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Incorporating a β-hairpin sequence motif to increase intracellular stability of a peptide-based PROTAC

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ABSTRACT

Proteolysis targeting chimeras (PROTACs) have emerged as a new class of therapeutics that utilize the ubiquitin-proteasome system (UPS) to facilitate proteasomal degradation of "undruggable" targets. Peptide-based PROTACs contain three essential components: a binding motif for the target protein, a short amino acid sequence recognized by an E3 ligase called a degron, and a cell penetrating peptide to facilitate uptake into intact cells. While peptide-based PROTACs have been shown to successfully degrade numerous targets, they have often been found to exhibit low cell permeability and high protease susceptibility. Prior work identified peptides containing a β-hairpin sequence motif that function not only as protecting elements, but also as CPPs and degrons. The goal of this study was to investigate if a β-hairpin sequence could replace commonly used unstructured peptides sequences as the degron and the CPP needed for PROTAC uptake and function. The degradation of the protein Tau was selected as a model system as several published works have identified a Tau binding element that could easily be conjugated to the β-hairpin sequence. A series of time- and concentration-dependent studies confirmed that the β-hairpin sequence was an adequate alternative CPP and degron to facilitate the proteasome-mediated degradation of Tau. Microscopy studies confirmed the time-dependent uptake of the PROTAC and a degradation assay confirmed that the β-hairpin conjugated PROTAC had a greater lifetime in cells.

1. Introduction

Drug discovery typically focuses on controlling protein activity by developing inhibitors for what is known as occupancy-driven pharmacology. The efficacy of these compounds, however, is diminished by off-target effects that result from the high concentrations necessary for the maximum effect of the inhibitor [1]. Additionally, drug targets are often focused on proteins with accessible active and regulatory sites, which can be selectively targeted against structurally similar proteins. This severely limits the potential target proteins which can be considered "druggable" [2]. Proteolysis targeting chimeras (PROTACs) have emerged as a novel approach that aims to eliminate aberrantly functioning proteins rather than inhibiting them by utilizing the ubiquitination proteasome system (UPS) to selectively degrade target proteins. The UPS involves a cascade of enzymes which begins when ubiquitin binds to an E1 ubiquitin activating enzyme. The ubiquitin is eventually transferred to the protein of interest to form a polyubiquitin chain so that it can be recognized and degraded by the proteasome [3]. PROTACs are bifunctional molecules that consist of a target binding moiety and a specific amino acid degradation sequence (commonly referred to as a degron) which is recognized by, and binds to, an E3 ligase to facilitate proteasomal degradation [4,5]. The first cell-permeable PROTACs exploited the interaction between hypoxia-inducible factor-1a (HIF-1α) and the von Hippel-Lindau E3 ligase (VHL). Schneekloth et al. developed a PROTAC using a HIF-1α derived heptapeptide (ALAPYIP) as the degron for their PROTAC and a poly-D-arginine cell penetrating peptide (CPP) sequence to facilitate entry into intact cells [6]. Peptide-based PROTACs have been used to target various proteins including α-synuclein [7], AR [6], β-catenin [8], ERα [9], and Tau [10,11]. They typically consist of three main components: (1) a target recognition sequence, (2) a degron sequence to recruit the E3 ligase, and (3) a cell penetrating peptide (CPP). Despite their success, peptide-based PROTACs often suffer from low cell permeability and high protease susceptibility which has resulted in a transition to small-molecule-based PROTACs [1]. However, most PROTACs which incorporate small molecules as the targeting warhead heavily rely on the binding pockets of the target protein and are thus limited in their ability to target "undruggable" proteins [12]. Therefore, exploring strategies to overcome the

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limitations of peptide-based PROTACs could expand the potential number of proteins which can be targeted by PROTAC degradation.

Peptide therapeutics typically demonstrate higher potency and lower toxicity compared to small-molecules; however, they have historically been limited by their instability and membrane impermeability [13]. Several strategies have been studied to address these weaknesses including incorporating protease-resistant non-natural amino acids [14] or by modifying the primary structure through cyclization or peptide stapling [15–17]. Recently, peptide-based PROTACs have begun incorporating these strategies to improve their efficacy. Jiang et al. developed a PROTAC using a binding sequence for ERα which was constrained into a helical structure [9]. The constrained PROTACs demonstrated greater uptake compared to linear PROTACs. Similarly, Liao et al. incorporated a stapled peptide that binds to β-catenin to develop a novel PROTAC with improved permeability and stability [8]. Incorporation of secondary structures, however, has mainly been limited to the binding sequence for the protein of interest, limiting their use to only the intended target. Additionally, several peptide-based PROTACs have utilized D-chirality arginines as a CPP, however they are typically combined with both target binding sequences and degrons that are easily degraded due to their unstructured, L-chirality sequences [6,10]. Additionally, D-chirality CPPs have been shown to be expelled from intact cells, potentially limiting the overall impact of the poly-D-arginine CPP [14]. To date, there have been no attempts to incorporate stabilizing features into the degron portion of the PROTAC. Previously, a library of peptides known as β-hairpin 'protectides' incorporating a D-Pro-Gly β-turn to establish secondary structure have been shown to provide increased protease resistance compared to unstructured peptides [18]. Houston et al. previously identified ornithine-containing peptides containing a β-hairpin sequence motif that function as degrons [19]. While the specific E3 ligase the peptides bind to is unknown, ubiquitination was significantly impaired by inhibitions of the SCF family of E3 ligases. Recently, Safa et al. demonstrated the ability of these β-hairpin protectides to function as cell penetrating peptides with enhanced stability, entering intact cells within 10 min with a half-life of over 400 min [20].

This work aims to explore if β-hairpin protectides could replace the degron and CPP in a peptide-based PROTAC to enhance intracellular stability and cellular permeability. The protein Tau was chosen as the target as it has previously been shown to be susceptible to peptide-based PROTAC degradation [10,11]. Tau is a protein that plays a role in mediating axonal transport, synaptic structure, and neuronal signaling pathways. Aggregations of Tau, however, have become a characteristic occurrence in Alzheimer's disease, increasing its prevalence as a therapeutic target [21]. The binding sequence which has previously been used in peptide-based PROTACs is derived from β-tubulin which was known to interact with Tau [22]. The previously identified Tau binding sequence was combined with two different β-hairpin sequences to create peptide-based PROTACs which were shown to facilitate the degradation of Tau in both a time and concentration dependent manner. This degradation was shown to be dependent on both the E1 enzyme and proteasome activity confirming their proposed function as a PROTAC. The β-hairpin containing PROTAC had a minimal effect on cell viability and, most importantly, the structured PROTAC demonstrated greater stability compared to the previously established unstructured version as well as time-dependent uptake into intact cells.

2. Materials and methods

2.1. Chemicals and reagents

Fmoc-protected amino acids, 2-(6-Chloro-1 H-benzotriazole-1-yl)−1, − 1,3,3-tetramethylaminium hexafluorophosphate (HCTU), trifluoroacetic acid (TFA), and rink amide SS resin, were purchased from Advance ChemTech, Louisville, KY, USA. Nα-Fmoc-Nδ-allyloxycabonyl-L-ornithine (Fmoc-Orn[Aloc]-OH) was purchased from Chem-Impex International, Inc, Wood Dale, IL, USA. 1-Hydroxy-6-(trifluoromethyl) benzotriazole (HOBt) and (Ethyl cyano[hydroxyimino]acetato)-tri-(1-pyrrolidinyl)-phosphonium (PyBOP) were purchased from Novabiochem, Billerica, MA, USA. Dimethylformamide (DMF) was purchased from Protein Technologies, Tucson, AZ, USA. Diisopropylethylamine (DIEA), triisopropylsilane (TIPS), tetrakis(triphenylphosphine) palladium (0) (palladium), 5(6)-carboxyfluorescein (FAM), chloroform ($CHCl_3$), methanol (MeOH), dichloromethane (DCM), and N-methylmorpholine (NMM) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Glacial acetic acid was purchased from Alfa Aesar, Ward Hill, MA, USA. Acetic anhydride was purchased from Fisher Scientific, Fair Lawn, NJ, USA. All the salts used for the preparation of assay buffers were purchased from Sigma Aldrich, St. Louis, MO, USA.

2.2. Peptide synthesis and purification

Peptides were synthesized as previously described by Safa et al. [20]. All peptides were synthesized on a Tribute peptide synthesizer (Protein Technologies, Tucson, AZ, USA) using a standard Fmoc peptide chemistry protocol on a 100 μmol scale using rink amide resin (189 mg, 0.53 mmol/g). Fivefold excess of Fmoc-amino acids (Fmoc-Arg(Pbf)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Val-OH, Fmoc-Orn-OH, Fmoc-D-Pro-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Gln(Trt)-OH, Fmoc-Orn(Aloc)-OH, and HCTU) in the presence of 10 equivalents of NMM were used for each of the amino acid coupling steps (10 min) with N-methyl-2-pyrrolidone (NMP) as the solvent. A solution of acetic anhydride, NMM, and NMP (1:1:3) was added to the deprotected resin and shaken for 30 min to acetylate the N terminus of the peptide. Once the peptide synthesis was complete, the resin was washed with DMF (3 × 30 s) and then DCM (3 × 30 s). The Aloc group was removed with 3-fold excess of palladium in 4 mL of CHCl3-HOAc-NMM (37:2:1) under nitrogen for 2 h. The resin was then washed with DCM (3 × 30 s) followed by DMF (3 × 30 s). In the dark, FAM was coupled to the delta nitrogen of the ornithine side chain with 4-fold excess of FAM, HOBt, PyBOP, and DIEA in 3 mL DMF for 24 h and then repeated for 8 h. The peptide resin was washed with DMF (3 × 30 s) and DCM (3 × 30 s). The peptide was cleaved from the resin and sidechain deprotected using TFA/water/ TIPS (4 mL, 95:2.5:2.5) for 3 h and collected in a 50-mL centrifuge tube. The cleavage reaction was repeated for 10 min. The cleavage solutions for the peptide were combined and concentrated in vacuo. Cold diethyl ether was then added to the peptide solution to precipitate the crude peptide. The peptide was centrifuged for 10 min (4000 rpm) and the ether layer decanted. Fresh cold diethyl ether was added, and the pelleted peptide was resuspended. The peptide was centrifuged again, and the procedure was repeated 5 times in total. After the final ether wash, the peptide pellet was dissolved in 5 mL water containing 0.1% TFA, frozen, and lyophilized.

High-performance liquid chromatography (HPLC) analysis was performed with a Waters 616 pump, Waters 2707 Autosampler, and 996 Photodiode Assay Detector which were controlled byWaters (Milford, MA, USA) Empower 2 software. The separation was performed on an Agilent (Santa Clara, CA, USA) Zorbax 300SB-C18 (5 μm, 4.6 × 250 mm) with an Agilent guard column Zorbax 300SB-C18 (5 μm, 4.6 × 12.5 mm). Elution was done with a linear 5–55% gradient of solvent B (0.1% TFA in acetonitrile) into A (0.1% TFA in water) over 50 min at a 1 mL/min flow rate with UV detection at 442 nm. Preparative HPLC runs were performed with a Waters prep LC Controller, Waters Sample Injector, and 2489 UV/Visible Detector which are controlled by Waters (Milford, MA, USA) Empower 2 software. The separation was performed on an Agilent Zorbax 300SBC18 PrepHT column (7 μm, 21.2 × 250 mm) with Zorbax 300SB-C18 PrepHT guard column (7 μm, 21.2 × 10 mm) using a linear 5–55% gradient of solvent B (0.1% TFA in acetonitrile) into A (0.1% TFA in water) over 50 min at a 20 mL/min flow rate with UV detection at 215 nm. Fractions of high (>95%) HPLC purity of each peptide and with the expected mass were combined and lyophilized. Representative HPLC purification and mass spectrometry validation of the peptide-based PROTAC containing a β-hairpin sequence are shown

in Supplementary Fig. S1.

2.3. Circular Dichroism

Peptides were tagged with 5,6-carboxyfluoroscein (FAM) and concentrations were determined based on the absorbance of FAM at 492 nm. Circular dichroism (CD) spectroscopy data were collected as previously described by Safa et al. [20] using a J-815 CD spectrometer (JASCO, Easton, MD, USA). Spectra were generated at 25 °C with a wavelength scan (260–185 nm) using a 50 nm/min scanning speed in a 0.1 cm cell. Data pitch and accumulation were 1 and 3 nm, respectively. The scan mode was continuous. The smoothing method was Savitzky–Golay with a convolution width of 7. All peptides were at a final concentration of 40 μM in a 10 mM sodium phosphate buffer.

2.4. Cell Culture and Lysate Generation

N2a cells (ATCC) were maintained in Minimum Essential Medium (MEM, Corning Inc., Corning, NY, USA) supplemented with 10% *v/v* HyClone Cosmic Calf Serum (VWR Life Sciences Seradigm). N2a lysates were generated by detaching cells with trypsin, resuspending in media, and pelleting the cells. The pellet was washed twice in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, and 1.75 mM KH_2PO_4 at pH 7.4). The pellet was then resuspended in an approximately equivalent volume of mammalian protein extraction reagent (ThermoFisher Scientific, Carlsbad, CA, USA) to the volume of the cell pellet, then vortexed for 10 min at 900 rcf. The samples were centrifuged at 14,000 rcf for 15 min at 4 °C. The supernatant was transferred to a new microcentrifuge tube and stored on ice until use. Total protein concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Madison, WI, USA).

2.5. Western Blotting Analysis

Two days prior to the experiment, N2a cells were seeded at a density of 5×10^5 cells/mL in 60 mm dishes. Peptide concentration was determined using the UV–vis function on a NanoDrop (Thermo Scientific). Peptide stocks were diluted in 6 M Guanidine hydrochloride and absorbance was measured at 280 nm (Trp $\varepsilon_{280} = 5690\ M^{-1}\ cm^{-1}$, Tyr $\varepsilon_{280} = 1490\ M^{-1}\ cm^{-1}$). On the day of the experiment, peptides were diluted to the desired final concentration with extracellular buffer (ECB; 5.036 mM HEPES pH 7.4, 136.89 mM NaCl, 2.68 mM KCl, 2.066 mM $MgCl_2{\cdot}6\ H_2O$, 1.8 mM $CaCl_2{\cdot}2\ H_2O$, 5.55 mM glucose). For concentration dependent evaluation of PROTAC efficacy, peptide solutions of various concentrations were incubated with the cells for 12 h at 37 °C. For time dependent evaluation of PROTAC efficacy, 200 μM peptide solutions were incubated with cells for 6, 12, 18, and 24 h at 37 °C. For experiments studying the effect of proteasome inhibition, cells were incubated with 200 μM of peptide for 24 h. 10 μM MG-132 was added to the cell cultures 6 h before harvest as described by Chu et al. [10]. For experiments studying E1 enzyme inhibition, cells were pre-treated with 10 μM PYR-41 for one hour, then co-incubated with 200 μM of peptide for 12 h. At the end of the desired peptide incubation times, cells were lysed with M-PER buffer (Thermo Fisher) containing protease inhibitor cocktail. Lysates were passed through a 23 G needle 5 times then centrifuged for 10 min at 14000 RCF and 4 °C. The protein concentration of the supernatant was quantified using a nano-drop. 50 μg of protein was separated using 4–15% Mini PROTEAN TGX Stain-Free Tris-Glycine gels (Bio-Rad), then transferred to nitrocellulose membranes at 300 mA for 2.5 h. Primary antibody incubation occurred overnight at 4 °C with the following antibodies: Tau (4019 S, Cell Signaling Technologies) at a 1:1000 dilution and β-actin (3700 S, Cell Signaling Technologies) at a 1:5000 dilution. Horseradish peroxidase-conjugated anti-mouse secondary antibody (115–03–003, JacksonImmuno Research) was incubated with the membrane for 45 min at room temperature. Chemiluminescent images were recorded using the ChemiDoc Imaging system (BioRad). Band intensity was quantified using ImageJ software and results were normalized by dividing the intensity of Tau bands by the corresponding β-actin bands.

2.6. Cell viability assay

Two days prior to the experiment, N2a cells were plated at a density of 1×10^4 cells/well in a 96 well plate. Cells were washed once with PBS and incubated with peptide solutions for 24 h at 37 °C. Following this incubation period, cells were washed with ECB and incubated with a mixture of 10 μL alamarBlue Viability Reagent (ThermoFisher Scientific, Carlsbad, CA, USA) in 90 μL serum-free media for 4 h at 37 °C. The fluorescent signal of alamarBlue was measured using fluorometry with an excitation filter of 532 nm and an emission filter of 535 nm. A no peptide control was also performed to normalize the fluorescent signals. Signals were normalized using Eq. (1):

$$Normalized\ Cell\ Viability = F/C \tag{1}$$

where F denotes the fluorescent signal of cells incubated with peptide and C denotes the fluorescent signal of cells incubated with no peptide. The reported data are the average of triplicate samples.

2.7. Fluorescent microscopy

One day prior to the experiment, N2a cells were seeded in 8-chambered Falcon Culture Slides (Corning) at a density of 2×10^4 cells/mL. On the day of the experiment, cells were washed once with ECB before incubation with 50 μM peptide solution fluorescently tagged with FAM (500 μL/well) for indicated time points at 37 °C. Following incubation, cells were washed twice with ECB then incubated with 8 μM Hoechst 3342 nuclei acid stain (ThermoFisher Scientific, Carlsbad, CA, USA) for 20 min at room temperature. Cellular fluorescence was visualized using a Leica DMi8 inverted microscope outfitted with a 40 × objective (Leica N PLAN L, 0.55 × correction). Images were acquired using the digital CMOS camera C11440 (Hamamatsu Photonics K.K.) with a fixed exposure time of 150 ms for the FITC filter, 100 ms for the DAPI filter, and 30 ms for brightfield. The following excitation/emission filters (Chroma Tech. Corp, Bellows Falls, VT, USA) were used to image the device: fluorescein isothiocyanate-FITC (λ_{ex} 440–520 nm and λ_{em} 497–557 nm) for capturing the signal from FAM and DAPI (λ_{ex} 335–385 nm and λ_{em} 405–465 nm) for capturing the signal from the Hoescht stain. Image acquisition was controlled using the Leica Application Suite software (LAS X, version 3.7.4.23463), where all images were recorded using the same parameters. The imaging was followed by quantitative analysis, where a manual line scan region of interest (ROI) was drawn across the center of the cells to quantify their fluorescent signals. Random ROIs were also drawn in free space not containing cells to measure background noise for each image. The fluorescent values were normalized by subtracting the noise from the measured signal.

2.8. Peptide degradation assay

Briefly, 30 μM of respective FAM-tagged peptide was incubated in N2a lysates diluted to a total protein concentration of 2 mg/mL in an assay buffer (10 mM sodium phosphate buffer, 100 mM NaCl, pH 7.6) at 37 °C in the dark. Aliquots of the reaction mixture were removed at set intervals, at which point further peptidase activity was quenched by heating the aliquots at 90 °C for 5 min followed by immediately freezing in liquid nitrogen and then storage at − 20 °C until analysis by HPLC. The zero-minute time point measurements were made using lysates that were heat killed immediately after peptide incubation. HPLC analysis was performed with a Waters 616 pump, Waters 2707 Autosampler, and 996 Photodiode Assay Detector, which are controlled by Waters Empower 2 software. The separation was performed on an Agilent Zorbax 300SB-C18 (5 μm, 4.6 × 250 mm) with an Agilent guard column

Zorbax 300SB-C18 (5 μm, 4.6 × 12.5 mm). Elution was done with a gradient resulting from mixing eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile). The gradient started from 5% B to 50% B in 45 min. The flow rate was 1.0 mL/min and the detected wavelength was 442 nm. Injection volume was 40 μL. A sample chromatogram for the degradation of Peptide 2 is shown in Supplementary Fig. S2. Peak areas were calculated by the Waters Empower 2 software by integration of peaks identified using a peak width of 30.00 and a peak threshold of 50.00. Percent intact peptide remaining was calculated by dividing the area of the parent peptide peak at each time point divided by the area of the parent peptide peak at the zero-minute time point. The identity of the parent peptide peak was confirmed using the parent peptide alone and verified with the t = 0-min chromatogram.

3. Results and discussion

3.1. Design and characterization of peptide-based PROTACs incorporating a β-hairpin sequence motif

To explore if the degron and CPP could be replaced by a single moiety, a library of PROTACs was synthesized incorporating peptides characterized by their β-hairpin secondary structure. The degron/CPP sequence was combined with an established binding sequence for the protein Tau (YQQYQDATADEQG), which has been previously shown to be susceptible to.

PROTAC-mediated degradation [10,11]. The library of PROTACs was synthesized by combining two variations of the β-hairpin sequences (RWRWR: RWVRWpGRWIRQ and OWRWR: OWVRVpGRWIRQ) with the binding moiety for Tau (Table 1). The degron/CPP sequence was synthesized on both the N- and C-terminus of the Tau binding sequence to determine if position affected the efficacy of the PROTAC. These PROTACs were compared to the previously reported unstructured PROTAC by Chu and colleagues, herein called Peptide 1, which includes the Tau binding sequence on the N-terminus of the peptide, a short linker sequence (GSGS) for increases flexibility, a degron to recruit the VHL E3 ligase (ALAPYIP) and a poly-D-arginine CPP (rrrrrrrr) [10]. Circular dichroism (CD) was used to confirm the β-hairpin PROTAC retained its secondary structure when the target binding sequence was added by comparing the similar shape of the CD spectra as the RWRWR sequence alone (Supplementary Fig. S3). The minimum is slightly shifted from 205 towards 200 nm, due to the presence of the unstructured Tau binding sequence [23]. However, the maximum at 226 nm which is likely due to the contribution of Trp-Trp excitation coupling between the sidechains is retained [18]. This confirms that the addition of the Tau recognition sequence does not alter the structure or stability of the β-hairpin sequence motif.

3.2. PROTACs containing a β-hairpin sequence motif induce concentration and time dependent degradation of Tau

To assess the functionality of the β-hairpin PROTACs, protein levels of Tau were analyzed by western blotting using both concentration and time dependent studies. For concentration dependent studies, N2a cells were treated with 0–200 μM of peptide solutions with the harvested lysates being analyzed with western blotting (Fig. 1). Peptide 1, the previously reported PROTAC, showed a decrease in Tau signal; however, the observed degradation was less potent than previously reported [10]. Peptides 2 and 3, which placed the β-hairpin sequence on the N-terminus of the Tau binding sequence, demonstrated concentration dependent degradation of Tau at levels superior to Peptide 1. Interestingly, Peptides 4 and 5, which placed the β-hairpin sequence on the C-terminus of the Tau binding sequence, showed no obvious degradation of Tau. This lack of degradation suggests that placement of the degron/CPP element is a key design consideration for peptide-based PROTACs. One possible explanation for the differences in Tau degradation could be the location of the CPP element. Kheradmand-Hajibashi et al. observed a difference in peptide uptake when a model cargo was fused to the N- or C- terminus [24]. Alternatively, placing the degron/CPP sequence on the C-terminus of the PROTAC may result in insufficient interaction with the Tau protein to facilitate ubiquitination. With an estimated 600 different E3 ligases with varying activity, degrons play an integral role in ensuring ubiquitylation occurs at the right time and place. Degron specificity is vital for proper function of the E3 ligase to facilitate the transfer of ubiquitin to a proximal lysine residue on the protein of interest [25,26]. This is in stark contrast to Peptide 1 which positioned the Tau recognition sequence at the N-terminus. This difference in PROTAC function can be attributed to the different E3 ligases used to facilitate Tau ubiquitination. For time dependent studies, only the PROTACs which had previously demonstrated concentration dependent degradation of Tau (Peptides 1, 2, and 3) were tested. Cells were treated with 200 μM PROTAC for up to 24 h before analyzing with western blotting (Fig. 2). All three peptides tested demonstrated time-dependent degradation of Tau. Overall, these time and concentration dependent studies confirm the potential of using the β-hairpin motif in next generation PROTACs based on the knockdown of the Tau protein. The enhanced degradation by Peptide 2 suggests that the RWRWR sequence provides a greater degree of Tau degradation when compared to the OWRWR sequence. This could be attributed to the fact that RWRWR exhibits enhanced uptake kinetics to OWRWR, likely due to the additional arginine residue in the former [20]. Additionally, the ornithine residue in the OWRWR sequence may affect the degradation efficiency of Peptide 3. The structure of ornithine differs from that of lysine, to which ubiquitin typically binds, by one CH_2 group on the amino acid side chain. Previous studies have demonstrated the potential self-ubiquitination of OWRWR, which may limit the ubiquitination of Tau by Peptide 3 [19]. RWRWR cannot be self-ubiquitinated, which may also contribute to the enhanced degradation by Peptide 2. One interesting finding is the limited degradation of Peptide 1 which was previously shown to be an effective PROTAC for Tau. One possible explanation for this discrepancy is that the prior studies characterizing Peptide 1 utilized N2a cells that were genetically modified to overexpress the Tau protein [10]. In a previous study, Wang et al. used a small molecule-based PROTAC to degrade Tau in a cell line with endogenously expressed Tau. Their study demonstrated that the potency of their PROTAC appeared much weaker compared to cells which were genetically modified to overexpress Tau [27]. Additionally, Lu et al. developed a similar peptide-based PROTAC, exchanging the HIF-1α degron in Peptide 1 with a degron to recruit the Keap1 E3 ligase [11]. This linear peptide was also found to degrade Tau in cells modified to overexpress the protein. Thus, the overexpression of Tau was likely necessary to achieve significant degradation of Peptide 1. This can potentially be attributed to poor intracellular stability of the unstructured PROTAC or weaker uptake kinetics. Moreover, the use of the D-chirality CPP could result in the removal of the PROTAC from the intact cells as prior work by Vaithiyanathan et al. showed that D-chirality CPPs were expelled from intact cells [14].

Table 1
Names and sequences of peptides. Lowercase letters are D-chirality.

Name	Sequence
Peptide 1	YQQYQDATADEQG-GSGS-ALAYIP-rrrrrrrr
Peptide 2	RWVRVpGRWIRQ -YQQYQDATADEQG
Peptide 3	OWVRVpGRWIRQ -YQQYQDATADEQG
Peptide 4	YQQYQDATADEQG- RWVRVpGRWIRQ
Peptide 5	YQQYQDATADEQG-OWVRVpGRWIRQ

3.3. PROTACs have a minimal effect on cellular viability

The desired effect of a PROTAC on cell viability often depends on what is being targeted. For PROTACs being used as therapeutics for cancers, degradation of the target protein often leads to inhibiting proliferation of cancer cells [28,29]. However, in the treatment of

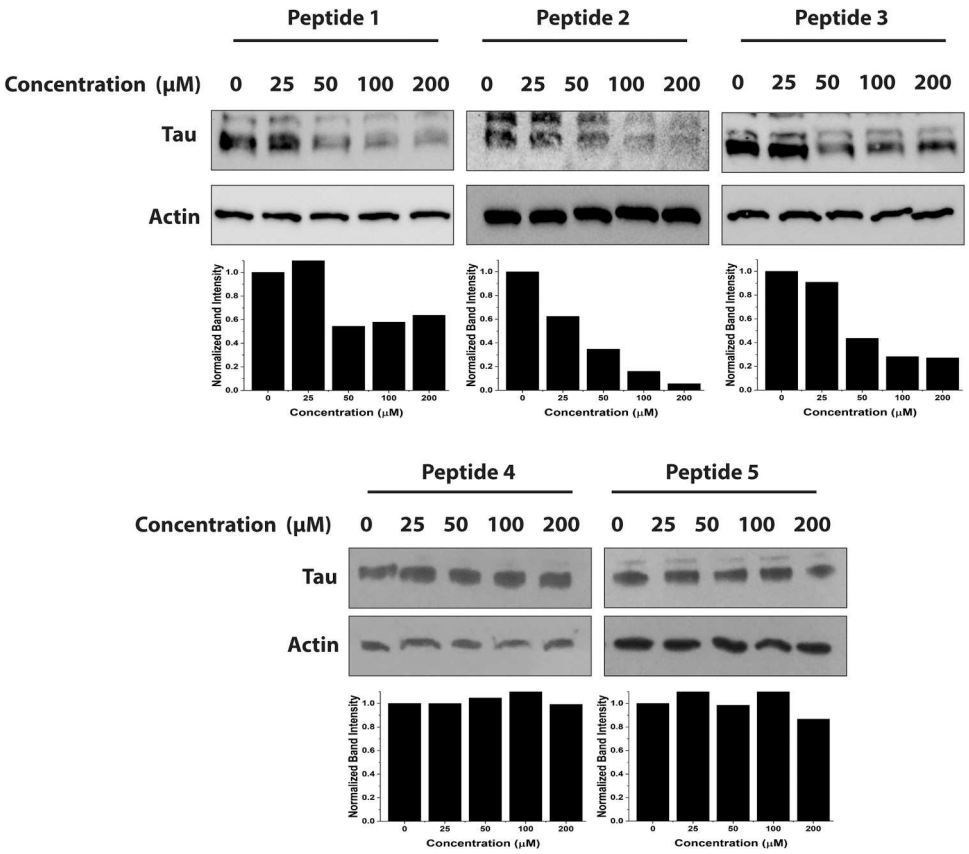

Fig. 1. Concentration-Dependent degradation of Tau. N2a cells were treated with increasing concentrations of PROTACs for 12 h. Lysates were analyzed with western blotting. Band intensity was quantified with ImageJ and Tau bands were normalized against Actin bands.

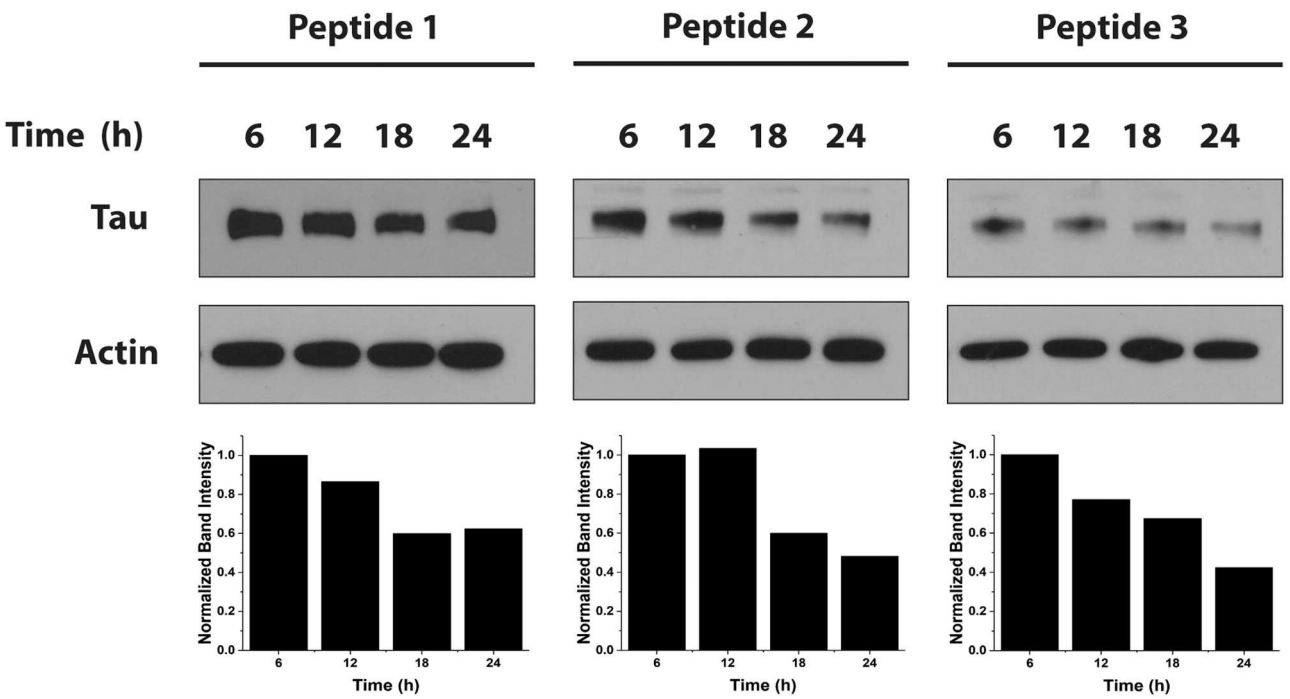

Fig. 2. Time-dependent Degradation of Tau. N2a cells were treated with 200 μM of PROTACs for up to 24 h. Lysates were analyzed with western blotting. Band intensity was quantified with ImageJ and Tau bands were normalized against Actin bands.

neurodegenerative diseases such as Alzheimer's, toxicity of therapeutics has historically been a limitation in the development of new treatments [30]. A major advantage of peptide therapeutics over small molecules is their lower toxicity in large part because they are degraded into amino acids [31]. Previous studies using peptide-based PROTACs to knock-down Tau have observed minimal effects on cell viability [10]. An alamarBlue study was used to determine if the β-hairpin PROTACs diminished cellular viability. Cells were incubated with the peptides at increasing concentrations for 24 h. All three peptides tested demonstrated a relatively small toxicity to N2a cells when tested with an alamarBlue stain (Fig. 3). For all three PROTACs, only 10% of the cells died with 200 μM peptide solution, similar to the previously reported viability studies for Peptide 1 [10]. While many peptide-based PROTACs have been developed as cancer treatments and thus result in decreased proliferation of cells, a previously reported peptide-based PROTAC developed as a treatment for liver fibrosis reported no significant decrease in cell viability after 24 h of treatment [32]. Furthermore, Qu et al. demonstrated that a peptide-based PROTAC designed to degrade α-synuclein for the treatment of Parkinson's disease was not only non-toxic but could rescue the decrease in cell viability that was observed in cells which overexpressed the target protein [7].

3.4. Incorporation of a secondary structure increases intracellular peptide stability

The intracellular instability of peptides is a significant limitation in the design of peptide-based PROTACs. Recently, Wang et al. developed a peptide-based PROTAC to degrade the E3 ligase Keap1 [32]. The PROTAC was found to induce degradation of the protein during the initial four hours of incubation; however, it became ineffective as the length of the incubation period increased, likely due to the low half-life of the peptide PROTAC. Several strategies exist to overcome these half-life limitations, including modifying the peptide structure. The RWRWR sequence has previously been shown to enhance stability compared to its linear counterpart [20]. To examine how the addition of a linear binding sequence affects the stability of the PROTAC, a degradation assay was performed by incubating peptides with N2a lysates to mimic the intracellular environment (Fig. 4). At each time point, further peptidase activity was quenched with a heat shock, and samples were analyzed using HPLC. Percent intact remaining peptide was determined by dividing the area of the peptide peak at each time point by the area of the peptide peak at the zero-minute time point. Peptides 1 and 2 were evaluated, as

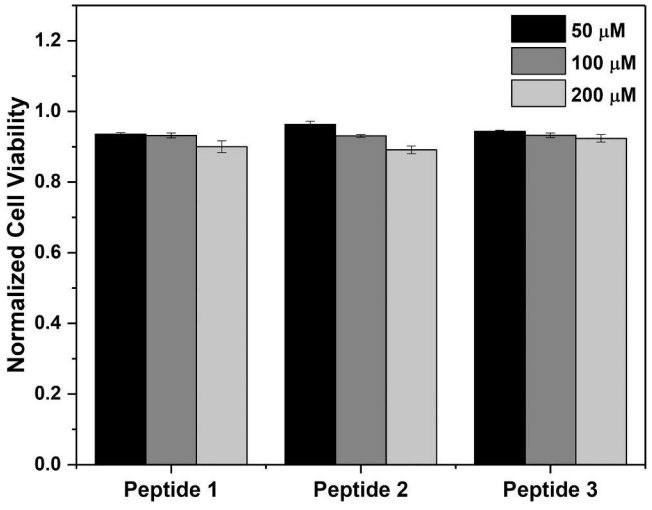

Fig. 3. PROTACs Have Minimal Effect on Cell Viability. N2a cells were incubated with various concentrations of peptide for 24 h. An alamarBlue assay was used to measure cell viability. All peptides demonstrated less than 10% decrease in viability.

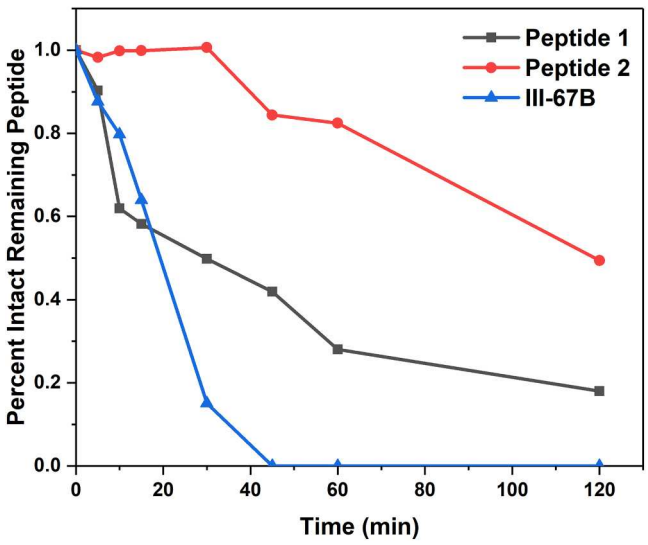

Fig. 4. Analysis of Peptide degradation in N2a Lysates. The stability of the peptides was evaluated by incubation with 2 mg/mL N2a lysates at 37 °C. The structured Peptide 2 (red circles) was more resistant to degradation than its unstructured counterpart Peptide 1 (black squares) as evidenced by a greater amount of intact peptide. An unstructured, rapidly degraded peptide (III-67B) was used as a positive control to demonstrate the activity of the N2a lysates (blue triangles). The zero-minute time point was achieved using heat shocked lysates and served as the baseline for intact peptide.

well as an unstructured positive control peptide, III-67B (GGAYAAPFKKKA). III-67B has previously been shown to rapidly degrade under cytosolic conditions [18]. As expected, this control peptide was fully degraded within 45 min. After two hours, approximately 20% of Peptide 1 was remaining, likely due to the unstructured Tau binding and degron sequences. In contrast, Peptide 2, which replaces both the degron and CPP with a structured sequence, was found to have approximately 50% of the peptide remaining. Thus, despite the incorporation of unnatural amino acids, Peptide 1 is found to have less cytosolic stability, possibly due to the additional linear degron sequence compared to Peptide 2 or the position in which the amino acids targeted by peptidases are located within the sequence [18]. However, the addition of the unstructured Tau binding motif does diminish the stability of Peptide 2 compared to the RWRWR peptide alone, which was previously reported to have approximately 80% peptide remaining after 2 h [20]. It is also worth noting that the PROTAC lifetime demonstrated here is shorter than which would be observed in intact cells. Proctor et al. demonstrated that the rate of peptide metabolism in cell lysates is significantly greater than in intact cells most likely due to the release of peptidases from ruptured organelles in the lysate [33]. However, lysates are used due to the sensitivity of the HPLC which is used to track protein degradation [34].

3.5. Peptide 2 exhibits time-dependent uptake with a uniform fluorescent distribution

While the RWRWR CPP was observed to rapidly enter intact cells, previous studies have shown the addition of cargo can change the uptake of the peptide [35]. To characterize the ability of Peptide 2 to penetrate cells, a fluorescent version of the peptide was synthesized by labeling it with 5,6-carboxyfluoroscein (FAM). Live-cell microscopy was used to visualize internalization of Peptide 2 over time. N2a cells were incubated with 50 μM FAM-tagged Peptide 2 for up to 24 h at 37 °C. Images show that Peptide 2 is not only cell permeable, but that uptake appears time-dependent, where after one hour, minimal peptide has entered the cells, while significant uptake observed by 12 h (Fig. 5). Time-dependent uptake was further confirmed by measuring the

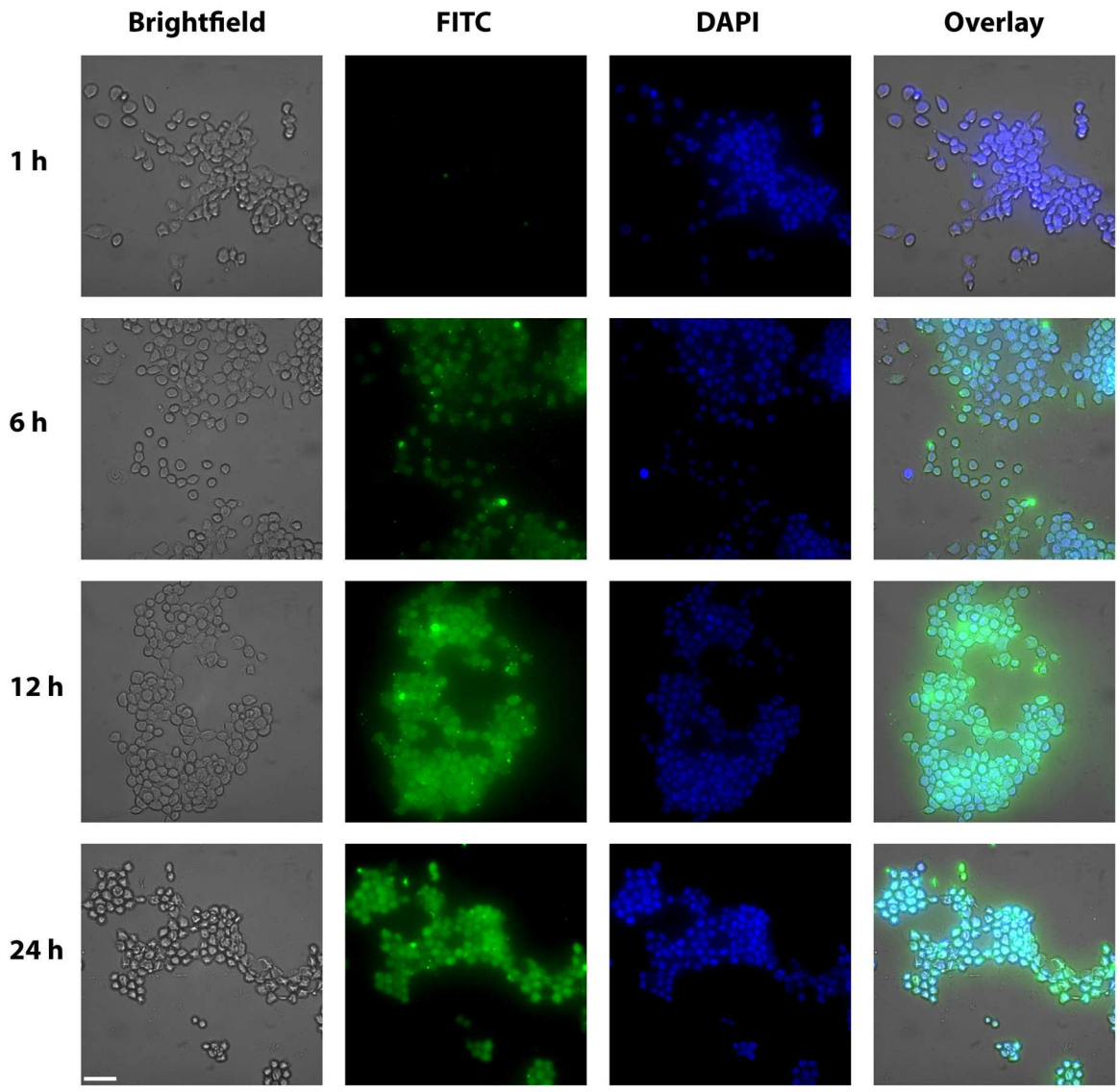

Fig. 5. Visualization of the Intracellular Distribution of Peptide 2 in N2a Cells. A total of 50 μM peptide solutions were incubated with cells seeded on glass imaging chambers at 37 °C. Cells were washed with ECB and incubated with 8 μM Hoechst stain for 20 min prior to imaging. Representative images include brightfield, FITC (for peptide uptake), and DAPI (Hoechst) filters. Scale bar is 50 μm. Images demonstrate time-dependent increased uptake of Peptide 2.

average fluorescent intensity in individual cells from a small population incubated with Peptide 2 (Fig. S4, Table S1). These results are similar to those previously published for Peptide 1, which demonstrated time-dependent uptake when tested by flow cytometry [10]. Additionally, a homogenous intracellular distribution was observed across all time points for Peptide 2. This distribution was confirmed by drawing line scans across individual cells for the 12- and 24-hour time points to quantify the fluorescent intensity across the cell (Fig. S5). The cell-to-cell variability in peptide uptake is to be expected, as single cell analysis has demonstrated the heterogeneity of cellular uptake of the RWRWR peptide [36]. While time dependent uptake of PROTACs is well established, variations in reported uptake time have been observed. For example, a peptide-based PROTAC using TAT as the CPP was observed to be internalized in four hours [7]. This difference is likely due to the CPPs each PROTAC incorporates. Safa et al. demonstrated that there is a tradeoff between stability and permeability for the RWRWR CPP, thus while TAT has greater permeability it is more rapidly degraded [20].

3.6. PROTAC-mediated degradation of Tau is dependent on the UPS

Thus far, Peptide 2 has been shown to degrade Tau in a concentration- and time-dependent manner. Additionally, Peptide 2 was shown to be cell permeable with enhanced intracellular stability. However, an important hallmark of PROTACs is their ability to utilize the existing ubiquitin proteasome system (UPS) to degrade proteins of interest [37]. To confirm the ability of Peptide 2 to function as a PROTAC, experiments were performed to observe degradation of Tau using inhibitors of the UPS. N2a cells were co-incubated with the E1 ubiquitin activating enzyme inhibitor PYR-41 or the proteasome inhibitor MG-132. Western blot analysis shows a decrease in Tau signal in the presence of just Peptide 2; however, that decrease is negated when Peptide 2 is co-incubated with PYR-41 or MG-132 (Fig. 6). This confirms that the decrease in Tau in the cells is due to UPS-mediated degradation induced by the β-hairpin containing PROTAC. Control samples with no peptide exhibited similar Tau levels to the samples incubated with inhibitor alone, confirming the inhibitors themselves do not induce Tau degradation. Experiments using inhibitors to confirm degradation of target proteins is dependent on the UPS is extremely common. Several studies

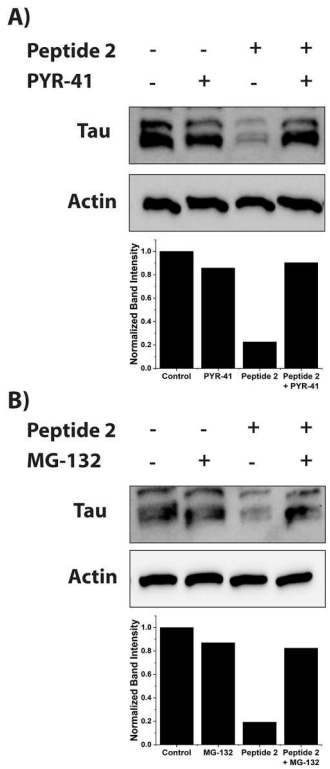

Fig. 6. Degradation of Tau is Proteasome-dependent. N2a cells were treated with 200 μM of PROTAC 2 and co-incubated with (A) PYR-41 and (B) MG-132. Lysates were analyzed with western blotting. Band intensity was quantified with ImageJ and Tau bands were normalized against Actin bands.

which utilized structured sequences in a peptide-based PROTAC have confirmed they are capable of degrading target proteins in a proteasome-dependent manner [9,38].

4. Conclusions

Peptide-based PROTACs have emerged as a promising therapeutic technology to target previously "undruggable" proteins. Despite advantages such as higher potency and lower toxicity, peptide PROTACs are often limited by poor membrane permeability and high protease susceptibility. This work aimed to incorporate a β-hairpin peptide sequence which had previously been shown to function as both a degron and CPP into a peptide-based PROTAC to address these limitations. To accomplish this, two variations of this peptide, RWRWR and OWRWR, were combined with an established binding motif for the protein Tau. When incorporated on the N-terminus of the binding sequence, both degron/CPPs were capable of degrading Tau in a concentration- and time-dependent manner. Additionally, this degradation was greater than that observed with the previously established linear sequence. Peptide 2, which contained the RWRWR sequence, was found to degrade Tau in both a proteasome and ubiquitin activating enzyme dependent manner. Viability studies showed uptake of Peptides 1, 2, and 3 had minimal effects on cell viability. Live-cell microscopy images demonstrated that Peptide 2 was cell permeable, and uptake occurred in a time-dependent manner. Finally, in a degradation assay Peptide 2 demonstrated greater stability compared to the previously established linear PROTAC. These findings indicate the β-hairpin sequence was capable of functioning as both the degron and CPP in a peptide-based PROTAC with enhanced stability and provide potential for incorporation of novel binding sequences to expand possible proteins degraded by peptide-based PROTACs.

CRediT authorship contribution statement

Hannah C. Hymel: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Jeffery C. Anderson:** Data curation, Formal analysis. **Dong Liu:** Data curation. **Ted J. Gauthier:** Data curation, Methodology, Validation, Writing – original draft. **Adam T. Melvin:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Adam Melvin reports financial support was provided by National Science Foundation Division of Chemical Bioengineering Environmental and Transport Systems. Hannah Hymel reports financial support was provided by Louisiana Board of Regents. Adam Melvin reports a relationship with Louisiana State University that includes: employment. None.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2023.109063.

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