Aβ self-assembly [9].The peptide worked to accelerate aggregation of the soluble Aβ oligomers into fibrils, reducing the concentrations of toxic putative intermediates. Rangachari and co-workers' dehydroalanine-bearing peptide (**II**, Fig. 1b) is able to efficiently reduce Aβ-induced cell toxicity [10], and Hecht and Klein's novel series of amphiphilic bis(thiourea)-hydrazide containing pseudopeptides (**III**, Fig. 1c) adhere to Aβ and inhibit its self-association [11]. Each of these peptidomimetics consist of a protein-recognition component and disruptive component. The disruption results from intermolecular interactions that work overall to disfavour amyloid formation, altering the physicochemical properties of the resultant protein-inhibitor complex, or by other chemical mechanisms.

Post-translational modifications (PTM) of α-syn, (proteolysis, phosphorylation, acetylation, glycosylation etc.) alter the chemical properties of the protein and are proposed to modulate Lewy pathology. The *O*-GlcNAc transferase catalyzed *O*-GlcNAcylation is a reversible PTM and several proteins are glycosylated including α-syn (see Fig. 2). Calpain-mediated *C*-terminal truncation is reported to yield aggregation-prone α-syn fragments which may promote seeded aggregation [13]. The *C*-terminal domain of α-syn is reported to stabilize the disordered conformation of the α-syn monomer *via* interactions with the *N*-terminal or NAC domains [14]. Calpain activity is reported to correlate with disease progression in PD mouse models [15,16]. Phosphorylation is suggested to play an important role in the regulation of α-syn aggregation, Lewy body formation and neuronal degeneration. Glycosylation of hydroxyl-amino acids with *N*-acetylglucosamine (*O*-GlcNAc) works competitively against phosphorylation, and it is speculated that this may constitute a protective mechanism against PD pathogenesis [17]. *O*-GlcNAc functionalization, at various native modification sites along the NAC domain of α-syn, reportedly alters the peptides self-assembling propensity and toxicity in a site-specific

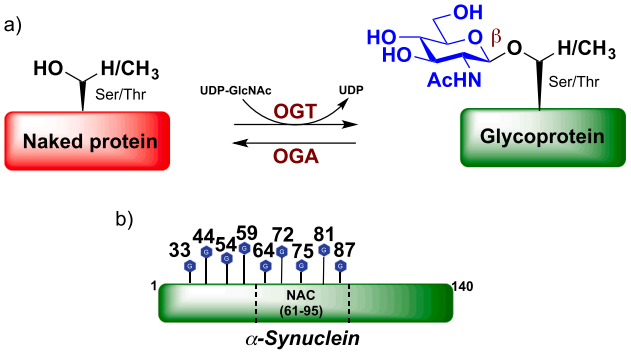


Fig. 2. (a) Reversible PTM *O*-GlcNAcylation of the side chain of serine; (b) α-Synuclein *O*-GlcNAcylation.

manner [17,18]. Results suggest that *O*-GlcNAcylated α-syn is incorporated into the aggregation reaction less efficiently than unmodified α-syn [17]. Interestingly, *O*-GlcNAcylation (*O*-GlcNAc addition) is reported to affect the proteins implicated in AD, ALS and HD in similar ways [19]. These findings support a model where *O*-GlcNAcylation prevents indiscriminate protein aggregation in general.

A truncated analogue of the NAC domain of α-syn has been reported previously to accelerate wild-type (wt) α-syn aggregation in a concentration-dependent manner upon co-incubation *in vitro* [20]. When modified with *O*-GlcNAc at a site analogous to Thr72, the truncated NAC segment (α-syn$_{68\text{-}77}$, Ac-^{68}GAVVT(*O*-GlcNAc)GVTAV^{77}-NH$_2$) did not increase, nor accelerate wt α-syn aggregation upon co-incubation. It was concluded that the glycopeptide was not participating in aggregation under the assay conditions. Glycoside addition can influence the capacity of a peptide to establish binding interactions with known substrates; it also affects its hydrophilic profile, steric bulk, and its resistance to metabolic processing [21].

Following from this, we sought to investigate whether PD-relevant NAC segments, known to bind α-syn selectively, would, when decorated with the disruptive *O*-GlcNAc unit, act as inhibitors of the pathogenic associative events that occur during the α-syn aggregation

reaction. As the effects of *O*-GlcNAcylation are site-specific [18], we were interested in developing a panel of small glycopeptides resulting from shifting the glycoside to sites presumed to be less mechanistically engaged in the recognition interaction, i.e. the *N*-terminal, to couple the disruptive nature of the *O*-GlcNAc residue with the associative nature of the sequence. The peptides examined here were *C*-terminally amidated and *N*-terminally acetylated, consistent with previous reports [20]. Here we describe efforts to construct and evaluate 10-/12-mer glycopeptides, derived from the NAC segment of α-syn, to probe their effects on wt α-syn aggregation. This study was conducted with a view to deciphering the role of *O*-GlcNAc on aggregation inhibition or mechanisms underlying the aggregation event.

2. Materials and methods

2.1. General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Bruker Avance DPX 400 spectrometer at a frequency of 400.2 MHz. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker Avance DPX 400 spectrometer at a frequency of 100 MHz. The spectra are reported as parts per million (ppm) downfield shift using the solvent peak as internal reference. The data are reported as chemical shift (δ), multiplicity, relative integral, coupling constant (JHz) and assignment where possible. Low resolution mass spectra were recorded on a Finnigan LCQ Deca ion trap spectrometer (ESI). High resolution mass spectra were recorded on a Bruker 7 T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR).

HPLC: Analytical reverse-phase HPLC was performed on a system consisting of a Shimadzu BM-20A Prominence communications bus control module, two Shimadzu LC-20 AD UFLC liquid chromatograph pumps fitted with a solvent mixer, a Shimadzu DGU-20A3 Prominence degasser, a Shimadzu SIL-20A HT UFLC Prominence chilled autosampler module, a Shimadzu CTO-20 AC Prominence column oven, a Shimadzu SPD-M20A Prominence Diode array detector module with an Alliance series column heater at 30 °C and 2996 photodiode array detector. A Waters Sunfire 5 μm, 2.1 × 150 mm column was used at a flow rate of 1 mL·min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B) and a linear gradient of either 0–100 %B or 0–50 %B over 10 min. The results were analysed using LabSolutions software. Preparative reverse-phase HPLC was performed using a the same Shimadzu system only coupled with a Shimadzu SPD-20AC dual wavelength detector operating at 214 and 254 nm and a Gilson FC 204 fraction collector calibrated to collect only peaks with intensities exceeding 100 mAU (λ = 214 nm) which were sub-fractionated into 0.2 min interval fractions. A Waters Sunfire 5 μm, 19 × 150 mm column was used at a flow rate of 7 mL min^{-1} using a mobile phase of 0.1% TFA in water (Solvent A) and 0.1% TFA acetonitrile (Solvent B) using a linear gradient of 0–100% B over 60 min unless mentioned otherwise. HPLC-ESI/MS (LCMS) was performed on an Agilent 1290 HPLC (with PDA) coupled in series to an Agilent 6530 Q-TOF operating in positive mode using Agilent Jet Stream ESI ion source. Separations were achieved using a Waters Sunfire 5 μm, 2.1 × 150 mm column and a flow rate of 1 mL·min^{-1}. A mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) using a linear gradient of 0–100% or 0–50% B over 8–10 min was used. Commercial materials were used as received unless otherwise noted. Amino acids, coupling reagents and resins were obtained from Mimotopes. Dichloromethane and methanol were distilled from calcium hydride. DMF was obtained as peptide synthesis grade from Auspep or Labscan.

2.2. Solid phase synthesis

All solid-phase peptide syntheses were carried out manually in disposable polypropylene syringes (Torviq) equipped with Teflon sinters.

Loading of Fmoc-amino acids onto Rink Amide (RAM) resin: Rink Amide (RAM) resin (0.450 mmol/g loading, 467 mg, 0.210 mmol) was swollen in dry DMF (5 mL) for 5 min at room temperature before being treated with 10 vol% piperidine/DMF (5 mL) solution and shaken for 5 min at room temperature. The procedure was repeated with a fresh portion, after which the resin was washed with DMF (5 × 4 mL), DCM (5 × 4 mL) and then DMF (5 × 4 mL). After, a solution of the Fmoc-protected amino acid (0.84 mmol, 4 equiv.) PyBOP (0.84 mmol, 4 equiv.) and *N*-methylmorpholine (1.68 mmol, 8 equiv.) in DMF (3 mL) was mixed with the resin and shaken for 1 h. After, the resin was washed with DMF (5 × 4 mL), DCM (5 × 4 mL) and then DMF (5 × 4 mL). The desired peptides were then assembled following iterative Fmoc-SPPS procedures. To determine resin loading, a solution of piperidine in DMF (4 mL, 10% v/v) was added to the resin before being shaken for 3 min. The drained Fmoc deprotection solution was retained in a 10 mL volumetric flask and the resin washed with fresh piperidine in DMF (10% v/v) such that the total volume did not exceed 10 mL. The efficiency of the initial loading was quantitatively determined by measurement of the dibenzofulvene-piperidine adduct using Varian Cary 4000- UV–Vis spectrophotometer (λ = 301 nm). Amino acid loading onto the resin was most often quantitative. The resin was subsequently washed with DMF (10 × 5 mL), DCM (10 × 5 mL), and DMF (10 × 5 mL).

Fmoc deprotection: Pre-loaded resin was treated with 10 vol% piperidine/DMF (5 mL) solution and shaken for 5 min at room temperature before being filtered and treated again with a fresh 10 vol% piperidine/DMF solution. The efficiency of the initial loading was quantitatively determined by measurement of the piperidine-fulvene adduct using UV–Vis spectrophotometry (λ = 301 nm). The resin was subsequently washed with DMF (5 × 4 mL), DCM (5 × 4 mL) and DMF (5 × 4 mL).

Glycosylated and unglycosylated amino acid coupling: Amino acids were coupled using a mixture of appropriate protected amino acid (0.84 mmol, 4 equiv.), PyBOP (0.84 mmol, 4 equiv.) and NMM (1.68 mmol, 8 equiv.) in DMF (3 mL) which was added to the resin and shaken. After 1 h the resin was washed with DMF (5 × 4 mL), DCM (5 × 4 mL) and then DMF (5 × 4 mL). Glycopeptides were synthesised at a 0.07 mmol scale, incorporation of the glycosylated amino acid building block **16** was conducted using a mixture of Fmoc-Ser(*O*-GlcNAc)-OH (0.091 mmol, 1.3 equiv), HATU (0.112 mmol, 1.6 equiv.), HOAt (0.112 , 1.6 equiv.), and NMM (0.224, 3.2 equiv.), in DMF (4 mL) which was added to the resin and shaken. After 16 h the resin was washed with DMF (5 × 4 mL), DCM (5 × 4 mL) and DMF (5 × 4 mL).

Capping: Following each coupling, unreacted sequences were capped by treating the resin with 10 vol% acetic anhydride/pyridine (5 mL) solution before being shaken for 5 min at room temperature, filtered and subsequently washed with DMF (5 × 4 mL), DCM (5 × 4 mL) and DMF (5 × 4 mL).

Resin cleavage and ether precipitation: The resin was washed thoroughly with DCM (10 × 4 mL) and subsequently treated with a solution of TFA/TIS/H_2O (90:5:5 v/v/v, 4 mL) and was shaken for 1 h at room temperature. The resin was filtered and washed with DCM (2 × 2 mL) before the resultant filtrate was evaporated to dryness. Cold diethyl ether (2 mL) was added to the precipitate, suspended, transferred to a 2 mL Eppendorf tube, and subsequently centrifuged at 1400 rpm for 1 min. The supernatant was decanted and discarded and the precipitate was dried under high-vacuum.

Glycopeptide deacetylation: Dry precipitate was suspended in dry methanol (1 mL) and mixed at room temperature before treatment with sodium methoxide in methanol (0.5 M, 50 μL, pH = 10). The solution was allowed to stir for 2 h before being neutralised with glacial acetic acid and evaporated to dryness. A solution of H_2O/ACN (1:1 v/v, 5 mL) was added to the precipitate, suspended, and centrifuged at 1400 rpm for 1 min. The supernatant was decanted and lyophilised to afford

precipitate ready for RP-HPLC purification. Details on the purity of the (glyco)peptides are presented in Table S1 and Figs. S1–S22 (analytical HPLC trace and HRMS data) of the supplementary material.

*2.2.1. Synthesis of Ac-^{71}Val-Thr-Gly-Val-Thr-Ala-Val-Ala-Gln-Lys-Thr-Ala^{82}-NH_2 (**1**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 19.7 min). The title compound (22.4 mg, 41%) was a white lyophilisate. Analytical HPLC: R_t 5.9 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1186.6817 ($[M+H]^+$), calcd. for $C_{51}H_{92}N_{15}O_{17}^+$ 1186.6790.

*2.2.2. Synthesis of Ac-Ser(O-GlcNAc)-^{71}Val-Thr-Gly-Val-Thr-Ala-Val-Ala-Gln-Lys-Thr-Ala^{82}-NH_2 (**2**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 18.2 min). The title compound (15.4 mg, 15%) was a white lyophilisate. Analytical HPLC: R_t 5.5 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1476.7922 ($[M+H]^+$), calcd. for 1476.7904$^+$ 1476.7904.

*2.2.3. Synthesis of Ac-^{68}Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val^{77}-NH_2 (**3**)*

The peptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 21.5 min). The title compound (26.2 mg, 40%) was a white lyophilisate. Analytical HPLC: R_t 7.4 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 936.5119 ($[M+Na]^+$), calcd. for $C_{40}H_{71}N_{11}NaO_{13}^+$ 936.5125.

*2.2.4. Synthesis of Ac-Ser-^{68}Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val^{77}-NH_2 (**4**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 20.6 min). The title compound (16.6 mg, 23%) was a white lyophilisate. Analytical HPLC: R_t 7.1 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1023.5452 ($[M+Na]^+$), calcd. for $C_{43}H_{76}N_{12}NaO_{15}^+$ 1023.5445.

*2.2.5. Synthesis of Ac-Ser(O-GlcNAc)-^{68}Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val^{77}-NH_2 (**5**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5 to 95 %B over 60 min; R_t 19.2 min). The title compound (5.3 mg, 6%) was a white lyophilisate. Analytical HPLC: R_t 9.7 min (0–100 %B over 12 min, λ = 214 nm); HRMS (ESI) 1226.6240 ($[M+Na]^+$), calcd. for $C_{51}H_{89}N_{13}NaO_{20}^+$ 1226.6239.

*2.2.6. Synthesis of Ac-Ser(O-GlcNAc)-Gly-^{68}Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val^{77}-NH_2 (**6**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5 to 95 %B over 60 min; R_t 19.3 min). The title compound (2.6 mg, 3%) was a white lyophilisate. Analytical HPLC: R_t 9.7 min (0–100 %B over 12 min, λ = 214 nm); HRMS (ESI) 1283.6461 ($[M+Na]^+$), calcd. for $C_{53}H_{92}N_{14}NaO_{21}^+$ 1283.6454.

*2.2.7. Synthesis of Ac-^{68}Gly-D-Ala-D-Val-D-Val-D-Thr-Gly-D-Val-D-Thr-D-Ala-D-Val^{77}-NH_2 (**7**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5 to 95 %B over 60 min; R_t 21.4 min). The title compound (5.1 mg, 8%) was a white lyophilisate. Analytical HPLC: R_t 7.4 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 936.5144 ($[M+Na]^+$), calcd. for $C_{40}H_{71}N_{11}NaO_{13}^+$ 936.5125.

*2.2.8. Synthesis of Ac-Ser(O-GlcNAc)-^{68}Gly-D-Ala-D-Val-D-Val-D-Thr-Gly-D-Val-D-Thr-D-Ala-D-Val^{77}-NH_2 (**8**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 19.4 min). The title compound (2.6 mg, 3%) was a white lyophilisate. Analytical HPLC: R_t 4.1 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1226.6243 ($[M+Na]^+$), calcd. for $C_{51}H_{89}N_{13}NaO_{20}^+$ 1226.6239.

*2.2.9. Synthesis of Ac-^{77}Val-Ala-Thr-Val-Gly-Thr-Val-Val-Ala-Gly^{68}-Ser(O-GlcNAc)-NH_2 (**9**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 20.1 min). The title compound (26.1 mg, 30%) was a white lyophilisate. Analytical HPLC: R_t 7.1 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1226.6244 ($[M+Na]^+$), calcd. for $C_{51}H_{89}N_{13}NaO_{20}^+$ 1226.6239.

*2.2.10. Synthesis of Ac-^{68}Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val^{77}-Ser(O-GlcNAc)-NH_2 (**10**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 19.2 min). The title compound (23.5 mg, 27%) was a white lyophilisate. Analytical HPLC: R_t 6.8 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1226.6243 ($[M+Na]^+$), calcd. for $C_{51}H_{89}N_{13}NaO_{20}^+$ 1226.6239.

*2.2.11. Synthesis of Ac-D-^{77}Val-D-Ala-D-Thr-D-Val-Gly-D-Thr-D-Val-D-Val-D-Ala-Gly^{68}-Ser(O-GlcNAc)-NH_2 (**11**)*

The glycopeptide was prepared following Fmoc-SPPS procedures and purified by preparative RP-HPLC (5–95 %B over 60 min; R_t 20.3 min). The title compound (15.3 mg, 18%) was a white lyophilisate. Analytical HPLC: R_t 7.1 min (0–100 %B over 10 min, λ = 214 nm); HRMS (ESI) 1226.6251 ($[M+Na]^+$), calcd. for $C_{51}H_{89}N_{13}NaO_{20}^+$ 1226.6239.

2.3. Expression and purification of α-synuclein

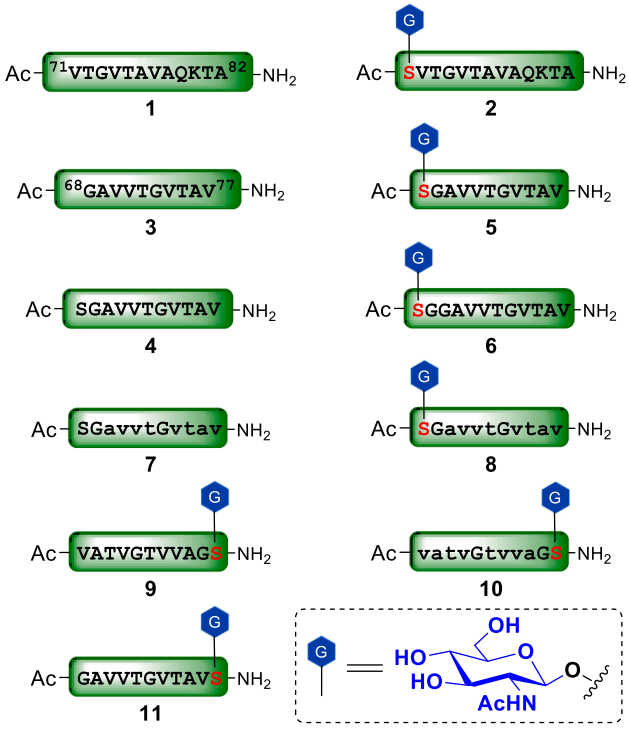
Escherichia coli BL21 (DE3) cells containing the human α-syn gene inserted into a pET-22b (+) vector (Novagen, Merck, MA, USA) were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside. α-syn was first purified following a non-chromatographic protocol reported by Volles and Lansbury [22]. Further purification using anion exchange followed protocols reported by Ventura and co-workers [23].

2.4. In vitro inhibition of α-synuclein aggregation

A stock solution of ThT (5 mM) in glycine-NaOH buffer (pH 8.0) was prepared and used with homogenous monomeric α-syn, made using commonly employed protocols described by Rahimi et al. [24]. Inhibition of α-syn aggregation was performed by incubation of a mixture of α-syn monomers (80 μM) suspended in aggregation buffer (20 mM Tris-HCl, pH 7.4, 200 mM $MgCl_2$ and 0.05% NaN_3) with or without candidate inhibitor compounds (80 μM) at 37 °C with constant shaking (1000 rpm in a thermomixer) for 48 h. For each treatment group, pre-incubated α-syn samples (60 μL) were mixed with a ThT solution (240 μL, 50 μM), the solution was dispensed in triplicate (n = 3) into 96-well plates which were analysed using a fluorimeter (ex. 440 nm/em. 500 nm). The fluorescence intensity of samples was compared to an untreated negative control and a positive control treated with epigallocatechin gallate (EGCG, 400 μM) to obtain percentage inhibition values.

2.5. Dot blot assay

Briefly, 2 μL samples were spotted on nitrocellulose membrane (Amersham Protran, GE Healthcare) and dried at room temperature.



Scheme 1. Contracted structures of peptide and glycopeptide modulators of α-syn aggregation. Compounds **1** and **2** are derived from the α-syn$_{71\text{-}82}$ stretch, the remaining compounds (**3–11**) are derived from the α-syn$_{68\text{-}77}$ stretch.

Nitrocellulose membrane was blocked at room temperature for 1 h with 3% BSA and then washed with Tris-buffered saline (TBST, 0.05% Tween 20, 50 mM Tris–HCl, 150 mM NaCl, pH 7.5). Each membrane was then incubated at 4 °C overnight with anti-α-synuclein antibody (1:2,000, BD Biosciences) and anti-α-synuclein filament antibody (1:8000, Abcam). Membranes were further washed in TBST and then incubated with secondary anti-mouse and anti-rabbit IgG (1:20,000, Thermo Scientific) for 2 h at room temperature. The blots were further incubated with ECL reagent (Millipore) for 5 min and developed.

3. Results and discussion

It has previously been shown that α-syn, when incubated in the presence of the NAC-derived sequence corresponding to residues 71–82 of α-syn (**1**, Scheme 1), undergoes aggregation at an accelerated rate [20]. We synthesised a peptide corresponding to this segment using iterative Fmoc-based solid-phase peptide synthesis (SPPS) with PyBOP/NMM. The ability of the peptide to promote aggregation was then verified by incubating α-syn (80 μM), expressed by *E. coli* and purified using anion exchange, with or without **1** (80 μM) under constant agitation for 120 h before being analysed by thioflavin T (ThT) fluorescence. We were particularly interested in the effect of the peptide at the end-point of the experiment rather than any effects on the rate of aggregation as ThT has been found previously to alter aggregation kinetics [25]. A selection of different conditions was used to compare the effect of different buffers on catalysing the reactions. Under all conditions, pH was maintained at 7.4 and NaN_3 was used as an antiseptic agent. It was surmised that **1** worked effectively at increasing fibril formation when using Tris(hydroxymethyl)aminomethane chloride (Tris-Cl) as a buffer (pH = 7.4), and $MgCl_2$ as an inducer of aggregation (see Fig. 3).

Given that **1** was able to significantly promote α-syn aggregation, we next set out to design and synthesise a selection of glycopeptides derived from segments spanning the NAC domain of α-syn. We first selected **2** (Ac-S(*O*-GlcNAc)VTGVTAVAQKTA-NH_2) to probe the effects

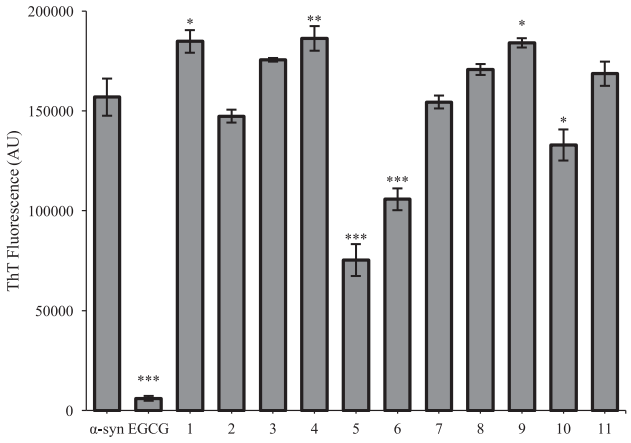
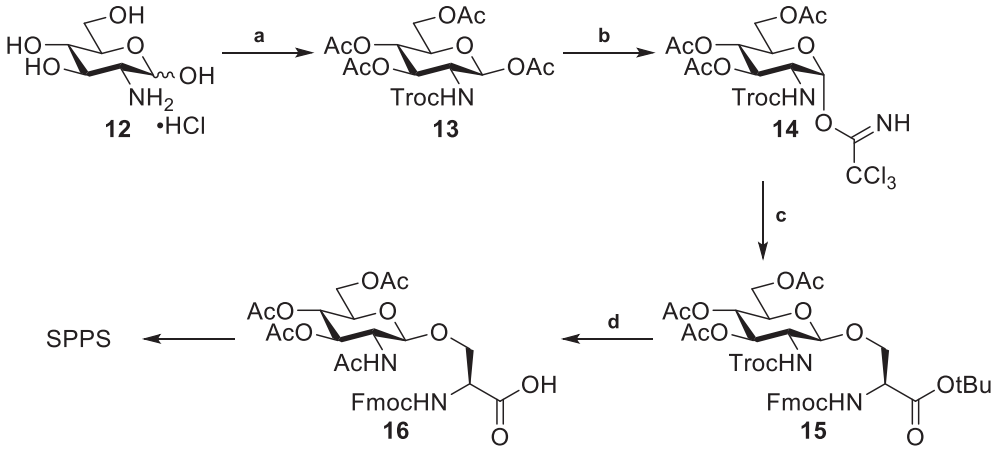


Fig. 3. Purified α-syn (80 μM) was incubated at 37 °C alone or with compounds (80 μM) before analysis by ThT fluorescence (λ_{ex} = 440 nm, λ_{em} = 500 nm) at 48 h. Representative results from 3 independent experiments are presented, error bars represent standard deviation analysed using one-way ANOVA with Dunnett's post hoc comparison (*P < 0.05 vs control; **p < 0.01 vs control; ***p < 0.001 vs control).

of *N*-terminal *O*-GlcNAcylation of the control α-syn$_{71\text{-}82}$ peptide. We then selected a panel of glycopeptides modified at various positions along the S68-V77 stretch (**3–11**, Scheme 1). To generate the glycopeptides, we incorporated a selectively-protected glycosylated amino acid building block, Fmoc-L-Ser((Ac)$_3$-β-D-GlcNAc)-OH (**16**), into Fmoc-SPPS (Scheme 2). Briefly, commercially available glucosamine hydrochloride salt (**12**) was selectively protected over two steps to form **13**. Removal of the anomeric acetyl group, followed by reaction with trichloroacetonitrile under basic conditions, afforded the glycosyl trichloroacetimidate **14**. The glycosyl acceptor, Fmoc-Ser(OH)-O^{t}Bu, synthesised in one step from commercially available Fmoc-Ser-OH, was then glycosylated stereoselectively using **14** with TMSOTf as a promoter to give **15**. Selective deprotection of **15** afforded the glycosylated amino acid **16**, usable in SPPS. Construction of the peptide sequences was undertaken using Fmoc-SPPS using Rink Amide (AM) resin to yield the *C*-terminal carboxamide. Protected amino acids were coupled using PyBOP/NMM, however coupling of **16** onto the *N*-terminally available peptide sequences was accomplished following a specialised procedure, optimised to minimise the D/L-epimerization of the glycosylated serine [26]. Serine was found to have a higher racemization rate than most other natural amino acids [27]. Specifically, a mixture of 1-hydroxy-7-azabenzotriazole (HOAt), (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), and NMM in DMF undergoes a rapid pre-incubation period prior to treatment of the peptides on solid-support. The reaction was accomplished over 16 h of mixing before the resin was washed with DMF and DCM. A molar ratio of 3.3:3.65:3.7:4:1 Fmoc-Ser(Ac$_3$GlcNAcβ)-OH/HATU/HOAt/NMM/peptide has been shown to afford a 96.8% yield with only a minimal (3.4%) amount of D-enantiomer produced [28]. Liquid Chromatography tandem Mass Spectrometry (LCMS) was employed to grade reaction success and confirm the identity of the glycopeptide products. Upon completion of the glycopeptides on-resin, they were released under acidic conditions and dried before the *O*-acetyl groups were removed using sodium methoxide in methanol, pH = 10. Each of the (glyco)peptides were purified by RP-HPLC and characterised by HRMS and analytical HPLC.

Following construction of the (glyco)peptides, their effects on α-syn aggregation was studied using ThT fluorescence assay. Mixtures of the candidate peptides (80 μM) were co-incubated with α-syn (80 μM) at 37 °C in a 1:1 ratio for 48 h with constant shaking. The fluorescence of the peptide analogues alone was also examined as some sequences were speculated to promote fluorescence even in the absence of α-syn, potentially skewing results. It was found that the fluorescence measured



Scheme 2. Reagents and conditions: (a) i: TrocCl, K_2CO_3, H_2O, rt, 1 h; ii: Ac_2O, pyridine, rt, 16 h, 61% over 2 steps; (b) i: ethylenediamine, AcOH, THF, rt, 15 h, 79%; ii: CCl_3CN, DBU, DCM, rt, 20 min, 60%; (c) Fmoc-Ser-*O*^{t}Bu, TMSOTf, DCM, −78 °C, 1 h, 97%; (d) i: Zn, Ac_2O, rt, 2 h, 78%; ii: TFA, DCM (1:1), rt, 2 h, quant.

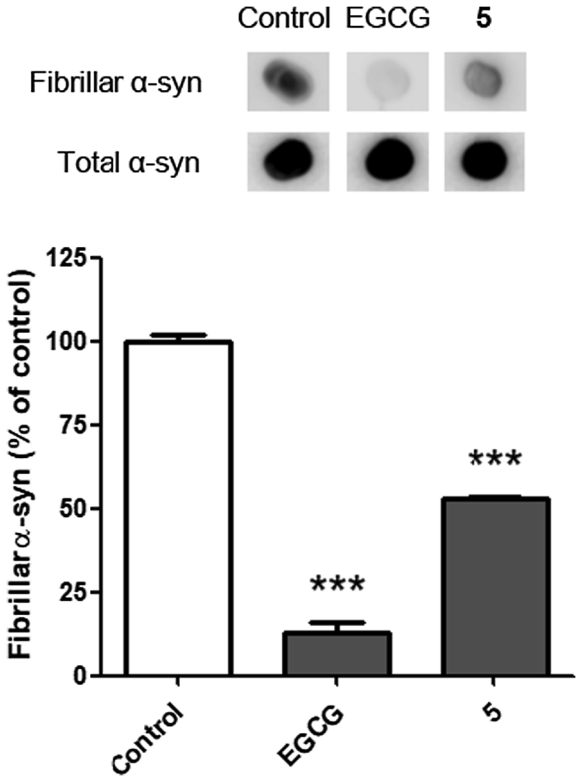


Fig. 4. Purified α-syn (80 μM) was incubated at 37 °C alone or with compounds (80 μM) before analysis by dot blot using a fibril-specific antibody at 48 h. Data were presented in mean ± standard error of the mean (SEM), ***p < 0.001 vs control, n = 3.

following incubation of the (glyco)peptide compounds alone was negligible compared to the fluorescence of the sample containing α-syn alone (See SI). Following incubation, each sample was mixed with ThT (50 μM final concentration), aliquoted into three wells each of a 96-well plate and read using a microplate reader.

As expected, **1** was found to significantly increase ThT fluorescence while its *N*-terminally *O*-GlcNAcylated analogue, **2** did not. Compound **2** was not able to significantly decrease ThT fluorescence relative to the untreated control either however. Indeed, no significant effect on ThT fluorescence was observed upon co-incubation of α-syn with **2**. Among the compounds derived from α-syn$_{68\text{-}77}$, unmodified **3** and serine-modified **4** both increased ThT fluorescence by 12% and 19% respectively relative to the control. This effect was only statistically significant in the case of co-incubation with **4**. When functionalised with Ser(*O*-GlcNAc), the effects of the α-syn$_{68\text{-}77}$ sequence on ThT fluorescence were significantly altered. *N*-terminally *O*-GlcNAcylated **5** and its analogue **6**, bearing a glycine spacer residue, reduced α-syn-mediated ThT fluorescence by 52% and 33% respectively. Though less effective, the *retro-inverso* analogue **10** was able to significantly decrease ThT fluorescence also. Finally, compounds **7**, **8**, **9** and **11** did not reduce ThT fluorescence under these conditions.

The effects displayed by **1** and **2** here are in agreement with observations made previously for a centrally *O*-GlcNAcylated peptide [20]. Findings suggest that, when *O*-GlcNAcylated, the α-syn protein is incorporated into the aggregation reaction with a reduced efficiency [17]. The results here suggest that *O*-GlcNAcylated **2** may be excluded from the aggregation reaction in a similar manner. It is possible therefore, that the decrease in fluorescence observed in samples containing either **5** or **6** results from their incorporation into the aggregation reaction. The decrease in fluorescence may result from the formation of heterogenous α-syn assemblies with decreased propensity to aggregate. We expect that the peptides may be arresting aggregation *via* interaction with the precursors to aggregation, species such as the oligomers. Mass spectra of the α-syn samples with and without compound were recorded and compared to determine whether the peptides were establishing strong binding interactions with the monomeric peptide, however the findings suggested that none of these peptides formed complexes with the monomer in any detectable manner (for spectra and methods, see SI).

The ability of **5** to inhibit α -syn fibril formation was validated using dot blot analysis, a non-denaturing method for examining protein epitopes. Dot blot analysis has been employed previously to study α-syn fibril formation and inhibitor-bound α-syn complexes structural conformations [29,30]. Equal volumes of each sample (2 μL) were spotted on nitrocellulose membrane before being treated with one of either the non-conformation dependent anti-α-syn antibody or the conformation dependent anti-α-syn filament antibody prior to further treatment with a detection antibody. Development of the blots confirmed that all samples contained equivalent concentrations of α-syn protein (see Fig. 4). Compound **5** was found to decrease the concentration of fibrillar α-syn by 50% relative to the untreated control.

Compounds **5** and **6** are the only glycopeptides identified to decrease α-*syn*-mediated ThT fluorescence to date. Compound **6** was notably less effective at reducing fluorescence than **5**. The structure of **6** differs from **5** by a single glycine residue, used as a conformationally unrestrained spacer residue. The glycine residue also switches the face of the peptide upon which the sugar is presented. In the structure of **6**, the glycoside is shifted further from the central binding residues, hampering its participation in the interactions between the peptide and

the native α-syn sequence. Similarly, shifting *O*-GlcNAc to the *C*-terminus, as was achieved in **11**, completely abolished inhibitory activity. These observations suggest that the effects of *O*-GlcNAcylation are site-dependent.

It is also interesting to note that **8** did not display similar inhibitory effects as its structure resembles the D-isomer of α-syn$_{68-77}$. Compound **8** was envisioned to effect α-syn aggregation in a similar manner to **5**. A number of D-isomeric Aβ-derived peptide sequences are reported to inhibit wt Aβ fibrillogenesis more potently than their L-isomer analogues [31]. Compound **9** was also unable to decrease ThT fluorescence, though its proximity pattern of side chains remains the same as **5**. Similarly, **10** bearing a *retro-inverso* sequence was designed to assume a side chain topology similar to that of the parent sequence yet it also failed to decrease fluorescence as effectively as **5** or **6**. Previous reports have found that *retro*-inverting Aβ-binding sequences can massively increase their association rate and binding affinity to Aβ while decreasing their dissociation rate [32]. *Retro-inverso* analogues are also known sometimes to fail at adopting structures that mimic the functions of the natural peptide, however. The lack of activity displayed by **9** and **10** could be due to a number of reasons. One explanation is that the inverted hydrogen bonding patterns may yield compensatory adjustments in the peptides local conformation which may render the sequence incompetent in participating in the aggregation reaction. The fact that peptides **8**, **9** or **10** were not effective at decreasing ThT fluorescence suggests that the effects of *O*-GlcNAcylation are highly dependent on the structural properties of the recognition sequence.

Similar studies have been conducted using *O*-glycosylated tau fragments, explored for their capacities to inhibit truncated tau PHF6 aggregation. It was found that inhibition displayed by the glycopeptides was dependent upon the nature of the glycoside unit [33]. Further studies found that the extent to which glycosides on glycopeptides affect the aggregation of the native counterparts also largely depends on the scaffold to which they are linked [34]. Due to the ability of sugars to hydrogen bond with hydrophilic amino acid side chains, they may prove useful as agents for disrupting the self-interactions crucial for amyloid formation.

4. Concluding remarks

Glycosylation bears the capacity to modulate the structure, function and stability of a protein. *O*-GlcNAcylation, in particular, is involved in an array of chronic conditions characterised by protein misfolding and aggregation. Upregulation of *O*-GlcNAcylation is suggested to be a viable strategy for the treatment of these disorders. Here we provide the first evidence that *O*-GlcNAc-modified α-syn sequences may qualify as inhibitors of pathological α-syn aggregation characteristic of synucleopathies such as PD. Our results suggest that rationally designed glycopeptides, where glycosides are conjugated to the self-associative core of α-syn, may produce useful inhibitors of homogenous α-syn aggregation for therapeutic purposes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Financial support to SR from an Australian Research Council - Discovery Early Career Research Award (ARC DECRA: DE140101632) is gratefully acknowledged. PR received Griffith University Postgraduate Research Scholarship (GUPRS). This work is supported by Menzies Health Institute Queensland (MHIQ), Griffith University Collaborative Interdisciplinary Grants.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103389.

References

[1] A. Hartmann, Postmortem studies in Parkinson's disease, Dialogues Clin. Neurosci. 6 (2004) 281–293.
[2] C. Wang, C. Zhao, D. Li, Z. Tian, Y. Lai, J. Diao, C. Liu, Versatile structures of α-synuclein, Front. Mol. Neurosci. (2016) article 48.
[3] C. Peng, R.J. Gathagan, V.M.Y. Lee, Distinct α-synuclein strains and implications for heterogeneity among α-synucleinopathies, Neurobiol. Dis. 109 (2018) 209–218.
[4] R. Melki, Role of different α-synuclein strains in synucleinopathies, similarities with other neurodegenerative diseases, J. Parkinson's Dis. 5 (2015) 217–227.
[5] B. Falcon, M. Masuda-Suzukake, M. Goedert, Like prions: the propagation of aggregated tau and α-synuclein in neurodegeneration, Brain 140 (2016) 266–278.
[6] Jing L. Guo, Dustin J. Covell, Joshua P. Daniels, M. Iba, A. Stieber, B. Zhang, Dawn M. Riddle, Linda K. Kwong, Y. Xu, John Q. Trojanowski, Virginia M.Y. Lee, Distinct α-synuclein strains differentially promote tau inclusions in neurons, Cell 154 (2013) 103–117.
[7] D.K.Y. Chan, Y.H. Xu, L.K.M. Chan, N. Braidy, G.D. Mellick, Mini-review on initiatives to interfere with the propagation and clearance of alpha-synuclein in Parkinson's disease, Transl. Neurodegener. 6 (2017) article 33.
[8] P. Ryan, B. Patel, V. Makwana, H.R. Jadhav, M. Kiefel, A. Davey, T.A. Reekie, S. Rudrawar, M. Kassiou, Peptides, peptidomimetics, and carbohydrate–peptide conjugates as amyloidogenic aggregation anhibitors for Alzheimer's disease, ACS Chem. Neurosci. 9 (2018) 1530–1551.
[9] T.L. Lowe, A. Strzelec, L.L. Kiessling, R.M. Murphy, Structure-function relationships for inhibitors of beta-amyloid toxicity containing the recognition sequence KLVFF, Biochemistry 40 (2001) 7882–7889.
[10] V. Rangachari, Z.S. Davey, B. Healy, B.D. Moore, L.K. Sonoda, B. Cusack, G.M. Maharvi, A.H. Fauq, T.L. Rosenberry, Rationally designed dehydroalanine (ΔAla)-containing peptides inhibit amyloid-β (Aβ) peptide aggregation, Biopolymers 91 (2009) 456–465.
[11] J.J. Klein, S. Hecht, Synthesis of a new class of bis(thiourea)hydrazide pseudopeptides as potential inhibitors of beta-sheet aggregation, Org. Lett. 14 (2012) 330–333.
[12] T.J. Gibson, R.M. Murphy, Design of peptidyl compounds that affect β-amyloid aggregation: importance of surface tension and context, Biochemistry 44 (2005) 8898–8907.
[13] A.J. Mishizen-Eberz, E.H. Norris, B.I. Giasson, R. Hodara, H. Ischiropoulos, V.M. Lee, J.Q. Trojanowski, D.R. Lynch, Cleavage of α-synuclein by calpain: potential role in degradation of fibrillized and nitrated species of α-synuclein, Biochemistry 44 (2005) 7818–7829.
[14] D.P. Hong, W. Xiong, J.Y. Chang, C. Jiang, The role of the *C*-terminus of human alpha-synuclein: intra-disulfide bonds between the *C*-terminus and other regions stabilize non-fibrillar monomeric isomers, FEBS Lett. 585 (2011) 561–566.
[15] M. Diepenbroek, N. Casadei, H. Esmer, T.C. Saido, J. Takano, P.J. Kahle, R.A. Nixon, M.V. Rao, R. Melki, L. Pieri, S. Helling, K. Marcus, R. Krueger, E. Masliah, O. Riess, S. Nuber, Overexpression of the calpain-specific inhibitor calpastatin reduces human alpha-Synuclein processing, aggregation and synaptic impairment in [A30P]αSyn transgenic mice, Hum. Mol. Genet. 23 (2014) 3975–3989.
[16] S.J. Crocker, P.D. Smith, V. Jackson-Lewis, W.R. Lamba, S.P. Hayley, E. Grimm, S.M. Callaghan, R.S. Slack, E. Melloni, S. Przedborski, G.S. Robertson, H. Anisman, Z. Merali, D.S. Park, Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease, J. Neurosci. 23 (2003) 4081–4091.
[17] N.P. Marotta, Y.H. Lin, Y.E. Lewis, M.R. Ambroso, B.W. Zaro, M.T. Roth, D.B. Arnold, R. Langen, M.R. Pratt, *O*-GlcNAc modification blocks the aggregation and toxicity of the protein α-synuclein associated with Parkinson's disease, Nat. Chem. 7 (2015) 913–920.
[18] P.M. Levine, A. Galesic, A.T. Balana, A.L. Mahul-Mellier, M.X. Navarro, C.A. De Leon, H.A. Lashuel, M.R. Pratt, α-Synuclein *O*-GlcNAcylation alters aggregation and toxicity, revealing certain residues as potential inhibitors of Parkinson's disease, Proc. Natl. Acad. Sci. USA 116 (2019) 1511–1519.
[19] P. Ryan, M. Xu, A.K. Davey, J.J. Danon, G.D. Mellick, M. Kassiou, S. Rudrawar, *O*-GlcNAc modification protects against protein misfolding and aggregation in neurodegenerative disease, ACS Chem. Neurosci. 10 (2019) 2209–2221.
[20] N.P. Marotta, C.A. Cherwien, T. Abeywardana, M.R. Pratt, *O*-GlcNAc modification prevents peptide-dependent acceleration of α-synuclein aggregation, ChemBioChem 13 (2012) 2665–2670.
[21] P. De Bona, M.L. Giuffrida, F. Caraci, A. Copani, B. Pignataro, F. Attanasio, S. Cataldo, G. Pappalardo, E. Rizzarelli, Design and synthesis of new trehalose-conjugated pentapeptides as inhibitors of Aβ(1–42) fibrillogenesis and toxicity, J. Pept. Sci. 15 (2009) 220–228.
[22] M.J. Volles, P.T. Lansbury Jr., Relationships between the sequence of alpha-synuclein and its membrane affinity, fibrillization propensity, and yeast toxicity, J. Mol. Biol. 366 (2007) 1510–1522.
[23] J. Pujols, S. Peña-Díaz, M. Conde-Giménez, F. Pinheiro, S. Navarro, J. Sancho, S. Ventura, High-throughput screening methodology to identify alpha-synuclein aggregation inhibitors, Int. J. Mol. Sci. 18 (2017) article 478.
[24] F. Rahimi, P. Maiti, G. Bitan, Photo-induced cross-linking of unmodified proteins (PICUP) applied to amyloidogenic peptides, J. Vis. Exp. 23 (2009) e1071.

[25] E. Coelho-Cerqueira, A.S. Pinheiro, C. Follmer, Pitfalls associated with the use of thioflavin-T to monitor anti-fibrillogenic activity, Bioorg. Med. Chem. Lett. 24 (2014) 3194–3198.
[26] T. Becker, A. Kaiser, H. Kunz, Synthesis of dendrimeric tumor-associated mucin-type glycopeptide antigens, Synthesis 2009 (2009) 1113–1122.
[27] A. Di Fenza, M. Tancredi, C. Galoppini, P. Rovero, Racemization studies of Fmoc-Ser(tBu)-OH during stepwise continuous-flow solid-phase peptide synthesis, Tetrahedron Lett. 39 (1998) 8529–8532.
[28] Y. Zhang, S.M. Muthana, D. Farnsworth, O. Ludek, K. Adams, J.J. Barchi Jr., J.C. Gildersleeve, Enhanced epimerization of glycosylated amino acids during solid-phase peptide synthesis, J. Am. Chem. Soc. 134 (2012) 6316–6325.
[29] L.B. Lassen, E. Gregersen, A.K. Isager, C. Betzer, R.H. Kofoed, P.H. Jensen, ELISA method to detect α-synuclein oligomers in cell and animal models, PLoS One 13 (2018) e0196056.
[30] M. Masuda, M. Hasegawa, T. Nonaka, T. Oikawa, M. Yonetani, Y. Yamaguchi, K. Kato, S.-I. Hisanaga, M. Goedert, Inhibition of α-synuclein fibril assembly by small molecules: analysis using epitope-specific antibodies, FEBS Lett. 583 (2009) 787–791.
[31] R.J. Chalifour, R.W. McLaughlin, L. Lavoie, C. Morissette, N. Tremblay, M. Boulé, P. Sarazin, D. Stéa, D. Lacombe, P. Tremblay, F. Gervais, Stereoselective interactions of peptide inhibitors with the β-amyloid peptide, J. Biol. Chem. 278 (2003) 34874–34881.
[32] B. Matharu, O. El-Agnaf, A. Razvi, B.M. Austen, Development of retro-inverso peptides as anti-aggregation drugs for beta-amyloid in Alzheimer's disease, Peptides 31 (2010) 1866–1872.
[33] M. Frenkel-Pinter, M. Richman, A. Belostozky, A. Abu-Mokh, E. Gazit, S. Rahimipour, D. Segal, Selective inhibition of aggregation and toxicity of a tau-derived peptide using its glycosylated analogues, Chem. Eur. J. 22 (2016) 5945–5952.
[34] M. Frenkel-Pinter, M. Richman, A. Belostozky, A. Abu-Mokh, E. Gazit, S. Rahimipour, D. Segal, Distinct effects of *O*-GlcNAcylation and phosphorylation of a tau-derived amyloid peptide on aggregation of the native peptide, Chem. Eur. J. 24 (2018) 14039–14043.