DEVELOPMENT OF PROTEASE-RESISTANT β-HAIRPIN PEPTIDES FOR THE DETECTION OF ENZYMATIC ACTIVITY IN CANCER CELLS

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ABSTRACT

Kaiulani Michelle Houston: Development of Protease-Resistant β-Hairpin Peptides for the Detection of Enzymatic Activity in Cancer Cells
(Under the direction of Marcey L. Waters)

Multiple myeloma (MM) is an incurable malignancy of the antibody-producing plasma cells in the bone marrow and is characterized by the targeted degradation of anti-tumor regulatory proteins by the ubiquitin proteasome system (UPS). The development of proteasomal inhibitors, such as bortezomib (Velcade®), has greatly improved the median survival time of MM patients. Inappropriate activation of protein kinase B (PKB or Akt) by bortezomib has been observed leading to refractory disease. Inhibitors of PKB activity are now in clinical trials for the treatment of MM. Chronic myelogenous leukemia (CML), the malignancy of myeloid cells in the bone marrow, is treated predominantly with Abl kinase inhibitors. We have designed protease-resistant fluorescent peptide probes that, once optimized, can be used to determine the effectiveness of these inhibitors by direct measurement of proteasomal and Abl kinase hyperactivity and PKB activation in patient cells.

Measuring intracellular UPS and kinase activity through the use of unstructured synthetic peptide substrates is limited by the vulnerability of these substrates to rapid

degradation in the cell by cytosolic proteases. Unstructured peptides in the cytosol of immune cells are threaded into protease active sites at the N-terminus.

We designed and synthesized short, well-folded protease resistant β-hairpin peptides through the incorporation of favorable cross-strand side chain interactions and a stabilizing turn nucleating sequence. Designing the β-hairpins to include ornithine, a noncanonical amino acid analog of lysine, and D-amino acids, produced long-lived peptides with half-lives of 2-6 hours and non-degradable peptides, in the presence of specific and nonspecific proteases *in vitro*. These β-hairpin peptides were covalently attached to the N-terminus of unstructured kinase substrate peptides resulting in increased stability to peptidases *in vitro* while maintaining efficacy as kinase substrates.

Here we also show that short β-hairpin peptides, not derived from natural protein targets, adequately served as substrates of the UPS. We demonstrated that ornithine can be ubiquitinated by UPS enzymes regardless of secondary structure in a site-specific manner. Finally we show the applicability of these hairpin degrons in determining UPS activity in diseases such as MM, where UPS inhibitors are part of mainline and emerging treatments.

To the Teacher, S. D. G.
To those who suffer from multiple myeloma and support medical and scientific research.
To the King of Salem

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LIST OF ABBREVIATIONS

Ac	Acetyl
Akt	Protein kinase B
Ala, A	Alanine
Alloc	Allyloxycarbonyl
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic Acid
Azk	Azidolysine
BME	2-mercaptoethanol, β-mercaptoethanol
Boc	tert-Butyloxycarbonyl
CD	Circular dichroism
CE-LIF	Capillary electrophoresis with laser induced fluorescence
Cys, C	Cysteine
DCM	Dichloromethane
DEDTC	Sodium diethyldithiocarbamate trihydrate
DIPEA	N,N'-Diisopropylethylamine
DMF	Dimethylforamide
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDT	1,2-Ethanedithiol
ESI	Electrospray ionization
FAM	5(6)-carboxyfluorescein, 5-carboxyfluorescein, 6-carboxyfluorescein

FlAsH	Fluorescein Arsenical Hairpin
FMOC	9-Fluorenylmethoxycarbonyl
Gln, Q	Glutamine
Glu, E	Glutamic Acid
Gly, G	Glycine
H_2O	Water
HBTU	O-(Benzotriazol-1-yl)-N,N,N'N'-tetramethyluronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
Ile, I	Isoleucine
ivDde	1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl
Leu, L	Leucine
Lys, K	Lysine
MALDI	Matrix-assisted laser desorption ionization
MeOH	Methanol
MeUb	Methylated ubiquitin
MM	Multiple myeloma
NMR	Nuclear magnetic spectroscopy
No Lys Ub	No lysine ubiquitin
Orn, O	Ornithine
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
PBS	Phosphate buffered saline
PDB	Protein data bank

PEG	Polyethylene glycol
Phe, F	Phenylalanine
PKB	Protein kinase B
Prg	Propargyl glycine
^{D}-Pro, p	^{D}-Proline
Pro, P	Proline
RFU	Relative fluorescence units
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SPPS	Solid phase peptide synthesis
tBu	Tert-butyl
TFA	Trifluoroacetic acid
Thr, T	Threonine
TIPS	Triisopropylsilane
Tris	Tris(hydroxymethyl)aminoethane
Trp, W	Tryptophan
Trt	Trityl
TUBEs	Tandem ubiquitin binding entities
Tyr, Y	Tyrosine
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
Val, V	Valine

Chapter I

PEPTIDES USED TO MONITOR ENZYMATIC ACTIVITY

A. Introduction

During the 20th and early 21st centuries, clinical diagnosis and treatment went from being predominantly macroscopic to being mostly microscopic. Nearly every medical diagnosis in well-developed countries is validated by one or more molecular tests that screen for a microscopic indication of disease.[1] The development and advancement of microscopy, spectroscopy, antibody-based assays, and the mapping of the human genome and proteome, has allowed for an increased understanding of disease causation and the determination of the mechanisms of action of drugs on their molecular targets. Knowledge of pharmaceutical targets and drug modes of action has led to the development of ways to monitor disease progression and remission at the molecular level.[2] Clinicians and bench scientists are designing ways to directly and molecularly measure the effects of therapeutic agents on their molecular targets during the course of the treatment.

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[1]Yager, P.; Edwards, T.; Fu, E.; Helton, K.; Nelson, K.; Tam, M.R.; Weigl, B.H. *Nature***2006**, 442, 412-18.

[2]Hudis, C.A. N Engl J Med **2007**, 357, 39-51.

The treatment of multiple myeloma (MM), an incurable hematologic malignancy of the plasma cells in the bone marrow, has greatly advanced with the FDA approval of molecularly targeted therapeutics.[3] However, none of the current diagnostic tests directly measure the activity of enzymes and protein-protein interactions targeted by these novel treatments.[3] In this chapter, we will briefly discuss the current state of MM diagnostics and the ways in which the effectiveness of the molecularly-targeted therapeutics used to treat the disease can be measured during the course of treatment. Specifically, we will highlight examples of how peptides can be used in the future to monitor disease progression and eventual remission by directly evaluating cytosolic enzymatic activity relevant to disease states. We will conclude with our proposal to design and use fluorescent peptide-based probes for the monitoring of therapeutic agent effectiveness during MM treatment with enzyme inhibitors.

B. Multiple myeloma diagnosis and treatment today

Multiple myeloma (MM) is the monoclonal proliferation of plasma cells, the antibody-producing white blood cells present in the bone marrow, and accounts for 10% of all hematologic malignancies in the Unites States.[4] MM arises from the accumulation of genetic errors in plasma cells that lead to constitutively active, hypoactive or dysregulated protein activity.[5]

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[3]Palumbo, A.; Anderson, K. *N Engl J Med***2011**, 364, 1046-60.

[4]Kyle, R.A.; Rajkuma, S.V. *Blood***2008**, 111, 2962-72.

[5]Ocio, E.M.; Mateos, M.V.; Maiso, P.; Pandiella, A.; San-Miguel, J.F. *Lancet Oncol***2008**, 9,1157-65.

Currently, a MM diagnosis depends on the combined outcome of several tests including, serum immunoglobulin levels (the heavy chain of antibodies), light chain protein concentration in the urine, bone marrow plasma cell population density, and the karyotyping of the plasma cells in the bone marrow.[3] High concentrations of bone marrow plasma cells and monoclonal antibodies, cytogenetic abnormalities within the nuclei of myeloma cells, and physical symptoms such as anemia and bone lesions constitutes a diagnosis. Further investigation of chromosome abnormalities using fluorescence *in situ* hybridization (FISH), is done to determine the prognosis of the patient, with certain karyotypes associated a better outcome than others. The high concentrations of proteins including beta$_2$-microglobulin and M-protein in the serum and urine are associated with more advanced stage disease.[3] Patients with a good immediate prognosis are first treated with an autologous stem cell transplant and then with a combination of chemotherapeutic agents targeting the catalytic activity of enzymes such as the 26S proteasome and histone deacetylases (HDACs), E3 ligase protein-protein interactions, and DNA replication.[5] Though all patients with treatable MM are prescribed pharmaceutical drugs that target protein activity and interactions, none of the above mentioned diagnostic tests directly measure the activity targeted by the prescribed drugs. As the knowledge of MM pathogenesis increases, the number of new potential drug targets also increases. Many FDA approved drugs and novel therapeutics currently being investigated in clinical trials are inhibitors of intracellular enzymes in signal transduction pathways that have been usurped by inappropriately regulated oncogenes, and thus contribute to tumor cell survival.[5] One such enzymatic signaling pathway is the ubiquitin-proteasome system (UPS), a family of nearly 1000 enzymes required for cell

homeostasis, namely through directing intracellular trafficking and protein degradation by the 26S proteasome.[6] Since the FDA approval of the 26S proteasome inhibitor bortezomib (Velcade) in 2003, the overall median survival of patients with MM has lengthened from 2-3 years to 5-10+ years depending on age and cytogenetic factors.[3,7] Several next generation proteasome inhibitors have been developed and targeting the entire (UPS) for the treatment of several diseases in addition to MM, is projected in the next decade to become as popular as targeting kinase/phosphatase signaling, which dominates 25% percent of the drug market today.[8]

Some clinical trials associated with inhibitors of kinases and UPS enzymes have failed in the late stages due to underwhelming effects on patient overall survival.[9] MM patients enrolled the Stage II/III clinical trial for perifosine, an allosterictic inhibitor of protein kinase B (PKB or Akt) activity, were screened for cytogenetic abnormalities associated with improper immunoglobulin production and excretion, and for their status as relapsed/refractory in response to combination therapies including bortezomib/thalidomide (immunomodulatory agent)/lenolidomide. The patient tumor cell-associated PKB activity was not determined prior to enrollment. Though aberrant PKB activity has been implicated in tumor growth presumably due to its roles in cellular metabolism, motility, proliferation, and angiogenesis, PKB may not be activated or hyperactive in all cancers.[10]

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[6]Nandi, D.; Tahiliani, P.; Kumar, A.; Chandu, D. *J Biosci***2006**, 31, 137-55.

[7]Samson, D. Postgrad Med J **1994**, 70, 404-10.

[8](a) Cohen, P.; Tcherpakov, M.; *Cell***2010**, 143, 686-93 (b) Gonzalez-Vera, J.A. *Chem Soc Rev***2012**, 41,1652-64.

.

Perifosine investigation was halted in Stage III though it showed synergistic activity with bortezomib.[9-10] Though bortezomib has been shown to activate PKB in some MM tumors, it is not known whether or not the activity of PKB was dysregulated in all MM patient tumors investigated and therefore the inhibitor may be useful for only a subset of patients.[11] Despite the short-term and long-term effectiveness of enzyme inhibitors in the treatment of MM, no diagnostic testing is associated with determining enzyme activity in biopsied myeloma cells. This gap in diagnostic testing is due to the absence of high throughput assays that can detect enzymatic activity in a small number of cells. As low as 10% of the bone marrow cells collected from patient biopsies must be plasma cells in order to constitute a possible malignant diagnosis.[3] Many techniques that measure enzymatic expression and activity such as Western Blotting, immunocoprecipitation, and ELISA require one million cells for an accurate readout while MM biopsies can sometimes yield less than 1000 malignant cells. Chemical cytometry assessing enzymatic activity relevant to disease progression in patient tumor cells at diagnosis would give clinicians the information needed to personalize treatments for their patients. Assessing targeted enzymatic activity prior to clinical trial enrollment would allow investigators the ability to enroll patients based on their aberrant enzyme activity status and thus more accurately determine who would most benefit from treatment.

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[9]Richardson, P.G.; Nagler, A.; Yehuda, D.B.; Badros, Hari, P.; Hajek, R.; Spicka, I.; Kaya, H.; Le Blanc, R.; Yoon, S.S.; Kim, K.; Martinez-Lopez, J.; Mittelman, M.; Shpilberg, O.; Tothova, E.; Laubach, J.P.; Ghobrial, I.M.; Leiba, M.; Gatt, M.E.; Sportelli, P.; Chen, M.; Anderson, K.C. *Blood***2013**, 122, 3189.

[10] Richardson, P.G.; Eng, C.; Kolesar, J.; Hideshima, T.; Anderson, K.C. Expert Opin Drug Metab Toxicol **2012**, 8, 623-33.

Another advantage of being able to directly measure enzymatic activity during treatment and/or clinical trial investigation of an enzyme inhibitor would be the ability to directly monitor the drug inhibitor's effect on its targeted activity. As in the case with MM where traditional treatment regiments include a combination of multiple enzyme inhibitors, a rapid high-throughput screening of several enzymes simultaneously would be ideal. Another factor to take into consideration is tumor heterogeneity. The enzymatic activity in tumor cells is not homogenous even when the tumor cells are all taken out of the same patient.[12] Early detection of treatment-resistant tumor cell populations would allow clinicians to implement an alternative treatment plan long before indirect measurements of relapse become detectable.

Below we will highlight a few types fluorescent peptide-based probes developed by others that are capable of detecting enzymatic activity *in vitro*, *in vivo,* and *ex vivo* in just a single cell. Our primary focus is on the detection of kinase activity and UPS enzymatic activity as these are relevant drug targets for the treatment of MM. We will briefly discuss the major challenges of using peptide-based probes to assess enzymatic activity in the cytosol of cells and introduce our proposed method to overcome these challenges. The use of the methods discussed is restricted mostly to bench research analysis rather than for a clinical testing. We hope the work described in the remaining chapters will be a foundation for the translation of direct enzymatic activity assays from bench top experimentation only to clinical diagnostics and disease-monitoring technology.

[11]Mahindra, A.; Laubach, J.; Raje, N.; Munshi, N.; Richardson, P.G., Anderson, K. *Nat Rev Clin Oncol***2012**, 9, 135-43.

C. Peptides as substrates for the detection of enzymatic activity *in vitro*

Commonly, enzyme expression, activation and regulation are determined by Western Blot, enzyme-linked immunosorbant assay (ELISA), small molecule activity-based probes, and peptide-modification based probes. All of the above methods rely on the mimic of protein-protein interactions essential to the maintenance of homeostasis in the body. While the above assays can give information about the activation, mutation and indirectly the catalytic activity of an enzyme, all require a large number of cells if used to investigate cell lysates and none are capable of giving real-time kinetic data.[8b] Assay sensitivity with fewer cells required for detection has been achieved through the use of fluorescent peptide-based probes. Peptides-derived from natural protein substrates have been successfully used as catalytic substrates of enzymes present in the cytosol of the cell.[13] Peptides can be designed to be recognized and modified by the enzyme of interest with high specificity and activity.[14] The addition of a fluorophore to the substrate peptide allows for quick sensitive detection of enzymatic activity that can be visualized by several types of instrumentation including fluorescence microscopy, fluorescence imaging of agarose gels, liquid chromatography coupled with mass spectrometry (LC/MS), and capillary electrophoresis with laser-induced fluorescence (CE-LIF). Below are four examples of how kinase and UPS activity have been detected *in vitro* using fluorescent peptides.

[12]Marusyk, A.; Almendro, V.; Polyak, K. *Nat Rev Canc***2012**, 12, 323-34.

[13] Kennelly P.J.; Krebs, E.G. *J Biol Chem***1991**, 266,15555-8.

[14]Wu, D.; Sylvester, J.E.; Parker, L.L.;, Zhou, G.; Kron, S.J. *Biopolymers***2010**, 94, 475-86.

i. *Chelation enhanced fluorescence (CHEF)*

One way to detect kinase activity is to measure a change in the fluorescence of a kinase substrate peptide due to phosphorylation of the substrate. This can be achieved by chelation enhanced fluorescence (CHEF) of the kinase substrate peptide through the phosphorylation-dependent coordination of a metal by a chromophore covalently attached to the kinase substrate peptide.[15] Notable examples of such probes were reported by the Imperiali Group whose probes included a cysteine conjugated sulfonamide-oxime (C-Sox) chromophore within the region C-terminal or N-terminal to the kinase substrate region.[16] Upon phosphorylation of the substrate peptide by kinases in in mammalian cell lysates, Mg^{2+} was simultaneously chelated by both the C-Sox chromophore and the phosphoryl group on the modified Ser/Thr residue which led to a 2-5 fold increase in fluorescence. Lukovic et al. were able to design CHEF-based probes with a higher degree of substrate specificity for several kinases including protein kinase C (PKC), Abelson kinase (Abl) and Akt1 (PKB) through a recognition-domain focused design strategy. In addition, they tailored the kinase activity detection to a 96-well format that allowed for kinetic data to be determined based on fluorescence enhancement *in vitro*, demonstrating the potential for this assay to evolve into a high-throughput technique.

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[15]Shults, M.D.; Janes, K.A.; Lauffenburger, D.A.; Imperiali, B. *Nature Methods* **2005**, 2, 277-84.
[16]Luković, E.; González-Vera, J.A.; Imperiali, B. *J Am Chem Soc***2008**, 130, 12821-27.

While the majority of the activity measured by these CSox probes was due to phosphorylation by the desired kinase as shown by the use of kinase inhibitors and immunodepletion of the kinase of interest, there is still some probe modification by other kinases. Since there are over 500 known kinases in the cell, cross-reactivity in not unexpected but not desired. Therefore continued improvement of substrate specificity is necessary before this technology can be applied to cell lysate assays in the clinic.

ii. Quencher dyes

Another strategy for specific kinase activity detection by a change in fluorescence is "Deep Quench" developed by the Lawrence Group. Deep quench detects the restoration of fluorescence of the kinase-specific peptide substrate upon modification by the desired kinase.[17] Kinase substrates designed for deep quench possessed a coumarin moiety conjugated to the positively charged peptide N-terminal to the modifiable Ser/Thr modification site. Prior to phosphorylation, coumarin fluorescence was quenched in the presence of the negatively charged Acid Green dye. Phosphorylation of the kinase substrate peptide and subsequent binding of the phosphorylated substrate to the 14-3-3 protein domain displaced the Acid Green dye and led to the fluorescence of modified substrates. This method allowed for the detection of protein kinase A (PKA) activity in mitochondrial membranes, with phosphorylation associated fluorescence observed over the autofluorescence associated with the organelles.

[17]Agnes, R.S.; Jernigan, F.; Shell, J.R.; Sharma, V.; Lawrence D.S. J Am Chem Soc **2010**, 132, 6075-80.

The dramatic fluorescence enhancement upon modification of these probes highlights the potential for similar peptide-based probes to be used ex vivo to monitor organelle specific-kinase activity in small sample sizes.

iii. Fluorescence detection by capillary electrophoresis

It is possible to detect kinase activity through the use of fluorescent peptide-based probes without monitoring a change in fluorescence. The Allbritton Group, in collaboration with the Lawrence Group, has reported the detection of Abl kinase activity in cell lysates using capillary electrophoresis coupled with laser-induced fluorescence (CE-LIF).[18] This technique allows for the separation and fluorescence detection of both the modified and unmodified reporters within a capillary based on the difference in electrophoretic mobility due to the differing net charge of the phosphorylated and nonphosphorylated substrates.

This method of detection is highly sensitive requiring only subnanomolar concentrations of modified peptides for fluorescence detection and was used to determine the catalytic efficiency of substrate phosphorylation.[18] This technique, is also amenable to the simultaneous screening of several substrates at once, allowing for the concurrent detection of the activity of multiple enzymes.

iv. Detection of UPS activity in vitro using short peptides

In addition to kinase activity, the activity of UPS enzymes, including the proteasome, can be detected using peptide substrates as well.

[18]Proctor, A.; Wang, Q.; Lawrence, D.S.; Allbritton, N.L. *Analyst***2012**, 137, 3028-38.

Recently, the Allbritton Group has examined the collective activity of E1, E2, and E3 UPS enzymes through the detection of ubiquitinated peptides bearing short modifiable recognition motifs known as degrons.[19] Short fluorescent peptide sequences, 9-26 amino acids long, derived from proteins regulated by UPS-directed ubiquitination in the cell, were modified by E1, E2 and E3 enzymes *in vitro*. Both monoubiquitination and polyubiquitination of the peptides substrates was observed by fluorescence imaging of the ubiquitinated substrates separated by gel electrophoresis. The kinetics of peptide substrate ubiquitination and the minimal sequence required for modification were also assessed. The Allbritton Group is currently adapting the assay for analysis by CE-LIF.

D. Peptides for the detection of intracellular enzymatic activity

The methods for kinase and UPS activity detection mentioned above as well as many other noteworthy approaches not mentioned here, mark great strides in the advancement toward enzymatic activity assessment in patient samples in the clinic. However, all of the above efforts were done in the context of purified enzymes or cell lysates and not within the cytosolic environment of intact cells. Detection of intracellular enzymatic activity requires the use of stable substrates and sensitive detection methods. Peptide-based fluorescent probes that meet the demands of *ex vivo* kinase activity detection have been developed by several groups using a variety of methodologies. Below we will briefly discuss three approaches used to successfully detect kinase or UPS enzymatic activity within intact cells.

[19]Melvin, A.T.; Woss, G.S.; Park, J.H.; Dumberger, L.D.; Waters, M.L.; Allbritton, N.L. *PLOS One***2013**, 8, e78082.

i. Caged compounds

A major challenge when measuring kinase activity inside of cells is the inability to accurately control and measure the start point of enzymatic activity. One way to gain control of the intracellular kinase activity initiation is to use "caged" substrates. Molecular caging involves masking the substrate functional group required for enzymatic activity with an optically silent moiety via a photocleavable bond. Photolysis of the caged compound at the desired zero time point, liberates the substrate to an optically active form.[20] The caging strategy was first applied to the masking of end phosphate groups on cyclic AMP and ATP with a photolabile nitromethoxy phenyl group. Upon photolysis biologically active forms of cyclic AMP and ATP were released and available for hydrolysis by cellular enzymes.[21]Recently, the caging strategy has been applied to the detection of PKA activity in erythrocytes by using a peptide-based substrate. The Lawrence Group reported controllable PKA activity detection through the combined strategies of fluorescence quenching and phosphorylation site caging.[22] The PKA substrate peptide contained a N-terminal fluorophore that was quenched in the presence of a negatively charged dye that noncovalently bound positively charged residues in the peptide chain via electrostatic interactions.

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[20]Lee, H.M.; Larson, D.R.; Lawrence, D.S. *ACS Chem Biol***2009**, 4, 409-27.

[21](a) Forbus III, B.; Kaplan, J.H.; Hoffman, J.F. *Biochemistry***1978**, 17, 3667-76. (b)Kaplan, J.H.; Ellis-Davies, G.C.; *Proc Natl Acad Sci USA***1988**, 85, 6571-75. (c)Nargeot, J.; Nerbonne, J.M.; Engels, J.; Lester H.A. Proc Natl Acad Sci USA 1983, 80, 2395-99.

[22]Oien, N.P.; Nguyen, L.T.; Jernigan, F.E.; Priestman, M.A.; Lawrence, D.S. *Angew Chem* **2014**, 126, 4056-59.

The phosphorylatable Ser residue was masked with the photolabile 4,5-dimethoxy-2-nitrobenzyl group prior to loading into intact erythrocytes (red blood cells). The reporter peptide was loaded into the red blood cells and fluorescence was detected only upon photolysis which liberated the Ser hydroxyl group for phosphorylation by PKA and the subsequent displacement of the negatively charged quencher dye, restoring the fluorescence of the N-terminal fluorophore. This strategy demonstrated the power of using temporal detection for the measurement of kinase activity *ex vivo*.

ii. Single cell CE

Collaborative efforts of both the Allbritton and Lawrence Groups has allowed for the detection of kinase activity in single cells using CE-LIF.[23] PKB activity was detected using peptide substrates with unnatural amino acids incorporated intermittently throughout the substrate peptide chain to increase resistance to degradation by proteases without greatly sacrificing substrate efficacy. These fluorescent probes were used to measure both the rates of phosphorylation and the rates of protease degradation based on the differences of the electrophoretic mobility of the phosphorylated substrates, nonphosphorylated substrates, intact reporter peptides and digestion fragments. These probes also gave information about differing sites of substrate hydrolysis by proteases in cell lysates versus intact cells. This report showed the potential of detecting kinase activity when the number of cells is the limiting factor such as the case with plasma cell analysis of MM patient tumors. Their results also showed the potential of redesigning substrates for increased stability to proteolysis without sacrificing the capability of being modified by the desired enzyme.

[23]Proctor, A.; Wang, Q.; Lawrence, D.S.; Allbritton, N.L. *Anal Chem***2012**, 84,7195-202.

iii. Detection of proteasome activity in live cells

The success of bortezomib in the clinic has increased the need for probes that can be used to detect proteasome activity inside intact patient cells. The proteasome utilizes three different types of catalytic activity to degrade proteins, trypsin-like activity, chymotrypsin-like activity, and caspase-like activity.[24] When screening potential proteasome inhibitors knowledge of which enzymatic activity is being blocked is highly valued. Several fluorescent peptide-based probes with terminal electrophiles have been used to label proteasome substrates. The general structure of several of these probes is a dye conjugated to the N-terminus of a tri-aminohexanoic acid (Ahx), tri-leucine (Leu), vinyl-sulfone (Dye-$(Ahx)_3$-L_3-VS) probe. One example reported by Verdoes et al. utilized a membrane-permeable Bodipy-TMR-Ahx_3L_3VS which they used to detect proteasome activity in cell lysates, intact cells and in mice.[24] When incubated with cell lysates in the presence of known proteasome inhibitors, the degree of proteasome labeling correlated to the degree of inhibitor. These probes were even used to determine which subunit of the proteasome was being inhibited. Though detection of proteasome labeling required cell lysis or fixation, the combination of membrane permeability and fluorescence detection of subunit specific labeling, lays a solid foundation for the use of these probes in a clinical setting.

[24]Verdoes, M.; Florea, B.I.; Menendez-Benito, V.; Maynar C.J.; Witte, M.D.; van der Linden, W.A.; van den Nieuwendijk, A.M.C.H.; Hofman, T.; Berkers, C.R.; van Leeuwen, F.W.B.; Groothuis, T.A.; Leeuwenburgh, M.A.; Ovaa, H.; Neefjes, J.J.; Filippov, D.V.; van der Marel, G.A.; Dantuma, N.P.; Overkleeft, H.S. *Cell Chem Biol***2006**, 13, 1217-26.

E. Purpose of this work

Though the use of peptides for the detection of enzymatic activity in the cell is advantageous due to their being excellent mimics of natural enzyme substrates, peptides comprised of canonical amino acids are also substrates for cytosolic peptidases. Peptide hydrolysis by aminopeptidases in the cytosol of immune cells such as plasma cells reduces peptides to their amino acid building blocks rapidly.[25] Peptide-based enzymatic activity probes without features to make them resistant to hydrolysis by cytosolic proteases, are subject to destruction in the cellular environment before an activity-based readout can be detected.

In the remaining three chapters we will describe our work to improve the protease resistance of fluorescent peptide probes used to detect kinase and UPS activity in malignant mammalian cell lines. Specifically we will describe the design, synthesis, and performance of orninthine-rich β-hairpin peptide probes of enzymatic activity and the advantages and of utilizing secondary structure and noncanonical amino acids to impart protease resistance.

[25]Yewdell, J.W., Reits, E., Neefjes, J. *Nat Rev Immunol***2003**, 3, 952-61.

Chapter II

DESIGN, SYNTHESIS, AND CHARACTERIZATION OF PROTEASE-RESISTANT β-HAIRPIN PEPTIDES CALLED PROTECTIDES

A. Background

Synthetic peptides have emerged as popular tools used to study cellular events, including molecular recognition, enzymatic activity, and membrane trafficking.[1] The natural existence of peptides in biological systems and their vast potential for diversification allows them to be used as pharmaceuticals and diagnostics as well. There is a need for diagnostic testing that can measure enzymatic activity associated with the effectiveness of drug inhibitors in patient cells *ex vivo*.[2] This need can be met through the use of fluorescently tagged peptide substrates that can be easily detected by spectroscopy, spectrometry, and/or electrophoresis, all methods already used in a clinical setting.[3]

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[1]Ruttekolk, I.R.; Witsenburg, J.J.; Glauner, H.; Bovee-Geurts, P.H.M.; Ferro, E.S.; Verdurmen, W.P.R.; Brock, R. *Mol Pharmaceutics***2012**, 9, 1077-86.

[2]Mahindra, A.; Laubach, J.; Raje, N.; Munshi, N.; Richardson, P.G., Anderson, K. *Nat Rev Clin Oncol***2012**, 9, 135-43.

[3]Gonzalez-Vera, J.A. *Chem Soc Rev***2012**, 41,1652-64.

The detection of kinase activity, the target of many pharmaceutical inhibitors, in live cells and in cell lysates has been reported using short unstructured peptides (6-30 residues) mimicking the native protein substrates.[3-4] Incorporating such reporters as part of regular diagnostic screening would be particularly helpful in cancers of the immune cells like multiple myeloma (MM) and chronic myelogenous leukemia (CML) where kinase and other enzyme inhibitors are part of the first line of treatment.[5] Currently, there are no diagnostic tests for either of these diseases that screen for the targeted enzyme activity, prior to treatment with drug inhbitors.[6]

While proteins, due to their tertiary structure, are not susceptible to degradation unless specifically targeted in live cells by degradation pathway proteins, unstructured peptides are vulnerable because of their shorter length and lack of secondary and tertiary structure.[7] In the cytosol of immune cells, aminopeptidases are predominantly responsible for peptidic digestion though the presence of endopeptidase activity has been reported.[8] The crystal structures of two aminopeptidases, tripeptidyl peptidase II (TPP II) and leucine aminopeptidase (LAP) reveal catalytic cleft entrance dimensions of 30 Å × 15 Å and 30 Å × 10 Å respectively, restricting access to short unstructured peptides.[9]

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[4]Kunkel, M.T.; Ni, Q.; Tsien, R.Y.; Zhang, J.; Newton, A.C. J Biol Chem **2005,** 280, 5581-7.

[5](a) Placzek, E.A., Plebanek, M.P.; Lipchik; A.M.; Kidd, S.R.; Parker, L.L. *Anal Biochem***2010**, 397, 73-8. (b) El-Amm, J.; Tabbara, I.A. *Am J Clin Oncol* **2013 Aug 7**, [Epub ahead of print].

[6]Palumbo, A.; Anderson, K. *N Engl J Med***2011**, 364, 1046-60.

[7](a)Reits, E.; Griekspoor. A.; Neijssen, J.; Groothuis, T.; Jalink, K.; van Veelen, P.; Janssen, H.; Calafat, J.; Drijfhout, J. W.; Neefjes, J. *Immunity***2003**, 18, 97-108. (b) Saric, T.; Graef, C.I., Goldberg, A.L. *J Biol Chem***2004**, 279, 46723-32.

Well-folded β-hairpin peptides at the N-terminus of kinase activity reporter peptides can be used to increase their resistance to such proteases.We recently reported that the attachment of β-turn peptides, also known as β-bends, to the N-terminus of an unstructured protein kinase reporter peptide, successfully slowed its degradation by cellular proteases.[10]

In this work, β-hairpin peptides were used to protect unstructured peptide-based probes from proteolytic degradation. A β-hairpin peptide consists of two parallel or antiparallel peptide strands connected by a rigid turn or an unstructured loop, forming the most basic unit of a β-sheet, one of the most prevalent types of secondary structure found in proteins.[11] In the past two decades, several studies have been dedicated to understanding the essential noncovalent and covalent interactions necessary to form stable well-folded β-sheets and β-hairpins outside of the context of full protein structure and tertiary contacts.[12] There are five basic features that allow for short peptides to adopt stable β-hairpin conformations; 1) a turn-nucleating sequence, 2) cross-strand backbone H-bonding, 3) sidechain noncovalent interactions, 4) backbone rigidity, and 5) terminal covalent and noncovalent interactions to prevent fraying (**Figure 2.1**).

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[8]Akkad, N.; Schatz, M.; Dengjel, J.; Tenzer, S.; Schild, H. *Med Microbiol Immunol***2012**, 201, 463-73.

[9]Rockel, B.; Kopec, K.O.; Lupas, A. N.; Baumeister, W. Biochim Biophys Acta 2012, 1824(1), 237-45. (b) Burley, S.K.; David, P.D.;, Taylor, A.; Lipscomb, W.N. *Proc Natl Acad Sci***1990,** 87, 6878-82.

[10](a) Eker, F.; Griebenow, K.; Schweitzer-Stenner, R. *J Am Chem Sci* **2003**, 125, 8178-85. (b) Yang, S.; Proctor, A.; Cline, L.L.; Houston, K.M.; Waters, M.L.; Allbritton, N.L. *Analyst* **2013**, 138, 4305-11.

Investigation of these key features has allowed for the design of well-folded β-hairpin peptides with multiple applications, including binding nucleic acids, mimicking protein-protein interactions, and imparting protease resistance.[13] Additionally, folded β-bend peptides which contain only the amino acid residues necessary to nucleate a β-turn,have been reported .[10]

β-hairpins were chosen as ideal protectides over other secondary structures such as alpha helices, due to their ability to tolerate diverse functionalization (including appending a peptide at the C-terminus) while maintaining their conformational folding in aqueous solutions. While the use of peptidomimetics, including the incorporation of β and ϒ amino acids, has allowed for the development of short, stable peptide mimics with remarkable resistance to proteolytic degradation, the building blocks are generally moreexpensive.[14] Also, we are designing protease resistant moieties to be covalently attached to L-amino acid peptide kinase substrates, and the use of peptidomimetics may necessitate additional synthetic steps.

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[11]Voet, D.; Voet, J. *Biochemistry*; 3rd ed.; John Wiley & Sons, Inc: Hoboken, NJ, **2004**, pg 227-9.

[12]Hughes, R. M.; Waters, M.L. *Curr Opin Struct Biol***2006**, 16, 514-24.

[13](a) Stewart, A.L.; Waters, M.L. *ChemBioChem***2009**,10, 539-44. (b) Wilger, D.J.; Park, J. H.:, Hughes, R.M.; Cuellar, M.E.; Waters, M.L. *Angew Chem***2011**, 50, 12201-4. (c) Cline, L.L; Waters, M.L. *Pept Sci***2009**, 92, 502-7. (d) Fasan, R.; Dias, R.L.A.; Moehle, K.; Zerbe, O.; Vrijbloed, J.W.; Obrecht, D.; Robinson, J.A. *Angew Chem***2004**, 43, 2109-12. (e) Cheng, Z.; Campbell, R.E. ChemBioChem 2006, 7, 1147-50. (f)Park, J.H.; Waters, M.L. *Org Biomol Chem***2013**, 11, 69-77.

Short, thermodynamically stable, β-hairpins have been designed and synthesized through the optimization of turn sequences, the utilization of favorable sidechain-sidechain interactions, the use of amino acids with high β-sheet propensity, and the use of salt bridges and capping motifs to prevent terminal fraying. [12,15] It has been reported by our group and others that optimization of beta-hairpin secondary structure conveys substantial resistance to proteolytic degradation.[13c,e-f]

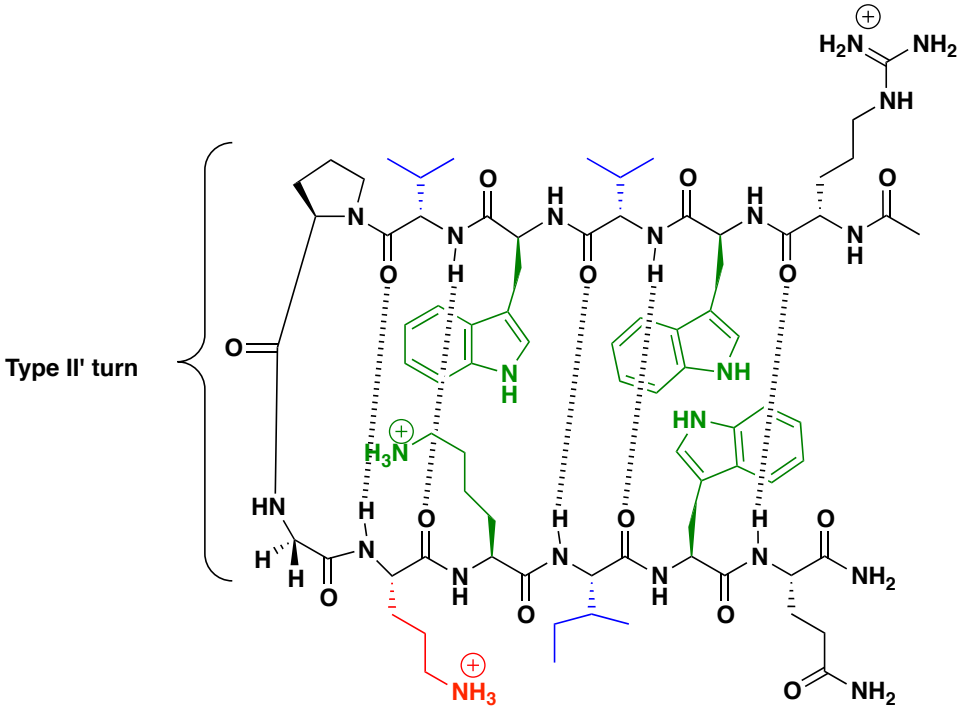

Figure 2.1 Anti-parallel β-hairpin peptide design features. Highlighted in green are residues participating in noncovalent sidechain interactions, in blue are residues with high β-sheet propensity, and the residue in red is the non-canonical amino acid ornithine. Backbone H-bonding is indicated by the hashed lines.

Previously, our group confirmed a direct correlation between secondary structure and the resistance of β-hairpin peptides to degradation by proteolytic enzymes.[13c,f] The **WKWK** and the **Trp Pocket**β-hairpin peptides (Table 2.1) were determined to be 95% and 99% folded in aqueous solvent, respectively, despite being only 12 amino acids in length.[16] Each contained 11 canonical amino acids and 1 non-canonical amino acid (ornithine, Orn) and were found to be substantially more resistant to degradation by specific and nonspecific proteases than the unstructured control.[13c]The sequence of **WKWK** was modified by replacing the turn sequence Asn-Gly (type I' turn) with D-Pro-Gly (type II' turn). The incorporation of the D-Pro-Gly turn sequence has been shown to enhance the stability of β-hairpins by nucleating a tighter turn.[17] The resulting **WKWK-pGturn** peptide (Figure 2.2 and Table 2.1), rich in basic amino acids (cleavage site of trypsin) and aromatic amino acids (cleavage site of α-chymotrypsin) showed resistance to degradation by trypsin, α-chymotrypsin and pronase E (a mixture of nonspecific proteases).[13c]The **WKWK-pGturn** peptide showed a 10-fold greater stability to proteolytic degradation by pronase E *in vitro* over the unstructured control with a similar primary amino acid sequence.[13c]This increased stability was attributed to its higher degree of fraction folded afforded by the type II' turn sequence.

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[14]Frackenpohl, J.; Arvidsson, P.I.; Schreiber, J.V.; Seebach, D. ChemBioChem **2001**, 2,445-55.

[15]Kier, B.; Shu, I.; Eidenschink, L.A.; Andersen, N.H. *Proc Natl Acad Sci***2010**, 107, 10466-71.

[16] (a) Butterfield, S. M.; Waters, M.L.; *J Am Chem Soc***2003**, 125, 9580-1. (b) Butterfield, S.M.; Sweeney, M.M.; Waters, M.L. *J Org Chem***2005**, 70 1105-14. (c) Rieman, A.J.; Waters, M.L. *Biochemistry***2009**, 48, 1525-31.

[17]Stanger, H.E.; Gellman, S.H. *J Am Chem Soc***1998**, 120, 4236-37.

The following report describes ours efforts to further increase the lifetime of **WKWK-pGturn** by modifying its amino acid structure to increase folding and to slow or prevent hydrolysis by cytosolic peptidases. The modified β-hairpin peptides were designed to last for hours *in vitro* under harsh proteolytic conditions. The resulting highly resistant β-hairpin peptides, which we call "protectides", were then covalently attached to the N-termini of unstructured kinase and proteasome substrates to slow their degradation in cell lysates. The synthesis and performance of these full-length reporters is discussed in Chapter III of this work.

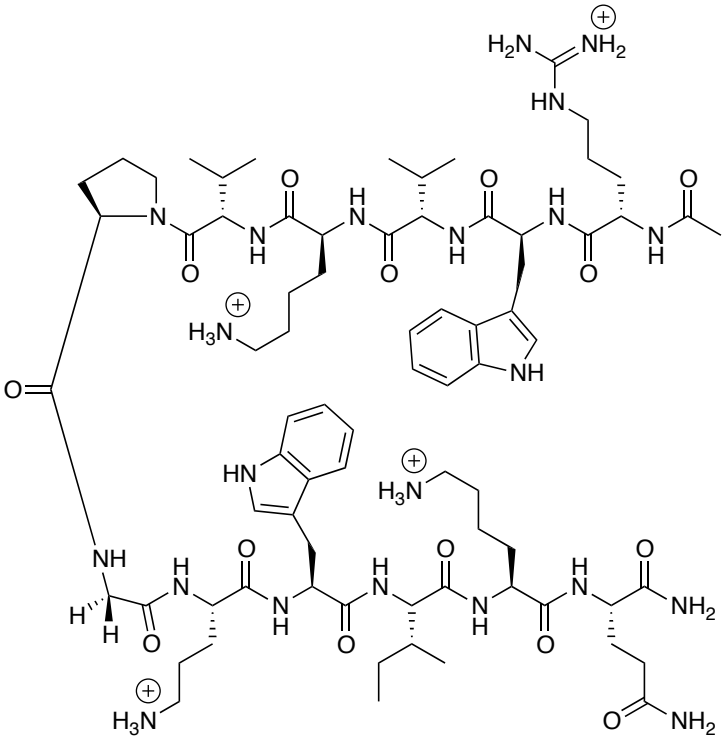


Figure 2.2 Structure of the **WKWK pGturn**β-hairpin peptide.[13c]

B. Design and Synthesis

Building on the work published previously by our group (Cline and Waters, 2009), we made key changes to the amino acid sequence of **WKWK-pGturn**, based on the five design features mentioned above as well as the addition of non-canonical amino acids. The **OWOWO** peptide has the same primary sequence as **WKWK pGturn** except all of the basic natural amino acids were replaced with the non-canonical amino acid analog of lysine, ornithine (Figure 2.3, Table 2.1). Although trypsin-like peptidases cleave at the carboxyl group of basic amino acids, the shortening of the Lys sidechain by one methylene group as is the case with Orn, slows the rate of hydrolysis by such enzymes.[18] Our group has demonstrated that Orn residues are capable of participating in diagonal cation-π interactions with aromatic sidechains and was therefore expected to participate in favorable interactions in the **OWOWO**peptide.[19] Since Orn carries a positive charge under physiological conditions, Orn residues were also added to increase the water solubility of otherwise hydrophobic peptides. The **ScramOWOWO**(Scrambled-OWOWO) peptide has the same primary amino acid sequence as the **OWOWO** peptide except the turn residues were positioned at the C-terminus of the peptide rather than in the middle (Figure 2.3). This peptide served as an unstructured control. The **Trp Pocket** peptide, which demonstrated proteolytic stability when tested previously, was also modified and investigated.[13c]The Asn-Gly turn sequence was replaced with the stronger turn-nucleating sequence $_{D}$-Pro-Gly, the N-terminal Arg was replaced with Orn, and 5(6)-carboxyfluorescein was attached to the sidechain of the Lys8 (Figure 2.3, Table 2.1).

[18]Seely, J.H.; Benoiton, N.L. *Can J Biochem***1970**, 48, 1122-31.

A long-standing challenge of designing and synthesizing short tightly-folded β-hairpin peptides is terminal fraying. To alleviate this problem, β-hairpins are commonly cyclized forming a covalent bond between the termini.[4] However, one solution to fraying using noncovalent interactions was reported by Kier et al. and is referred to here as the Andersen capping motif.[15]The Andersen capping motif, "acyl-W-loop-WTG", added +6 kJ/mol of stability to the β-hairpins reported.[15]This capping motif confers additive stability through a face-to-edge Trp-Trp interaction, bifurcated hydrogen bonding of the Thr residue with the N-terminal acyl group and the H_N of Gly, and a CH-π interaction between the N-terminal Trp and the C-terminal Gly (Figure 2.4).[15] The Andersen capping motif was incorporated into the primary amino acid sequences of the **AC1F**, **AC2F**, **AC4F**, and **AC5F**β-hairpin peptides (Figure 2.4, Table 2.1) The cross-strand cation-π sidechain-sidechain interaction that was present in the **WKWK-pGturn** peptide and the **Trp Pocket** peptide was incorporated into the **AC4F** peptide by positioning a Lys residue opposite a Trp residue on the non-hydrogen bonding face of the peptide. The "tryptophan zipper" (trpzip) motif reported by Cochran and coworkers was incorporated into the **AC5F** peptide through the placement of four Trp residues cross-strand from each other on the non-hydrogen bonded face of the β-hairpin.[20] The edge-to-face π-π interaction between the indole sidechains of the trpzip motif have allowed for short β-hairpin peptides to be over 99% folded under physiological conditions.[16c]

[19]Hughes, R.M.; Benshoff, M.L.; Waters, M.L.*Chem-Eur J***2007** 13, 5753-64.

[20] (a) Cochran, A.G.; Skelton, N.J.; Starovasnik, M.A. *Proc Natl Acad Sci***2001**, 98, 5578-83. (b) Tatko, C.D.; Waters, M.L.; *J Am Chem Soc***2002**, 124, 9372-3.

Finally, the $_D$-isomer of the **WKWK-pGturn** peptide was made by replacing all of the L-amino acids with their $_D$-isomer and the $_D$-Pro residue was replaced with $_L$-Pro. As Gly is achiral it remained the same. The $_D$-isomer of **WKWK-pGturn**, referred to as the**Mirror**β-hairpin peptide, was expected to have the greatest resistance to degradation since proteases are highly enantiospecific. The scrambled version of the **Mirror** peptide, the **ScramMirror** peptide, was synthesized as an unstructured control (Figure 2.3).

The fluorophore 5(6)-carboxyfluorescein (FAM) was appended to the sidechain of the Lys or Orn at the i+4 position just after the turn sequence of the β-hairpin peptides and to Orn7 (Lys7) of the scrambled peptides. The fluorophore was attached next to a turn residue of all β-hairpin peptides while all of the aromatic residues were placed on the opposite face to prevent the quenching of the FAM.[21]We expected that the incorporation of the fluorophore FAM to the sidechain of Lys or Orn, would also increase protectide resistance to trypsin-like peptidases, as the FAM would likely prevent proteolytic degradation due to steric hindrance. The attachment of FAM to each protectide allowed for their detection in cell-based assays described in Chapters III and IV of this work.

[21]Cooper, W.J.; Waters, M.L. *Org Lett***2005**, 7, 3825-8

Table 2.1 Name, amino acid sequence and secondary structure of peptides investigated for protease resistance[a]

Peptide Name	Sequence	2° Structure
ScramOWOWO	Ac-OWVOWIO(FAM)QVOpG-NH_2	Random Coil
OWOWO	Ac-OWVOVpGO(FAM)WIOQ-NH_2	β-Hairpin
OWOC	Ac-COWVOVpGO(FAM)WIOQC-NH_2	β-Hairpin
WKWK.[13c]	Ac-RWVKVNGOWIKQ-NH_2	β-Hairpin
WKWK-pGturn.[13c]	Ac-RWVKVpGOWIKQ-NH_2	β-Hairpin
AC4F**	Ac-WVWVpGK(FAM)KIWTGOO-NH_2	β-Hairpin
Trp Pocket[13c]	Ac-RWVWVNGOKILQ-NH_2	β-Hairpin
Trp Pocket dPro	Ac-OWVWVpGK(FAM)OILQ-NH_2	β-Hairpin
Mirror	Ac-^{D}R^{D}W^{D}V^{D}K^{D}VPG^{D}K(FAM) ^{D}W^{D}I^{D}O^{D}Q-NH_2	β-Hairpin
ScramMirror	Ac-^{D}K^{D}W^{D}V^{D}R^{D}W^{D}I^{D}K(FAM) ^{D}Q^{D}V^{D}OPG-NH_2	Random Coil
AC1F**	Ac-WIpGK(FAM)WTGO-NH_2	β-Hairpin
AC2F**	Ac-WIpGK(FAM)WTGPSO-NH_2	β-Hairpin
AC5F**	Ac-WTWIpGK(FAM)WTWTGOO-NH_2	β-Hairpin

[a]O represents the non-canonical amino acid ornithine. "Ac" represents acetylation of the N-terminus and C-terminus of each peptide was amidated. "AC" denotes peptides containing the Andersen Capping Motif. Secondary structure was determined by circular dichroism.

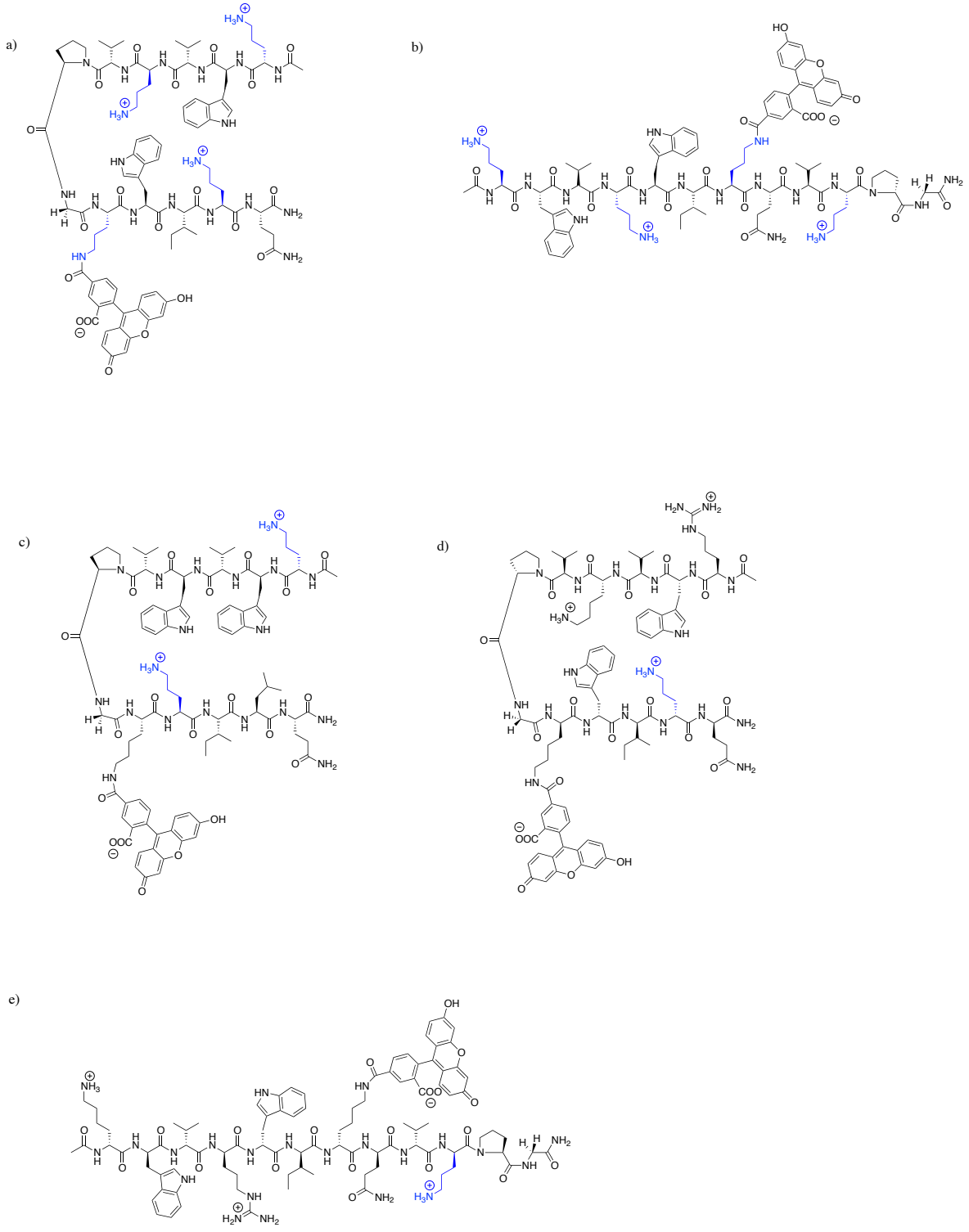


Figure 2.3 Structured protectides and the unstructured control peptide evaluated in this work. (a) **OWOWO** (b) **ScramOWOWO** (c) **Trp Pocket dPro** (d) **Mirror** (e) **ScramMirror**. Ornithine residues are highlighted in blue.

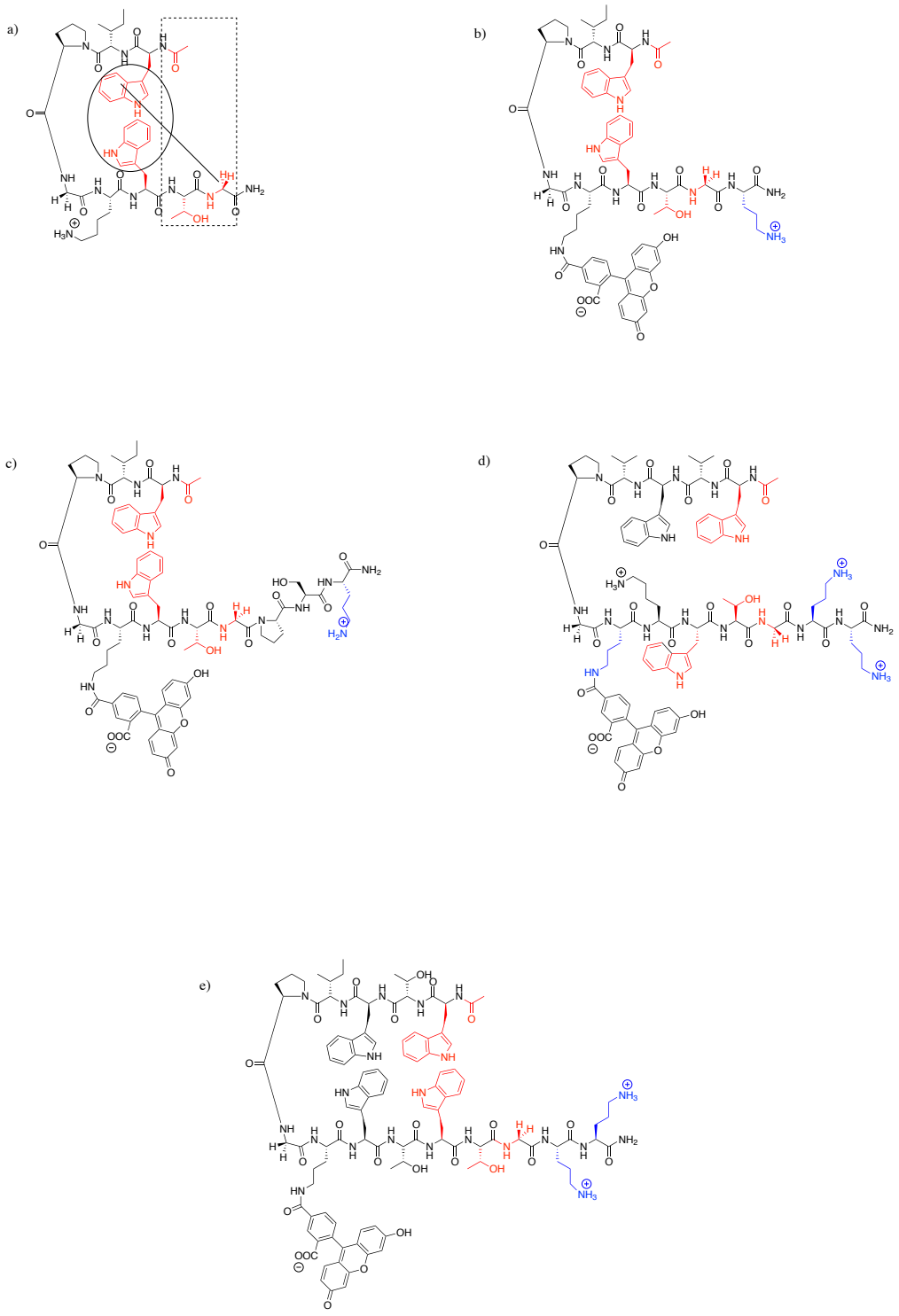


Figure 2.4 Structures of β-hairpin protectides containing the Andersen capping motif. (a) Example peptide highlighting the noncovalent interactions of the Andersen capping motif. (b) **AC1F** (c) **AC2F** (d) **AC4F** (e) **AC5F**. Residues part of the capping motif are highlighted in red. Ornithine residues are highlighted in blue.

C. Results and discussion

i. Structural characterization by circular dichroism

The secondary structure of the **WKWK-pGturn** peptide was characterized previously using circular dichroism (CD) and NMR. This 12-mer was folded greater than 98% at 25°C in aqueous solvent.[13c] The secondary structure of the peptides listed in Table 2.1 was determined CD spectroscopy (Figures 2.5, 2.6, 2.7). The **OWOWO**peptide had a local minimum near 218 nm indicative of a β-hairpin fold but it also had a minimum near 205 nm which has been reported in the spectra of other β-hairpin peptides with type II' turns and aromatic residues (Figure 2.5).[22] The exciton coupling between the indole rings of the Trp residues was observed by the presence of a local maximum at 225 nm and may be responsible for the shift in the minimum at 205 nm. The minimum at 205 nm may also represent a distortion of the fold to accommodate the cross-strand cation-π interaction between Orn and Trp residues on the non-hydrogen bonded face. The scrambled peptide, **ScramOWOWO**, had a minimum near 195 nm which is characteristic of a random coil (Figure 2.5).[13c] This observation confirmed that moving the turn sequence to the C-terminus of the peptide prevented the formation of any secondary structure though the identification of the amino acids and the chain length of the primary sequence was identical to that of the **OWOWO**β-hairpin peptide. The **Trp-Pocket dPro** peptide had a minimum near 218 nm and maximum near 230 nm indicative exciton coupling between the cross-strand Trp-Trp pair (Figure 2.5). The precedence of this strong Trp-Trp interaction in CD spectroscopy masked the minimum near 215 nm characteristically found in the CD spectra of β-hairpins and β-sheets.[20a]

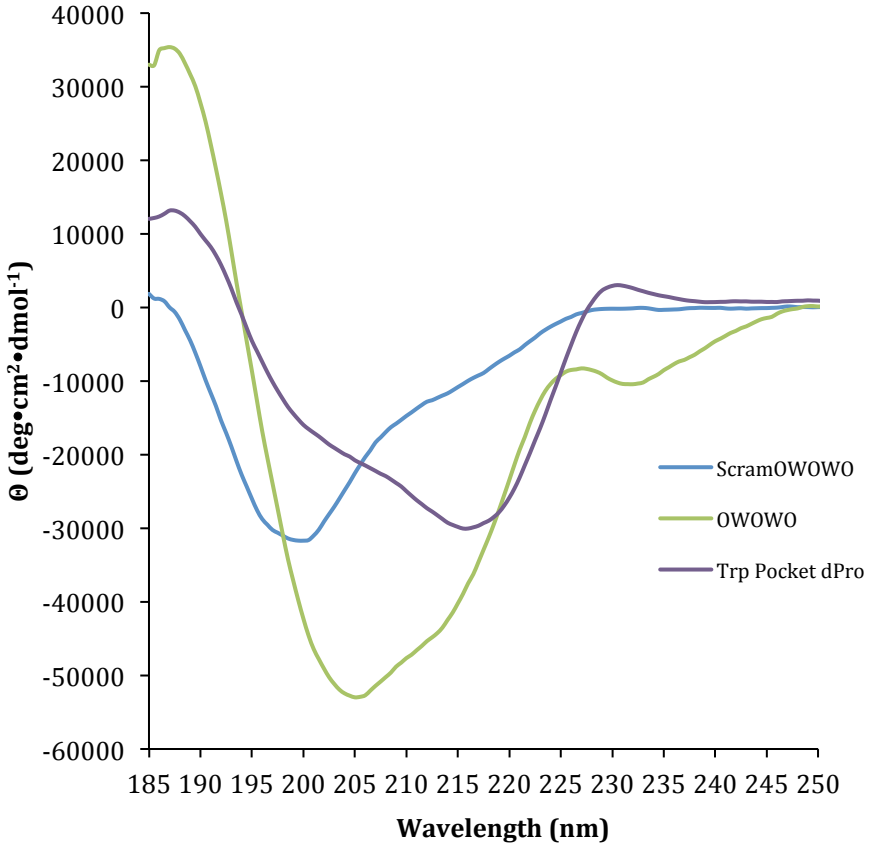


Figure 2.5 Circular dichroism spectra of the **ScramOWOWO**, **OWOWO**, and **Trp Pocket dPro** peptides. Experiments were done at 40 μM peptide concentration in 10 mM sodium phosphate buffer, pH 8 at 25°C. **ScramOWOWO** (blue) was found to be a random coil while **OWOWO** and **Trp Pocket dPro** (green and purple respectively) both fold into a β-sheet conformation.

The CD spectra of the Andersen capped peptides was similar to that of the trizip peptides reported by Cochran and coworkers (Figure 2.6).[20a] Since a major component of the noncovalent capping motif is a cross-strand face-to-edge Trp-Trp interaction, the similarity between the spectra was expected. Exciton coupling again was observed by the presence of a minimum near 218 nm and a local maximum near 230 nm. The Trp-Trp interactions masked the β-sheet peak at 215 nm in the spectra of all the Andersen capped β-hairpins (Figure 2.6).

[22]Mahalashmi, R.; Shanmugam, G.;Polavarapu, P.L.; Balaram, P. Chembiochem **2005**, 6, 2152-8.

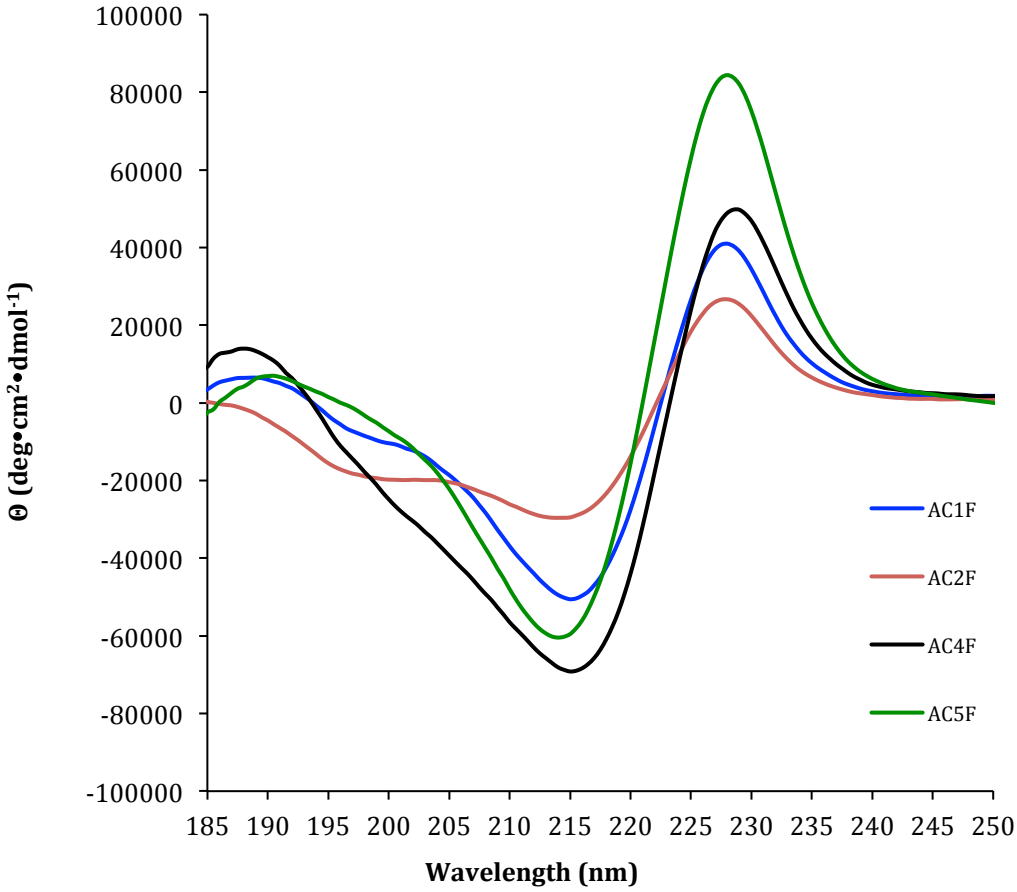


Figure 2.6 Circular Dichroism Spectra of the Andersen Capped peptides, **AC1F** (blue), **AC2F** (red), **AC4F** (black), and **AC5F** (green). Experiments were done at 40 μM peptide concentration in 10 mM sodium phosphate buffer at 25°C. All capped peptides fold into a β-sheet conformation and display strong Trp-Trp interactions with a minimum mean residue ellipticity near 218 nm and a maximum near 230 nm.

The secondary structure of the **ScramMirror** peptide and the **Mirror** peptide was determined using CD as well (Figure 2.7). Since the **WKWK-pGturn** peptide was confirmed to be a well-folded β-hairpin by both CD and NMR, it was expected that its predominantly D-amino acid analog, the **Mirror** peptide would fold as β-hairpin as well. The CD spectra of the **ScramMirror** and **Mirror** peptides were mirror images of the CD spectra of the **Scrambled-WKWK** and **WKWK-pGturn** peptides.[13c]The maximum near 200 nm in the CD spectrum of the **ScramMirror** peptide is indicative of a random coil conformation. The maximum near 218 nm in the spectrum the **Mirror** peptide indicates β-hairpin formation. The local maximum near 230 nm isdue to Trp-Trp interactions.

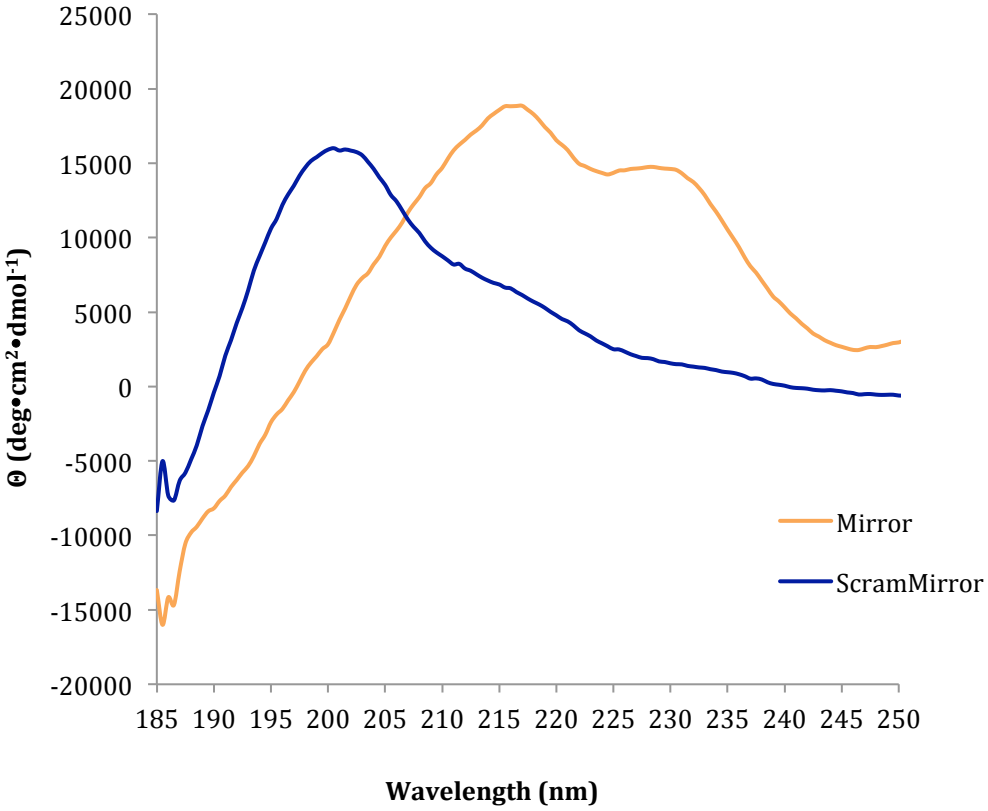


Figure 2.7 Circular dichroism Spectra of the **Mirror** and **ScramMirror** peptides. Experiments were done at 40 μM peptide concentration in 10 mM sodium phosphate buffer, pH 8 at 25°C. The **Mirror** (orange) peptide folds into a β-sheet conformation indicated by a maximum near 215 nm. The local maximum near 230 nm is due to Trp-Trp interactions. The **ScramMirror** (orange) peptide has a random coil conformation shown by the maximum near 195 nm.

ii. Determination of proteolytic stability in vitro

Based on the work published by Cline and Waters as well as ease of synthesis, we selected six peptides to be tested *in vitro* for resistance to proteolytic degradation by a mixture of peptidases. Pronase E, a mixture of endopeptidases and exopeptidases isolated from *Streptomyces griseus*, was chosen because of the wide range of targeted cleavage sites. Most peptidases in the cell are contained within organelles and not free in the cytosol, thus pronase E *in vitro* conditions were expected to be harsher than that of the cytosolic environment. The **WKWK-pGturn**β-hairpin peptide showed substantially greater resistance to pronase E than its unstructured counterpart even though its secondary structure was not locked by covalent cyclization.[13c]The peptides chosen for the pronase E degradation assay are listed in Table 2.2. The **AC4F** peptide was tested to determine the feasibility of using noncovalent capping motifs to increase resistance of β-hairpin peptides to degradation by proteases. The **OWOWO** peptide was tested to determine if any resistance to proteolytic degradation could be gained by the incorporation of a noncanonical amino acid with the same stereochemistry found in natural proteins. The **ScramOWOWO** peptide was included to determine the necessity of secondary structure when nearly half of the primary amino acid sequence contained noncanonical amino acids. While the four peptides mentioned above were tested in triplicate, the **ScramMirror** and **Mirror** peptides were tested in duplicate and served as negative degradation controls since natural enzymes are highly stereospecific and thus these peptides were expected to be resistant to degradation. Since there was one natural amide bond in both peptides, ($_{L}$-Pro-Gly), these peptides would also serve to give insight on whether or not secondary structure was necessary to protect that natural peptide bond.

Each peptide was incubated with pronase E at 37°C for 24 hours, at the enzyme concentration necessary for the **ScramOWOWO** peptide to have a half-life of less than 10 minutes. At 16 different time points, aliquots of the reaction mixtures were quenched with acetic acid and the amount of peptide degradation at each time point was determined by analytical RP-HPLC. The acetic acid used to quench the reaction also served as an internal standard. To determine the fraction of intact peptide remaining, the parent peak was integrated in the Empower Pro 2 analytical RP-HPLC software and was graphed as a function of time (Figure 2.8).

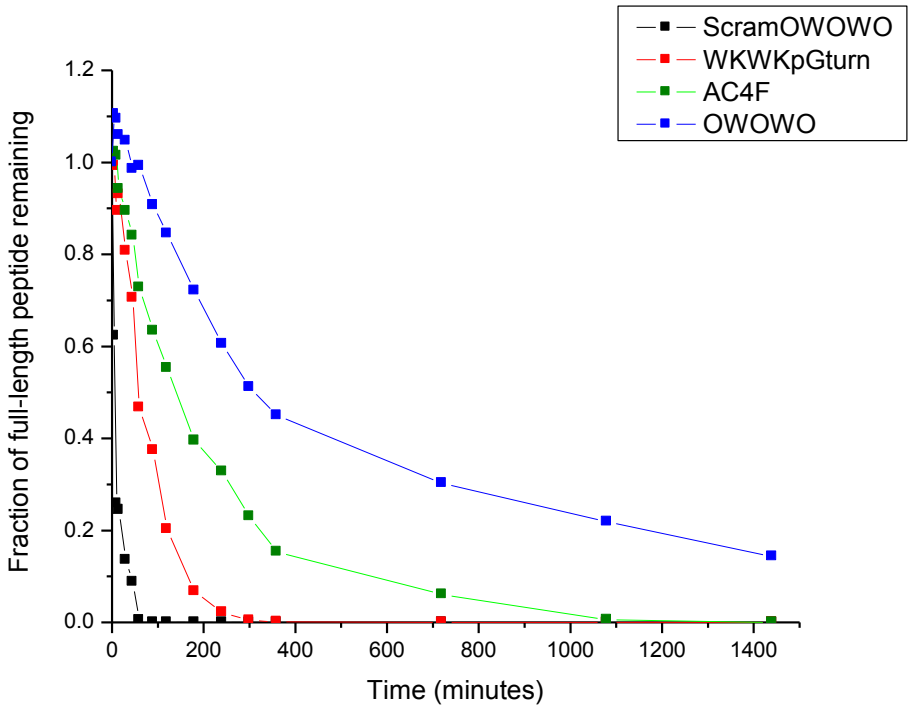


Figure 2.8 Analytical RP-HPLC analysis of peptide degradation by pronase E. Experiments performed with 100 μM initial peptide concentration, in 10 mM phosphate buffer, 100 mM NaCl, pH 7.6 at 37°C. The **ScramOWOWO** peptide degraded fully after 15 minutes. The **WKWK pGturn**, **AC4F**, and **OWOWO**β-hairpin peptides show greater resistance to proteolysis. Graph represents the average peptide degradation of experiments done in triplicate.

The unstructured **ScramOWOWO** degraded quickly relative to the β-hairpin peptides including relative to the **OWOWO** peptide, as expected with half-lives of 7 minutes and 315 minutes, respectively(Figure 2.8). Unexpectedly, the **ScramOWOWO** peptide with four Orn residues and a bulky fluorophore attached which were absent from the **WKWK-pGturn** peptide, still degraded much faster with a half-life of 7 minutes in comparison to 40 minutes for **WKWK pGturn**. This suggests that the inclusion of sidechain unnatural functional groups alone, in the absence of increased secondary structure, is not enough to impart substantial resistance to proteolytic degradation.

The **AC4F** and the **OWOWO**β-hairpin peptides both contain residues capable of engaging in cross-strand cation-π interactions to impart stability just like those found in **WKWK pGturn**, however these two peptides have additional features to increase stability to proteolytic degradation. The **AC4F** peptide contained the Andersen capping motif, which includes a number of energetically favorable noncovalent terminal interactions.[15] These additional terminal contacts, which presumably reduced terminal fraying,are likely responsible for the increased half-life of **AC4F** over **WKWK pGturn**. Since the addition of Orn residues and a fluorophore alone did not make much difference in the rate of degradation of the **ScramOWOWO** peptide, this greater half-life of **AC4F** over **WKWK pGturn** can be attributed to its improved folding. The most stable peptide however was the **OWOWO** peptide, with 4 internal and terminal Orn residues. Though it lacks the capping motif, the combination of non-canonical amino acids and secondary structure proved to be ideal, resulting in half-life of 315 minutes.

The **Mirror** and **ScramMirror** peptides appeared to show no degradation over 24 hours in their analytical RP-HPLC chromatograms (Figures 2.10, 2.11). However,

without mass spectrometry confirmation, it cannot be said with certainty that these two peptides were not cleaved. The only scissile bond of the **Mirror**β-hairpin available to the proteases is in the middle of the peptide. If the proteases had cleaved the Mirror peptide, the resulting fragments would have differed significantly in polarity and size and therefore would have likely had an elution time different from the intact parent peptide. Since only one peak was seen throughout the 24-hour study, it is highly likely that no degradation took place. The native amide bond of the **ScramMirror** peptide however was at the C-terminus and could have been readily accessed by the pronase E proteases. Cleavage at this bond would have resulted in a peptide fragment that would be similar in size and polarity to the parent peptide and could therefore co-migrate with the parent peptide. Any future studies done with these peptides would require mass spectrometry confirmation of their resistance to proteolytic degradation.

Table 2.2. Peptide Dimensions and Resistance to proteolysis by pronase E[a]

Peptide Name	**β-Hairpin Dimensions (Å)**	**$t_{1/2}$ (min)**
ScramOWOWO	**----**	7
WKWK-pGturn	**21 x 13**	40
AC4F	**----**	143
OWOWO	**21 x 12**	315
Mirror	**21 x 13**	ND
ScramMirror	**----**	ND

[a] $t_{1/2}$ time necessary for 50% of peptide to be degraded by pronase E; ND denotes No Degradation observed; Studies with **ScramOWOWO**, **WKWK pGturn**, **AC4F** and **OWOWO** were done in triplicate; **ScramMirror** and **Mirror** were tested in duplicate.β-hairpin dimensions were determined using Spartan'04 software

D. Conclusions

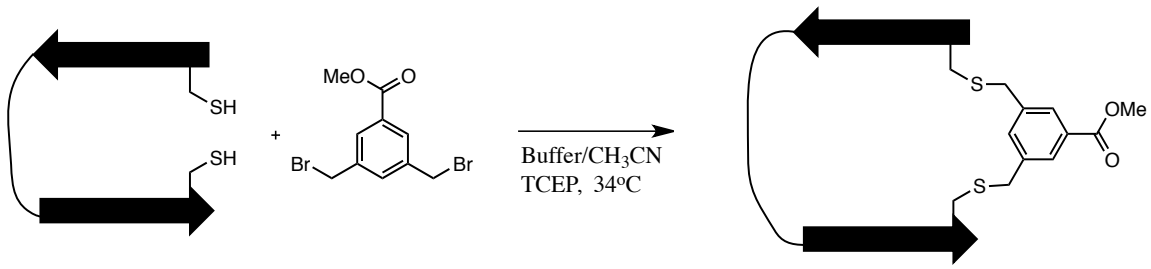
We successfully designed short (9-14 residues) well-folded β-hairpin peptides that showed resistance to rapid degradation by proteolytic enzymes. By using a strong turn-nucleating sequence, cross-strand noncovalent interactions, and strong noncovalent interactions between the termini, all of the peptides designed showed the desired β-hairpin or random coil structure (Figures 2.5, 2.6, 2.7). The redesigns of **WKWK pGturn** increased its resistance to degradation by pronase E by 7-fold as shown in Figure 2.8 and Table 2.2. The **OWOWO** peptide was found to have the longest half-life, with the exception of the D-amino acid peptides**Mirror** and **ScramMirror**, with 50% degradation occurring after 5 hours. The **WKWK pGturn** peptide exhibited a half-life of 40 minutes, demonstrating that the ornithine substitutions and FAM significantly increase the resistance of the peptide to proteolytic degradation. The **AC4F** peptide, with the noncovalent capping motif reported by Kier et al., showed greater protease resistance than the **WKWK pGturn** peptide with a half-life of 143 minutes. Resistance to proteolytic degradation may not correlate with degree of folding and the number of unnatural amino acids equally. **AC4F** with its terminal capping motif may be a better folded β-hairpin than the **OWOWO**peptide, though this cannot be confirmed without further investigation with NMR and/or CD thermal melts. Replacing all of the basic amino acids with Orn could possibly increase the resistance of the **AC4F** peptide. The **Mirror** and **ScramMirror** peptides did not appear to be degraded at all when analyzed by analytical RP-HPLC, however these results must be confirmed by mass spectrometry

where the presence of degradation products similar in mass and polarity would be revealed.

E. Ongoing Work

While a half-life of 315 minutes is a 45-fold improvement over the control peptide, an ideal protectide would be made of predominantly L-amino acids and would have a half-life of at least 24 hours. Recently, the cyclized version of **OWOWO**was synthesized in an attempt to achieve this goal. The **OWOC** peptide (Table 2.1, Figure 2.9) includes a cysteine residue at both termini whichallows for covalent cyclization through reaction with methyl 3,5-dibromobenzoate (Scheme 2.1). This method of cyclization was adapted from the work done independently by Timmerman and coworkers and Heinis coworkers used to cyclize linear peptides.[23] The advantage of this method of cyclization as opposed to a disulfide bridge between the two termini is that the thioether bonds are resistant to reduction in the cytosolic environment. Cyclization of **OWOC** using a dibromoxylene is expected to also provide increased resistance to hydrolysis by peptidases at the termini. Comparisons between noncovalent and covalent solutions to terminal fraying and the positioning of non-canonical amino acids can be made based on the half-lives of the **OWOCyc** (Figure 2.9) peptide and the **AC4F** peptide in the presence of proteases. Based on the long half-life of the uncyclized **OWOWO**β-hairpin peptide in the presence of endopeptidases and exopeptidases from pronase E, the cyclized version is expected to remain intact even longer. The **ScramOWOWO**, **WKWK pGturn**, **AC4F**, **OWOWO**, and **OWOCyc** peptides will be incubated with HeLa whole cell lysates at 37°C and their lifetimes will be compared. It will be interesting to compare the differences in the

lifetimes of these peptides between cell lysates and pronase E. Currently, work is also being done to characterize the secondary structure of the **OWOCyc** peptide by CD.



Scheme 2.1. Cyclization of **OWOC** to form **OWOCyc**

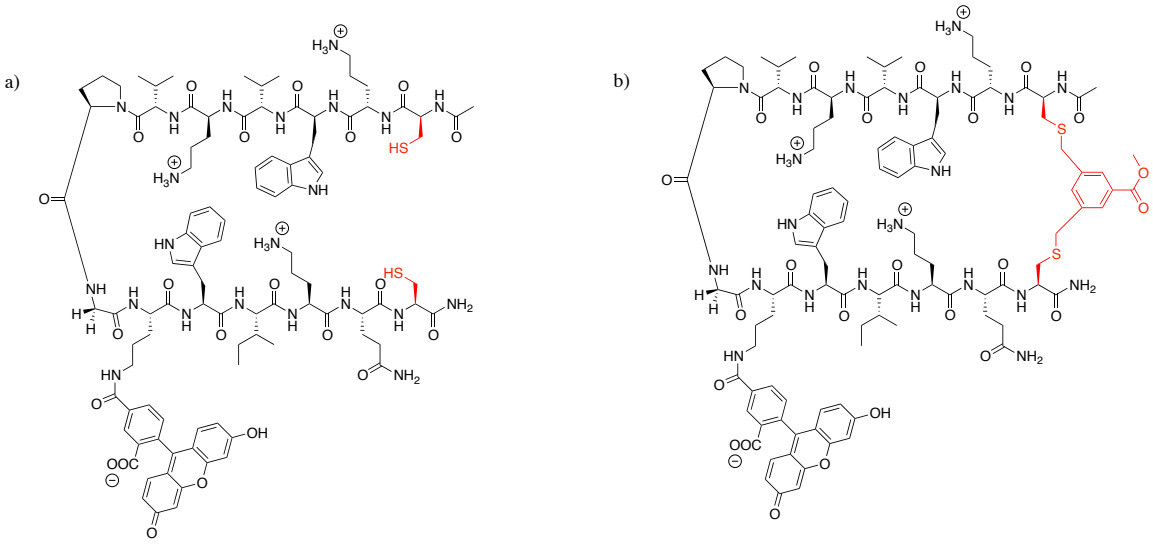


Figure 2.9. Structures of the **OWOC** and the **OWOCyc** peptides. (a) **OWOC** peptide. (b) **OWOCyc**. The functional groups participating in the cyclization are highlighted in red.

[23](a) Timmerman, P.; Beld, J.; Puijk, W.C.; Meleon, R.H.; *ChemBioChem***2005**, 6, 821-4. (b) Chen, S.; Morales-Sanfrutos, J.; Angelini, A.; Cutting, B.; Heinis, C. *ChemBioChem***2012**, 13, 1032-38.

F. Experimental Section

Peptide Synthesis and Purification

Peptides were synthesized using manual or automated standard solid phase peptide synthesis (Thuramed Peptide Synthesizer, CEM Liberty1 Microwave Peptide Synthesizer) using Fmoc protected amino acids on 0.06-0.08 mmol of CLEAR Amide resin from Peptide International. Four equivalents of standard amino acids were used for each peptide synthesis while 1.5-4 equivalents of noncanonical amino acids and fluorophores were used. All peptides were acetylated at the N-terminus by treating the resin with 5% acetic anhydride and 6% 2,6-lutidine in 5 mL of DMF bubbling with N_2 for 40 minutes. 5(6)-Carboxyfluorescein (FAM) was coupled to the ε-NH of lysine or the δ-NH of ornithine at the i+4 position of the beta-hairpins or the centrally located amino acids of the scrambled sequences. The sidechains of these amino acids were orthogonally protected with an allyloxycarbonyl group (N-Alloc) or with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-3-methylbutyl (N-ivDde). Alloc removal was done based on the protocol published by Pazos et al.[24] The removal of ivDde from the sidechain of lysine or ornithine was accomplished by treating the peptide resin 3 x 2 mins with 20 mL of 2% hydrazine monohydrate in DMF bubbling with N_2 followed by washing with DMF, MeOH, DCM. Following deprotection, FAM was coupled using 2-4 equivalents of FAM, 5 equivalents of PyBOP/HOBt and 8 equivalents of DIPEA in 5 mL of DMF and allowed to bubble with N_2 for 4-8 hours.

[24]Pazos, E.; Jiménez-Balsa, A.; Maszareñas, J.L.; Vázquez, M.E. *Chem Sci***2011**, 2, 1984-87

Double coupling was sometimes required. Kaiser testing was done after the capping, deprotection and coupling steps. Peptides without cysteine were cleaved from the resin in 9.5:2.5:2.5 trifluoroacetic acid (TFA), TIPS and water respectively for 3-4 hours. Peptides containing cysteine were cleaved using 9.4:2.5:2.5:1 TFA, EDT, water and TIPS respectively for 3-4 hours. The TFA was evaporated and the cleaved peptides were precipitated using cold ethyl ether and extracted with water. Extracted peptides were lyophilized and then purified using semi-preparative RP-HPLC on a Vydac C18 semi-preparative column with a gradient from '0 to 100% B' in 45-120 minutes. Solvent A was 95% water, 5% acetonitrile and 0.1% TFA and Solvent B was made of 95% acetonitrile, 5% water and 0.1% TFA. Purified peptides were lyophilized and their synthesis and purity confirmed by ESI-TOF or MALDI-TOF using 2,5-dihydroxybenzoic acid matrix.

Peptide Concentration Determination

UV/Vis was used for the concentration determination of each peptide in 10 mM sodium phosphate buffer (100 mM NaCl, pH 7.48 for protease degradation studies) pH 8 using FAM abosorbance at 492 nm, ε_{492} = 78,000 M^{-1} cm^{-1}, for peptides with FAM or by 6M guanidinium chloride denaturation and tryptophan absorbance at 280 nm ε_{280} = 5690 $M^{-1}cm^{-1}$ for peptides without FAM.

Circular Dichroism Spectroscopy

CD spectroscopy data was collected using an Applied Photophysics Chiroscan Circular Dicroism Spectrophotometer. Spectra were generated at 25 °C with a wavelength scan 260 nm to 185 nm, with 0.5 second scanning in a 0.1 cm cell. Peptide concentrations of 40 μM in 10mM sodium phosphate buffer, pH 8.0 (peptides with FAM) or pH 7.4 (peptides without FAM) were used.

Peptidase Concentration Determination

Pronase E enzymes from *Streptomyces griseus* were purchased as a lyophilized powder from Sigma-Aldrich (EC 3.4.24.31). Stocks were made by bringing up 5 mg of enzyme in 15 mL of 10 mM sodium phosphate buffer, 100mM NaCl pH 7.48 (0.33 mg/mL). 1 ml aliquots were stored in the -80°C freezer for future use. As 7 μL of enzyme stock in 1.3 mL of peptide solution completely digested the **ScramOWOWO** peptide in 15 minutes, this was the volume of enzyme used in all protease studies.

Peptide Degradation by Pronase E

The enzymatic proteolysis procedure was adapted from Cline and Waters.[13c] The reaction was initiated by the addition of 7 μL of pronase E stock solution to 1.293 mL solution of 100 μM peptide in 10 mM sodium phosphate buffer, 100 mM NaCl pH 7.5 at 37°C . 75 μL aliquots were taken at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 720, 1080, 1440 minutes and quenched with 10 μL of acetic acid and 15 μL of buffer generating a total volume of 100 μL. 25 μL of each quenched sample was injected on the analytical RP-HPLC (Waters) and the fraction of peptide remaining at each time point

was determined based on the absorbance of each peptide at 214 nm and/or 280 nm. Peak integrations were done using Empower Pro 2 software. The acetic acid peak was used as an internal standard. The fraction of full-length peptide at each time point was determined by dividing the total peak area of the full-length peptide at each time point by the area of the acetic acid peak. This number was divided by the corresponding value of the zero time point.

Peptide Cyclization

To a solution of **OWOCys** peptide (20 μM final concentration) in 20 mM $NH_4HCO_3/(NH_4)_2HCO_3$, pH 9.64, methyl 3,5-bis(bromomethyl)benzoate (in 80:20 buffer and CH_3CN) was added slowly (20%/hr, 224 μM final concentration) in the presence of 500 μM TCEP . The reaction proceeded at 34°C in the dark for 6 hours and monitored by MALDI mass spectrometry using 2,5-dihydroxybenzoic acid as the matrix. The total reaction volume was 250 μL, containing 150 μL peptide and 100 μL methyl 3,5-(dibromomethyl)benzoate.

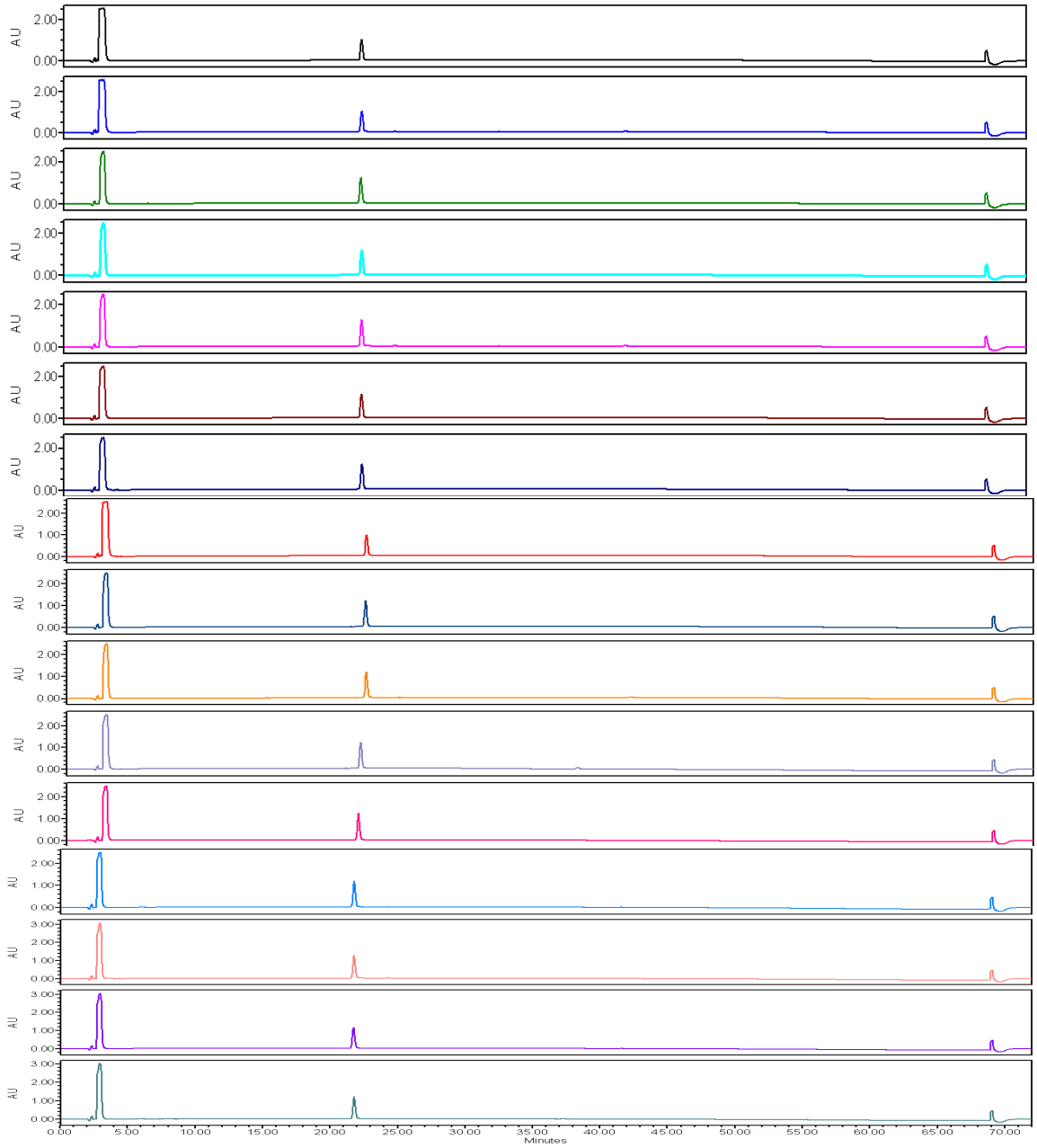


Figure 2.10. Analytical RP-HPLC chromatograms of the **Mirror** peptide incubated with pronase E for different time periods. Reactions were quenched with acetic acid (peak migration time around 2 minutes) at (black) **0 m**, (blue) **5 m**, (green) **10 m**, (cyan) **15 m**, (magenta) **30 m**, (burgundy) **45 m**, (midnight blue) **60 m**, (red) **90 m**, (denim blue) **120 m**, (orange) **180 m**, (periwinkle) **240 m** (pink) **300 m**, (sky blue) **360m**, (peach) **720 m**, (purple) **1080 m**, (sea green) **1440 m** (Time points listed from top to bottom). Peptides were detected by absorbance at 220 nm.

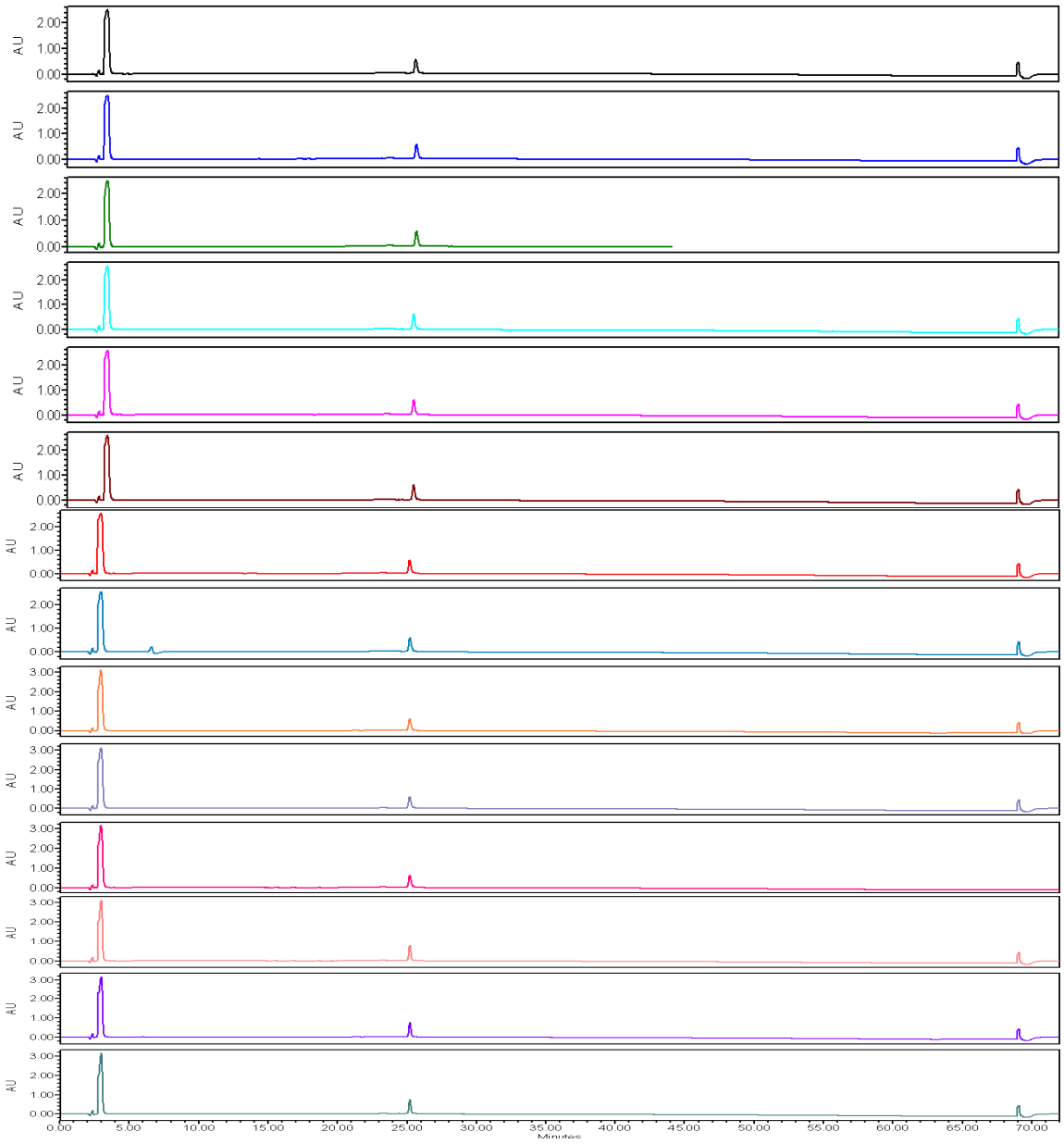


Figure 2.11. Analytical RP-HPLC chromatograms of the **ScramMirror** peptide incubated with pronase E for different time periods. Reactions were quenched with acetic acid (peak migration time around 2 minutes) at (black) **0 m**, (blue) **5 m**, (green) **10 m**, (cyan) **15 m**, (magenta) **30 m**, (burgundy) **45 m**, (midnight blue) **60 m**, (red) **90 m**, (denim blue) **120 m**, (orange) **180 m**, (periwinkle) **240 m** (pink) **300 m**, (sky blue) **360m**, (peach) **720 m**, (purple) **1080 m**, (sea green) **1440 m** (Time points listed from top to bottom). Peptides were detected by absorbance at 220 nm.

Chapter III

COVALENT LINKAGE OF PROTECTIDES TO UNSTRUCTRURED KINASE AND PROTEASOME SUBSTRATES FOR ENHANCED PROTEASE STABILITY

A. Background

Emergence of molecularly targeted therapeutics

Increased awareness of the complexity of healthy cell homeostasis and diseased cell dynamics has led to the emergence of disease-specific molecularly targeted therapeutics, particularly in the treatment of hematologic cancers such as multiple myeloma (MM) and chronic myelogenous leukemia (CML). The development and FDA approval of a 26S proteasome inhibitor for the treatment of MM in the past decade and a half, have extended the median survival from 2-3 years after diagnosis to 5-7 years.[1] The existence of the Philadelphia chromosome, a chromosomal translocation gene fusion product (t(9;22)(q34;q11)) which results in a constitutively active oncoprotein, BCR-Abl tyrosine kinase, has long since been known to be the culprit of most cases of CML.[2] The development of Abl tyrosine kinase inhibitors for the treatment of CML has extended patient prognosis from 3-4 years in 1999, to 10+ years in 2014.[2]

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[1] (a) Samson, D. Postgrad Med J, 1994, 70, 404-10. (b) Mahindra, A.; Laubach, J.;Raj, N.; Munshi, A.; Richardson, P.G.; Andersen, K. Nat Rev Clin Oncol, **2012**, 9, 135-43.

[2]Jabbour, E.; Kantarjian, H. *Am J Hematol*, **2014**, 89, 547-556.

Combination therapy, involving the use of multiple inhibitors simultaneously to treat a patient has been a long-standing practice even prior to knowledge of inhibitory mechanism of action. Perifosine and TAS-117, allosteric inhibitors of protein kinase B (PKB also known as Akt), have shown synergistic anti-MM tumor activity when coupled with proteasome inhibitors and other immunodulatory drugs used to treat MM.[3] Though the treatments described above have been very successful in the treatment of MM and CML, the success has been dampened in recent years by the formation of drug-resistant tumor cell populations.[1,2]

The development of diagnostic tests specific for determining the outcome of patients treated with targeted therapeutics is lagging far behind that of drug development. Diagnosis of MM and CML primarily involves detection of cytogenetic abnormalities in biopsy samples and detection of disease-specific proteins in the blood and urine.[4] Ideally, diagnostic testing would include direct testing for aberrant enzymatic activity in biopsied cells to determine which protein inhibitors would most benefit the patient. Also, these assays could be used to test patient cells during follow-up visits to directly determine if the inhibitors are successfully blocking enzymatic activity and if resistant tumor cell populations have arisen.

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[3](a) El-Amm, J.; Tabbara, I.A. *Am J Clin Oncol*, **2013** [Epub ahead of print]. (b)Mimura, N.; Hiseshima, T.; Shimomura, T.; Suzuki, R;, Ohguchi, H.; Rizq, O.; Kikushi, S.; Yoshida, Y.; Cottini, F.; Jakubikova, J.,;Cirstea, D.; Gorgun, G.; Minami, J.; Tai, Y.T.; Richardson, P.G.; Utsugi, T.; Iwama, A.; Anderson K.C. *Cancer Res*, **2014**, [Epub ahead of print].

[4](a) Kyle, R.; Rajkumar, A.V. *Blood*, **2008**, 111, 2962-72,(b) Bataille, R.; Annweiler, C.; Beauchet, *O. Clin Lymphoma Myeloma Leuk*, **2013**, 13, 635-7.

Molecular activity screening would be useful in clinical trials and in the early stages of testing new drugs as well. [5] The goal of many clinical trials goes beyond detection of anti-tumor activity. In addition to tumor growth, researchers and clinicians are monitoring the correlation, if any, between the anti-tumor response and change in biomarker levels, particularly plasma protein levels.[2] Clinicians in each trial try to match patient outcome with an easily detected change in molecular events. The Allbritton Group in collaboration with the Lawrence Group at UNC have done extensive work to develop assays that would allow rapid intracellular enzymatic activity analysis in single intact patient cells collected from biopsy samples.[6] The foundation of these assays is the use of fluorescent peptide substrates that can be recognized and modified specifically by the protein of interest and then detected by single cell capillary electrophoresis coupled with laser-induced fluorescence (CE-LIF). While single cell CE-LIF is a highly sensitive semi-automated technique that can be used to detect subnanomolar concentrations of modified peptides rapidly after single cell injection, rapid degradation of reporter peptide by cytosolic peptidases in the cell has hampered progress toward marketing this technique.

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[5] Rodon, J.; Dienstmann, R.; Serra, V.; Tabernero, J.*Nat Rev Clin Oncol*, **2013**, 10,143-53.

[6](a) Sims, C. E.; Allbritton, N.L. *Curr Opin Biotechnol*, **2003**, 14, 23-8. (b) Kovarik, M.L.; Allbritton, N.L. *Trend Biotechnol*, **2011**, 29, 222-30. (c) González-Vera, J. A. *Chem Soc Rev*, **2012**, 41, 1652-64.

The fate of peptides in the immune cell

One of the biggest obstacles in studying real-time cellular events using unstructured peptides is their susceptibility to degradation by cytosolic peptidases.[7] The cytosol of immune cells has particularly high protease activity due because of their roles in recognizing and signaling the presence of foreign invaders including viruses and bacteria. Degradation of inactivated cellular proteins or of foreign invader proteins usually begins with a big proteolysis complex known as the 26S proteasome. Proteosomal degradation takes place in both the nucleus and cytosol of the cells, allowing for rapid protein turnover regardless of protein location in the cell. Proteins in immune cells are first tagged for degradation by the 26S proteasome or the immunoproteosome, a large degradation complex very similar to the 26S proteasome, but has a few differing subunits in the catalytic cleft.[8] Proteasomal degradation of the targeted protein is initiated at the C-terminus of the protein and results in the emergence of oligopeptides of various lengths, usually between 4-20 amino acids long. The proteasome is the only confirmed carboxypeptidase in the cytosols of immune cells. Oligopeptides are further digested in the cytosol only (no peptidase other than the proteasome is known to exist in the nucleus) by aminopeptidases and a few endopeptidases.[7] Most peptides are rapidly hydrolyzed down to their individual amino acids but a few escape degradation and are used for immune cell presentation.[7]

[7]Yewdell, J.W.; Reits, E.; Neefjes, J. Nat Rev Immunol, **2003**, 952-61.

[8]Kramer, H.B.,;Nicholson, B.; Kessler, B.M.; Altun, A. *Biochim Biophys Acta*, **2012**, 1823, 2029-37.

These few surviving peptides are thought to be bound by chaperones that protect them from complete degradation. The peptides are trimmed further at their N-termini to make them the correct size for antigen presentation, and then they are trafficked to the cell surface where they are bound and presented to sentinel T cells by the major histocompatibility complex (MHC).[7]

N-terminal protection

While trying to elucidate the mechanism of MHC class I antigen presentation on immune cells, Mo et al. reported that N-terminal acetylation of antigen peptides prevented their presentation by the MHC proteins because they could not be trimmed to the appropriate length for presentation by aminopeptidases.[9] (Evidence of antigen peptide cleavage by endopeptidases was observed however.) Building on their work, Reits et al. designed a peptide that was resistant to hydrolysis by aminopeptidases found in human melanoma cell line, by capping it at the N-terminus with an Fmoc group.[10] Shibata et al. attached the 10-amino acid cyclic RES-703-1 peptide to the N-terminus of biologically active unstructured substrate peptides, and observed up to a 4-fold increase in resistance in the presence of trypsin and chymotrypsin in vitro over 4 hours.[11]

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[9] Mo, X.Y.' Cascio, P.; Lemerise, K.; Goldberg, A.L.; Rock, K. *J Immunol*, **1999**, 163, 5851-9.

[10]Reits, E.; Griekspoor, A.; Neijssen, J.; Groothius, T.; Jalink, K.; van Veelen, P.; Jansssen, H.; Calafat., J.; Drijfhout, J.W.; Neefjes, J. *Immunity*, **2003**, 18, 97-108.

[11]Shibata, K.; Suzawa, T.; Soga, S.; Mizukami, T.; Yamada, K.; Hanai, N.;Yamasaki, M. *Bioorg Med Chem Lett*, **2003**, 13, 2583-86.

Inspired by these results, recently we published Abl kinase peptide substrates with increased stability due to their being capped at their N-termini with a cyclized β-bend peptide.[12] We found that appending short cyclized and uncyclized β-bend peptides (5-12 amino acid peptides with a reverse turn nucleating sequence) to the N-terminus of an Abl kinase reporter extended its half-life in the presence of cytosolic proteases by 10-40 fold. The substrate stability increased proportionally with the size of the β-bend protecting peptide and the cyclized β-bends provided the greatest improvement in stability over the unprotected control. The β-bend protected Abl kinase reporter was then microinjected into Ba/F3 BCR-Abl cells and phosphorylation was observed. The unprotected substrate, when microinjected into cells, degraded too rapidly for phosphorylation to be observed. These results showed the true potential of employing a β-bend N-terminal protection strategy to increase enzyme substrate stability in diagnostic assays. Continuing with this strategy, we designed β-hairpin peptides (similar to β-bends except they are longer to utilize more cross-strand sidechain interactions to increase folding) that are highly resistant to degradation under harsh proteolytic conditions in vitro (Chapter II of this work). Below we describe the fate of kinase substrate peptides with our protease-resistant β-hairpin peptides appended to their N-termini.

[12] Yang,S.; Proctor, A., Cline, L.L.; Houston, K.M.; Waters, M.L., Allbritton, N.L. *Analyst*, **2013,** 138, 4305-11.

B. Design and Synthesis

The β-hairpin protectide

To begin probing the possibility of using β-hairpins as tools to study enzymatic events within cells, their stability in the presence of proteolytic enzymes was determined *in vitro*. Select β-hairpin peptides were incubated for 24-hours with pronase E, a cocktail of endopeptidases and exopeptidases from *Streptomyces griseus*. The proteolysis reaction was quenched at different time points and the amount of intact β-hairpin peptide remaining was monitored by analytical RP-HPLC (Chapter II, Figure 2.9, Table 2.2). The most stable β-hairpin peptide was **Mirror** peptide (no degradation observed over 24-hours). This β-hairpins along with **ScramMirror** (a random coil peptide that also showed no degradation in the presence of pronase E as monitored by analytical RP-HPLC), were selected to protect unstructured kinase and proteasome substrate peptides from degradation, and were henceforth referred to as protectides.

Kinase and proteasome substrate peptides

Protectides were attached to the N-termini of two different peptide substrates of clinically relevant enzymes, including protein kinase B (PKB) and BCR-Abl kinase (Abl). Each of the peptide substrates were investigated previously for their enzyme modification specificity and efficacy.[12-13]

[13] (a) Kunkel, M.T.,;Ni, Q.; Tsien, R.Y.; Zhang, J.; Newton, A. *J Biol Chem*, **2005**, 280, 5581-87. (b) Bonger, K.M.;Chen, L.C.; Liu, C.W.; Wandless, T.J. *Nat Chem Biol*, **2011**, 7, 531-7.

The kinetics of degradation by cytosolic enzymes has also been reported for the PKB substrate peptide and the Abl kinase substrate.[12] Listed in Table 3.1 are the amino acid sequences of the protectides and of the kinase and proteasome substrates investigated here. These two enzyme substrate peptides were chosen because inhibitors of their corresponding enzymes are currently in use as therapeutics or are in advanced stages of clinical trial investigation .[1-3]

Protectide-substrate synthesis

Full-length reporter peptides complete with a protectide region, a polyethylene glycol (PEG) spacer, an enzyme substrate region, and a fluorophore (FAM) attached to the protectide for detection, were synthesized linearly by solid phase peptide synthesis (SPPS) using standard Fmoc chemistry (Scheme 3.1). Each peptide was purified by semi-preparative RP-HPLC and purification was confirmed by ESI or MALDI mass spectrometry. The structures of the full-length reporters are shown in Figure 3.1. Work done previously by Dr. Shan Yang in the Allbritton Group evaluated the necessity of including a spacer between the protecting N-terminal group and the C-terminal substrate portion of kinase reporter peptide.[14] She found that a flexible space is required to allow unhindered phosphorylation by the desired enzyme and that the spacer length significant. Building on her work, we chose to synthesize reporters with PEG_2, $(PEG_2)_2$, or PEG_4 spacers (Figure 3.3).

[14]Yang, S. Development of Peptidase Resistant Reporters for Intracellular Enzymatic Activity. University of North Carolina at Chapel Hill, Dissertation, **2011**.pg 220

Amino acid spacers such as polyglycine, poly-β-alanine, and poly-aminohexanoic acid were considered but failure to synthesize these full-length reporters with reasonable yields caused us to proceed only with PEG spacers. The sequences of the protectide-substrate reporter investigated for resistance to proteolytic degradation *in vitro* are listed in Table 3.2.

Table 3.1 Sequences of Protectides and Kinase Substrates[a]

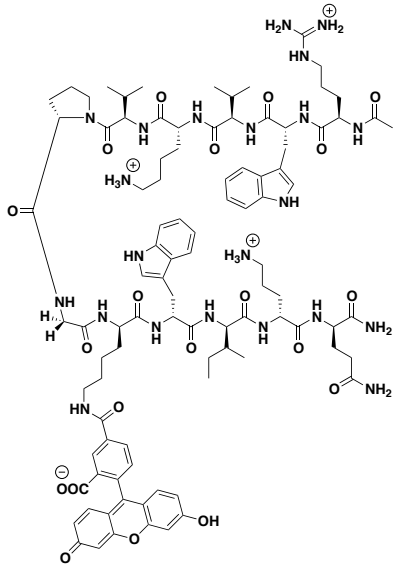
Name	Sequence	Protectide or Substrate
Mirror	Ac-^{D}R^{D}W^{D}V^{D}K^{D}VPG^{D}K(FAM)^{D}W^{D}I^{D}O^{D}Q-NH_2	Protectide
ScramMirror	Ac-^{D}R^{D}W^{D}V^{D}K^{D}VPG^{D}K(FAM)^{D}W^{D}I^{D}O^{D}Q-NH_2	Protectide
FPKB[13]	FAM-RKRDRKG**T**LGI-NH_2	Protein kinase B substrate
Abl[12]	FAM-EAI**Y**AAPFAKKK-NH_2	Abl kinase substrate

Table 3.2 Names and Sequences of Protected and Unprotected Kinase Reporter Peptides

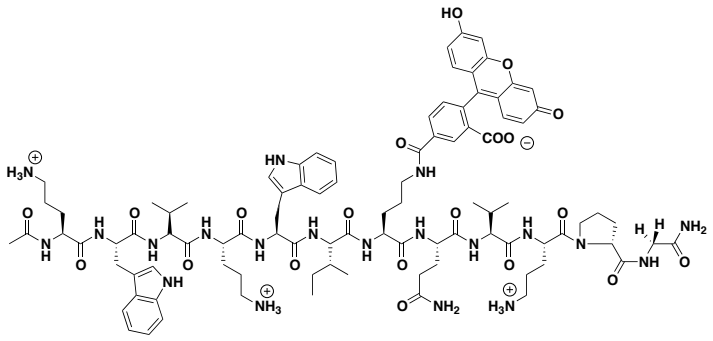
Name	Sequence
MP4B	Ac-^{D}R^{D}W^{D}V^{D}K^{D}VPG^{D}K(FAM)^{D}W^{D}I^{D}O^{D}Q-PEG_2-PEG_2-**RKRDRKG<u>T</u>LGI**-NH_2
SMP4B	Ac-^{D}R^{D}W^{D}V^{D}K^{D}VPG^{D}K(FAM)^{D}W^{D}I^{D}O^{D}Q-PEG_2-PEG_2-**RKRDRKG<u>T</u>LGI**-NH_2
ScramOWOWO	Ac-OWVOWIO(FAM)QVOpG-NH_2
MD-PEG4-Abl	Ac-^{D}R^{D}W^{D}V^{D}K^{D}VPG^{D}K(FAM)^{D}W^{D}I^{D}O^{D}Q-PEG_4- **EAI<u>Y</u>AAPFAKKK**-NH_2

[a]Kinase substrate in bold; Phosphorylatable residue highlighted in red; FAM-6-carboxyfluorescein fluorophore for detection; p denotes ^{D}Proline; M/MD denote the Mirror protectide, SM denotes the **ScramMirror** protectide.

a)



b)



c)

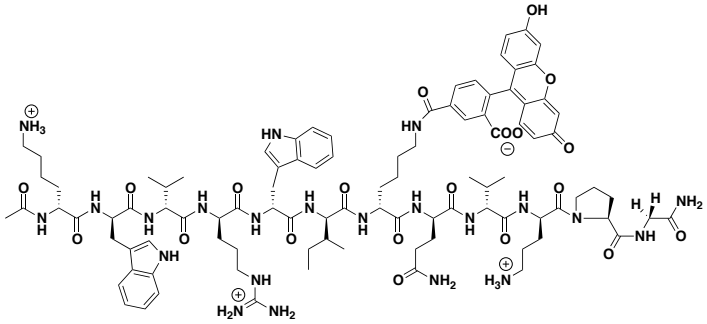
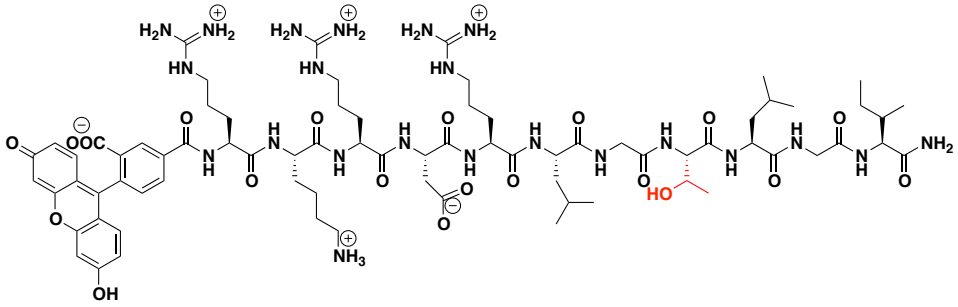


Figure 3.1 Structures of protectides,a) **Mirror** b) **ScramOWOWO** d) **ScramMirror**.

a)



b)

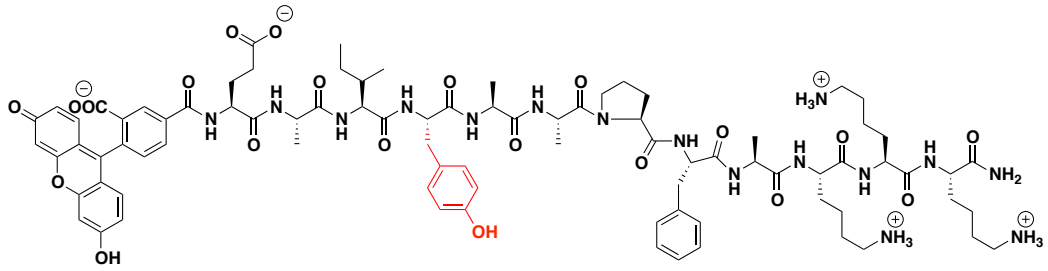


Figure 3.2 Structures of kinase substrates a) Protein kinase B substrate (**PKB**), Abl kinase substrate (**Abl**). Phosphorylatable residues are highlighted in red.

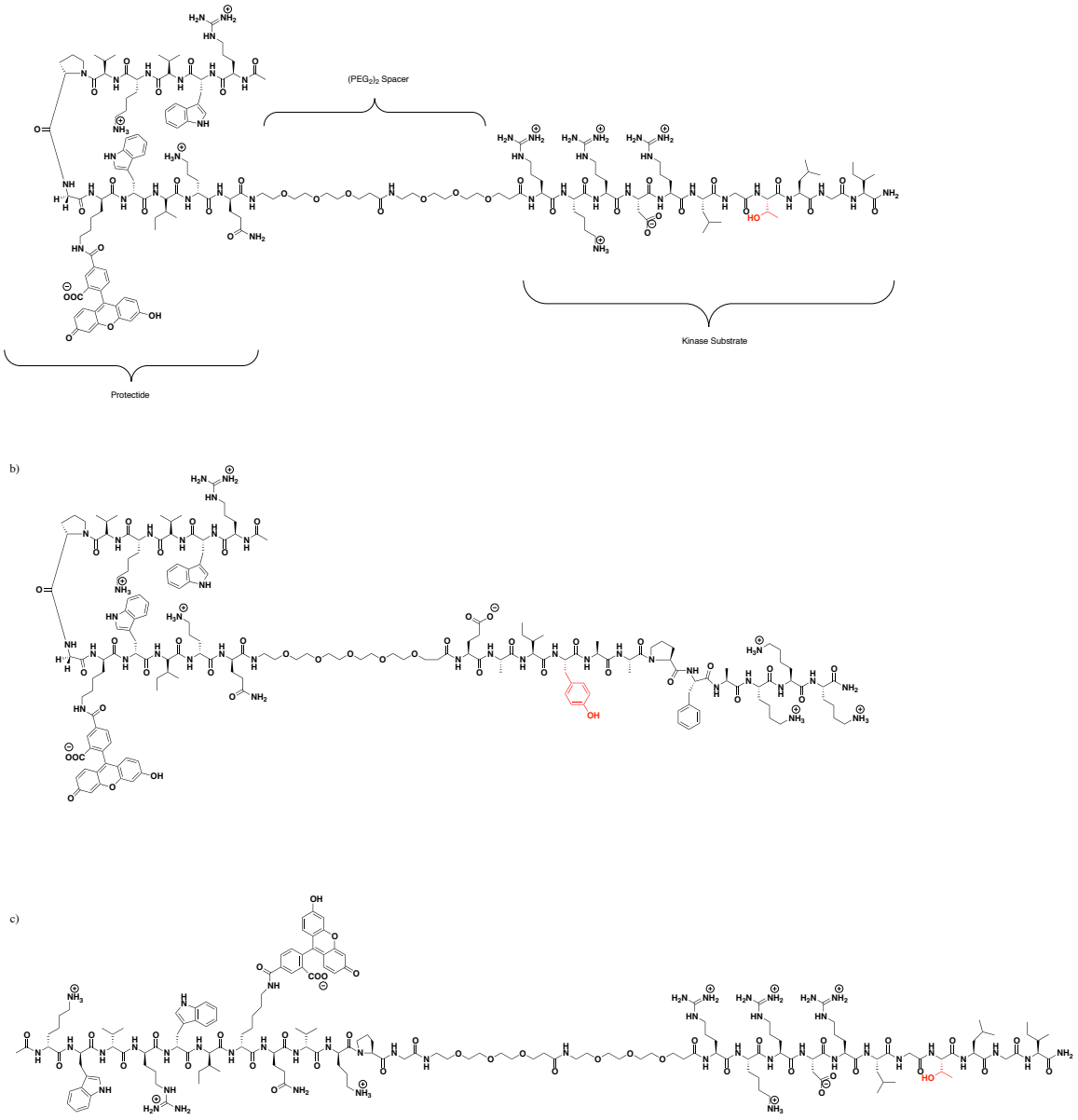


Figure 3.3 Structures of protectide-kinase substrate full-length reporter peptides. a) **MP4B** with the protectide, PEG spacer, and kinase substrate regions labeled. b) **SMP4B** c) **MD-PEG4-Abl**. Phosphorylatable residues are highlighted in red.

C. Results and Discussion

i. Secondary structure characterization of PKB reporter peptides

The **FPKB** peptide, a substrate for phosphorylation by PKB previously reported by Kunkel et al. and Proctor et al., was protected with the**Mirror** and **ScramMirror** protectides (Figure 3.1, Table 3.2). The secondary structure of the **PKB** peptide, **MP4B** peptide and the **SMP4B** peptide was determined by circular dichroism (CD). The CD spectrum of the **FPKB** peptide showed a minimum near 195 nm confirming that this peptide was indeed unstructured, and thus susceptible to rapid degradation by cytosolic proteases (Figure 3.2). The spectrum of the **MP4B** (Mirror-$(PEG_2)_2$-FPKB) reporter peptide was very similar to that of the Mirror protectide (Chapter II, Figure 2.8) with a maximum near 215 nm, characteristic of β-sheet confirmation. The peak near 230 nm can be attributed to Trp-Trp interactions. The peaks present in the **MP4B** CD spectrum are more shallow that those of the **Mirror** protectide only spectrum (Chapter II, Figure 2.8). This could mean that there is some terminal fraying caused by the C-terminal extension. However, since the CD spectra were reported using mean residue ellipticity (MRE, θ), and **MP4B** has twice as many residues as **Mirror**, the degree of folding of the protectide is likely very similar in the presence or absence of substrate. The CD spectrum of the **SMP4B** (Scrambled Mirror- $(PEG_2)_2$-PKB) reporter peptide is quite interesting. Unexpectedly, it doesn't display the characteristic random coil maximum peak near 195 nm as was seen in the **Scrambled Mirror** peptide spectrum (Chapter II, Figure 2.8). It appears as though a mixture of conformations may be present. There is a shallow minimum near 190 nm that could be due to the C-terminal portion of the reporter which lacks secondary structure on its own, though this peak is slightly blue-shifted in

comparison to the **FPKB** peptide CD spectrum. The broad peak between 200 nm and 205 nm could be due to the random coil confirmation of the Scrambled Mirror peptide alone, although this peak is slightly red shifted (Chapter II, Figure 2.9). Ironically, there is a maximum peak near 215 nm indicative of a β-hairpin conformation. The C-terminal amino acids of the **ScramMirror** portion of the full-length reporter are Pro-Gly. Placement of this reverse turn inducing sequence at the C-terminus of **ScramMirror** as opposed to in the middle of the sequence (like in the **Mirror** protectide) resulted in a random coil confirmation. However, the Pro-Gly sequence is not at the C-terminus of the **SMP4B** peptide but in the middle, followed by a flexible PEG spacer that could form a long loop, making it possible for the reporter fold back on itself. If this is the case, the contacts are likely to be weak given that the two strands have opposite stereochemistry and were not designed to have optimal cross-strand noncovalent interactions. To accurately determine the lack or presence of secondary structure in the **SMP4B** reporter peptide, further investigation by NMR is required. Further structural characterization was not deemed necessary for our purposes but may be considered in the future.

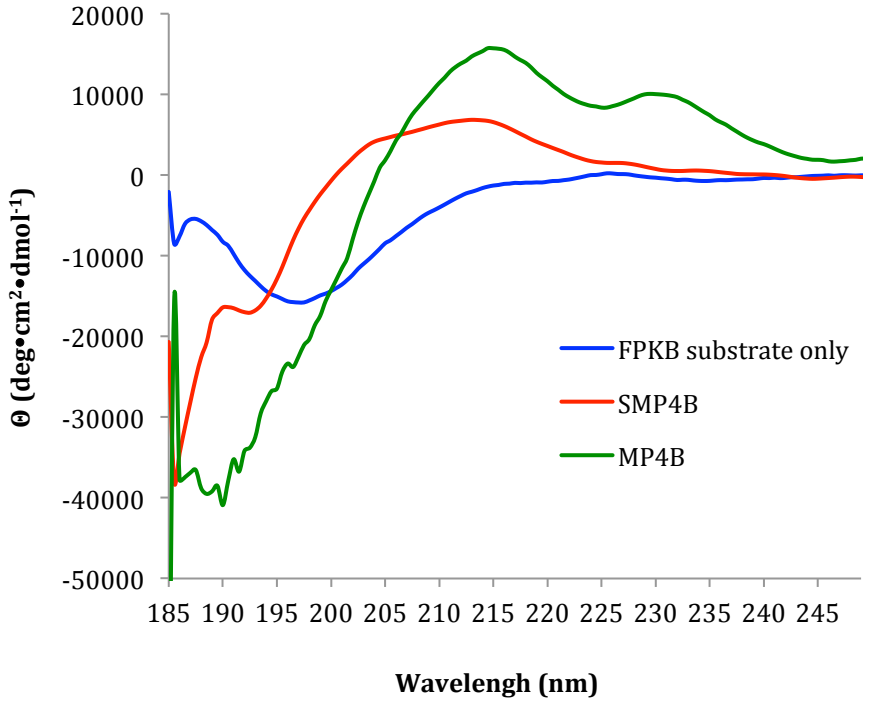


Figure 3.4 Circular dichroism spectra of the **FPKB**, the **MP4B**and the **SMP4** reporter peptides. Experiments were done at 40 μM peptide concentration in 10 mM sodium phosphate buffer, pH 8 at 25°C. The **FPKB** (blue) peptide has a random coil conformation shown by the minimum near 195 nm. The **MP4B** (green) peptide displays β-sheet character indicated by a maximum near 215 nm. The local maximum near 230 nm is due to Trp-Trp interactions. The **SMP4B** (red) peptide has an unconfirmed but likely mixed conformation.

ii. Investigation of protectide-PKB substrate reporter in vitro by pronase E

The first protectide-substrate reporter to be tested for resistance to proteolytic degradation was the **MP4B** reporter peptide. This peptide was incubated in the presence of pronase E from 24-hours and was quenched and monitored by analytical RP-HPLC at different time points. The **ScramOWOWO** peptide was also incubated with pronase E in this assay to serve as a known positive degradation control. The analytical RP-HPLC chromatograms of the assay are shown below in Figures 3.5 and 3.6. Within 5 mins at least one degradation product was seen in the chromatogram of the **MP4B**reporter peptide. Over the course of 24 hours, a few more peaks were seen at different time points. Without mass spectrometry confirmation, it was speculated that the two major degradation products corresponded to hydrolysis of the peptide bond between the PEG spacer and the protectide and possibly cleavage of the bond between the PEG spacer and the substrate. It is also possible that the Pro-Gly peptide bond in the protectide portion of each could have be hydrolyzed. Mass spectrometry is needed to identify the degradation products present. Currently, we are using MALDI mass spectrometry to correctly identify the fragments. Knowledge of the pronase E generated degradation products will be useful in the redesign strategy for making more resistant substrates. These results did not deter us from examining **Mirrror**-protected substrates in cell lysates because pronase E contains exopeptidases and endopeptidase that are not present in the cytosol and in whole cell lysates.

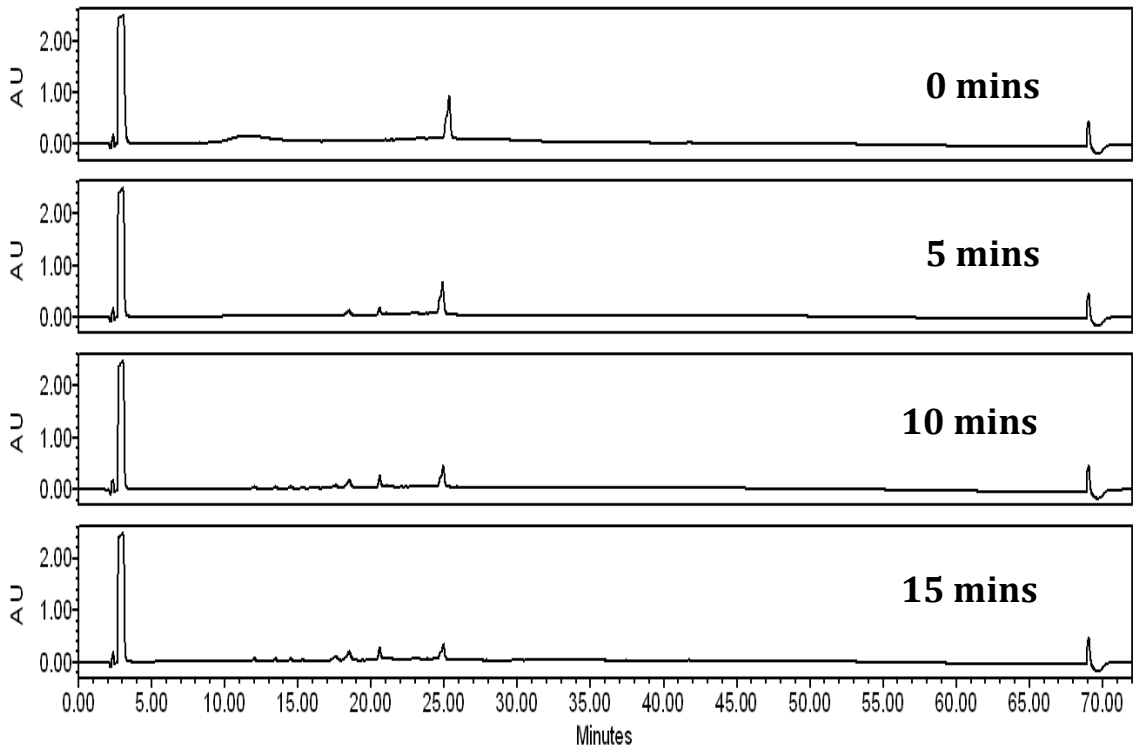


Figure 3.5 Degradation of the **ScramOWOWO** peptide in the presence of pronase E. Peptide concentration was 100 μM and degradation was observed over 15 minutes. Time points were quenched with acetic acid (peak at 2 minutes) and determination of intact peptide remaining was done using analytical RP-HPLC, monitoring at 220 nm.

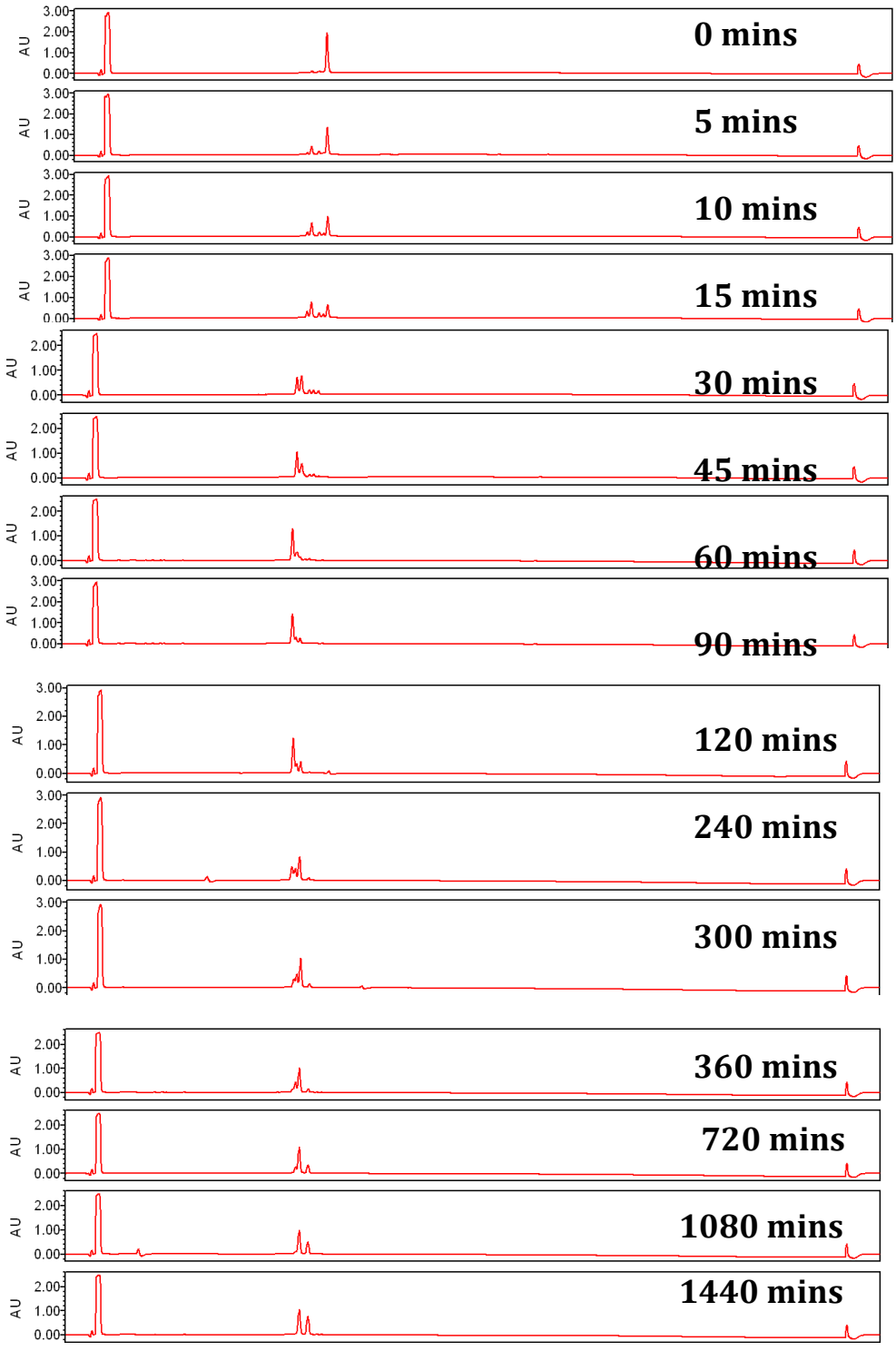


Figure 3.6 Degradation of the **MP4B** peptide in the presence of pronase E. Peptide concentration was 100 μM and degradation was observed over 24-hours. Time points were quenched with acetic acid and determination of intact peptide remaining was done using analytical RP-HPLC, monitoring at 220 nm.

iii. Investigation of SMP4B and MP4B in OPM-2 cell lysates

Next **MP4B** and **SMP4B** were incubated with OPM-2 cell lysates. OPM-2 cells are a multiple myeloma cell line that we used for several assays as a model system for the optimization of the protectides for intracellular assays. The protectide-PKB reporter peptides were incubated with OPM-2 whole cell lysates for 2 hours, quenching at various time points and analyzed to determine the amount of intact peptide remaining over the time course. The results of this cell lysate degradation assay were analyzed by capillary electrophoresis coupled with laser-induced fluorescence (CE-LIF). There were three advantages to using CE-LIF as opposed to analytical RP-HPLC: 1)fluorescence detection, 2) tunable separation of the fragments, and 3)less sample required for analysis. Fluorescence detection allows for only peptides with FAM conjugated to be detected, therefore there was no signal interference from other molecules present in the cell lysates. Optimizing CE electrophoretic separation is easier, faster, and requires far less solvent than the optimization of analytical RP-HPLC solvent systems. Finally, limit of detection of CE-LIF is much lower (subnanomolar) than that of analytical RP-HPLC (low micromolar), which is required with single-cell analysis and preferred with cell lysates as well. Cleavage of reporter peptides was observed in the early time points (data not shown). Currently, we are optimizing CE electrophoretic buffer conditions to separate all of the degradation products to allow us to calculate the amount of intact peptide remaining. We are also utilizing MALDI mass spectrometry to determine which peptide bonds of the full-length reporter peptides are most susceptible to hydrolysis. Once optimal separation conditions are found and CE and the fragments are confirmed by MALDI mass spectrometry, the reporters will be redesigned and resynthesized to increase the resistance of the vulnerable bonds.

It must be noted that these lysates were generated using the mammalian-protein extraction reagent (MPER). It was later determined after the assay that this reagent possibly contained protease inhibitors and therefore an alternative lysis protocol was used in subsequent studies.

iv. Investigation of protectide-Abl kinase reporter peptide

Work done by Dr. Imola Zigoneanu, a post-doctoral fellow in Allbritton Group, Department of Chemistry, University of North Carolina at Chapel Hill on protectides developed by Kaiulani Houston.

To further investigate the versatility the protectide strategy, an Abl kinase substrate peptide was synthesized with a PEG_4 spacer and the Mirror protectide attached (**MD-PEG_4-Abl**). First the **MD-PEG_4-Abl** reporter was incubated with purified Abl kinase *in vitro* in the presence of ATP. Phosphorylation of the protectide-reporter was observed by CE-LIF (Figure 3.7). Complete phosphorylation of **MD-PEG_4-Abl** was not observed however. This may be due the PEG4 spacer not being long enough to prevent steric hindrance during phosphorylation or it may be due to efficacy of the substrate. The degree of phosphorylation of the Abl peptide alone was not observed at the time under the same conditions, therefore a comparison could not be made. Though further optimization is required, Figure 3.7 gives strong evidence that the protectide-substrate can be designed to maintain its efficacy as a kinase substrate.

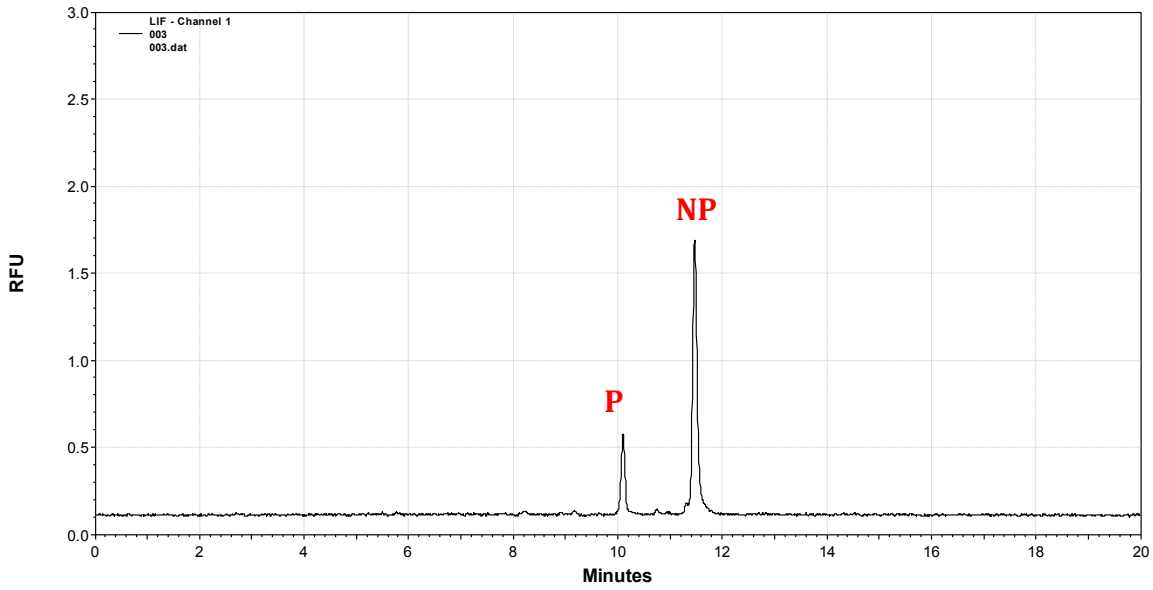


Figure 3.7 Phosphorylation of the **MD-PEG4-Abl** reporter peptide by Abl kinase. Experiments done at 37°C with 30 μM peptide concentration and 30 μM enzyme concentration for 30 minutes. Results observed by CE-LIF using 150 mM sodium phosphate, 5 mM SDC, pH 7.3 as the electrophoretic buffer in a 50 μm capillary.

To determine if the incorporation of protectide increased the stability of the Abl kinase reporter in the presence of cellular proteases, the **MD-PEG$_4$-Abl** peptide was incubated with Ba/F3 BCR-Abl cells lysates . The Ba/F3 BCR-Abl cell line are murine pro-B cells that express the oncogene BCR-Abl kinase whose constitutive activity is responsible for most cases of CML. The stability of the protectide-Abl peptide reporter was monitored at various time points between 0-30 minutes (Figure 3.8). The **Abl** peptide alone degraded quickly in lysates with a half-life around 3 minutes. The addition of the Mirror protectide and the PEG4 spacer positively impacted the stability of the Abl kinase reporter. The half-life of the **MD-PEG4-Abl** reporter was around 20 minutes, showing approximately a 7-fold improvement in stability. We believe that this increase in stability is mostly attributed to the protectide rather than the PEG4 spacer as the protectide provided the bulkiness needed to hinder entrance into the catalytic cleft of the proteases. We therefore conclude that the addition of a protectide to the N-terminus of a kinase substrate is a promising method for the generation of protease resistant reporters of enzymatic activity *in vitro*.

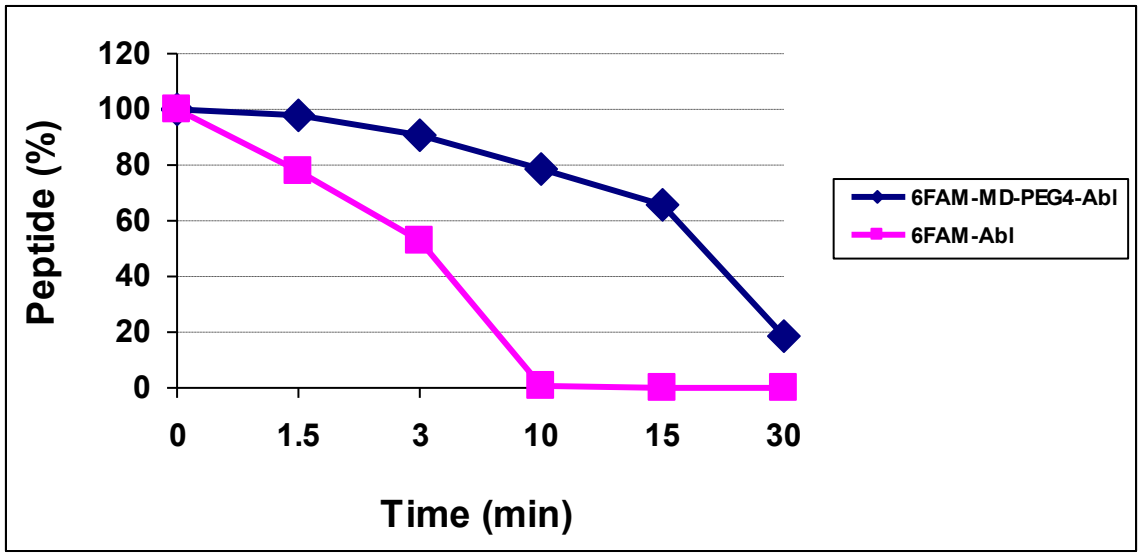


Figure 3.8 Assessment of the stability of the **Abl** kinase reporter peptide (6FAM-Abl, pink) and the **MD-PEG$_4$-Abl** reporter peptide (blue) in the presence of Ba/F3 BCR-Abl cell lysates. The y-axis represents the % of intact peptide remaining. Experiments were done at a 1 μM peptide concentration in 3mg/mL lysate at 37°C. Results observed by CE-LIF using 150 mM sodium phosphate, 5 mM SDC, pH 7.3 as the electrophoretic buffer in a 50 μm capillary.

D. Conclusions

Above we described our efforts to extend the lifetimes of kinase substrates by attaching a β-hairpin D-amino acid protectide to their N-termini. When the protectide-PKB substrate was incubated with nonspecific proteases from pronase E degradation was seen within 5 minutes. This was not unexpected given that exopeptidases that are not found in the cytosols of immune cells were present in the pronase E cocktail. Dr. Imola Zigoneanu in the Allbritton Lab synthesized a protectide-Abl kinase reporters. When incubated with cell lysates degradation was seen within 15 minutes though the protected substrate did last longer in the presence of cell lysates than the unprotected substrate .

Based on these results, we conclude that while the **Mirror** protectide is protease-resistant, it does not effectively protect C-terminal substrates from degradation. One possible explanation for our observations is that while the protectides are considered well-folded, there is a dynamic equilibrium between a folded and unstructured state. It is possible that the protectide-substrates can be woven into the catalytic clefts of proteases while the protectide was sampling an unfolded state. Another possibility is that the lysate endopeptidases began hydrolysis at the C-terminal portion of the protectide reporters since there was no negative charge from a C-terminal carboxylate at the end of the reporter peptide to deter catalytic cleft entrance. Efforts to attach cyclized protectides to kinase substrates are underway. Also, we are exploring ways to incorporate N-terminal and C-terminal negative charges in the sequences of the protectide-kinase reporters to minimize threading into the catalytic sites of proteases.

E. Ongoing work

The kinase activity reporters described above were synthesized by SPPS in a linear fashion generating the full-length protected substrate at the end of synthesis. Linear synthesis of the combined protectide, PEG linker, and PKB substrate peptide was both synthetically challenging and hard to purify. In addition, the protectide would have broader applications if libraries of protectides and substrates were synthesized separately and stored, then conjugated by a quick one-step reaction at the time of the assay. To circumvent the problematic linear synthesis of the protected PKB substrates and to expand the application of the protectide, the protectides and the PEG_2-PKB substrates peptides are being synthesized individually but are functionalized for click chemistry (Scheme 3.1). Prior attempts do to a fully solution phase click chemistry were unsuccessful. However, solid phase copper-catalyzed click chemistry, with the substrate peptide bearing the azide immobilized on resin and the protecting group bearing an alkyne free in solution will likely be more successful. Currently, we are attempting to synthesize full-length protected PKB activity reporters by adding a solution containing protectide and a copper source to a PEG_2-PKB substrate peptide with an azidolysine as the N-terminal residue, which is immobilized on CLEAR-Amide beads and allowing them to mix for several hours. The protected substrate will then be cleaved from the resin and purified if necessary by RP-HPLC. The ultimate goal is to be able to quickly protect, inject, and detect multiple enzymatic activities inside a patient cells using these protectide reporter peptides coupled with the power of single cell CE-LIF. Reaching this goal would allow us to quickly screen for enzymatic activity prior to treatment to determine what available enzyme inhibitor would be post beneficial to a patient. Such a

multi-dimensional assay would be beneficial post-treatment as well for the determination of how effectively the inhibitors are blocking enzymatic activity in patient cells and would allow the early detection of therapy-resistant cell populations. Optimization of this assay would expand capability of detecting enzymatic activity in any population of diseased cells that are diagnosed or observed *ex vivo*.

Scheme 3.1 Using click chemistry to conjugate kinase substrates to cyclized protectides. A propargyl glycine residue on the cyclized protectide is reacted with an azidolysine residue on the kinase substrate immobilized on resin, via a copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC, click chemistry). The protectide-substrate is then cleavage from the resin.

F. Experimental

Peptide Synthesis and Purification

Peptides were synthesized using manual or automated standard solid phase peptide synthesis (Thuramed Peptide Synthesizer, CEM Liberty1 Microwave Peptide Synthesizer) using Fmoc protected amino acids on 0.06-0.08 mmol of CLEAR Amide resin from Peptide International. Four equivalents of standard amino acids were used for each peptide synthesis while 1.5-4 equivalents of noncanonical amino acids and fluorophores were used. All peptides were acetylated at the N-terminus by treating the resin with 5% acetic anhydride and 6% 2,6-lutidine in 5 mL of DMF bubbling with N_2 for 40 minutes. 5(6)-Carboxyfluorescein (FAM) was coupled to the ε-NH of lysine or the δ-NH of ornithine at the i+4 position of the beta-hairpins or the centrally located amino acids of the scrambled sequences. The sidechains of these amino acids were orthogonally protected with an allyloxycarbonyl group (N-Alloc) or with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-3-methylbutyl (N-ivDde). Alloc removal was done based on the protocol published by Pazos et al.[15] The removal of ivDde from the sidechain of lysine or ornithine was accomplished by treating the peptide resin 3 x 2 mins with 20 mL of 2% hydrazine monohydrate in DMF bubbling with N_2 followed by washing with DMF, MeOH, DCM. Following deprotection, FAM was coupled using 2-4 equivalents of FAM, 5 equivalents of PyBOP/HOBt and 8 equivalents of DIPEA in 5 mL of DMF and allowed to bubble with N_2 for 4-8 hours.

[15]Pazos, E.; Jiménez-Balsa, A.; Maszareñas, J.L.; Vázquez, M.E. *Chem Sci***2011**, 2, 1984-87

Double coupling was sometimes required. Kaiser testing was done after the capping, deprotection and coupling steps. Peptides without cysteine were cleaved from the resin in 9.5:2.5:2.5 trifluoroacetic acid (TFA), TIPS and water respectively for 3-4 hours. Peptides containing cysteine were cleaved using 9.4:2.5:2.5:1 TFA, EDT, water and TIPS respectively for 3-4 hours. The TFA was evaporated and the cleaved peptides were precipitated using cold ethyl ether and extracted with water. Extracted peptides were lyophilized and then purified using semi-preparative RP-HPLC on a Vydac C18 semi-preparative column with a gradient from '0 to 100% B' in 45-120 minutes. Solvent A was 95% water, 5% acetonitrile and 0.1% TFA and Solvent B was made of 95% acetonitrile, 5% water and 0.1% TFA. Purified peptides were lyophilized and their synthesis and purity confirmed by ESI-TOF or MALDI-TOF using 2,5-dihydroxybenzoic acid matrix.

Peptide Concentration Determination

UV/Vis was used for the concentration determination of each peptide in 10 mM sodium phosphate buffer (100 mM NaCl, pH 7.48 for protease degradation studies) pH 8 using FAM abosorbance at 492 nm, ε_{492} = 78,000 M^{-1} cm^{-1}, for peptides with FAM or by 6 M guanidinium chloride denaturation and tryptophan absorbance at 280 nm ε_{280} = 5690 $M^{-1}cm^{-1}$ for peptides without FAM.

Circular Dichroism Spectroscopy

CD spectroscopy data was collected using an Applied Photophysics Chiroscan Circular Dicroism Spectrophotometer. Spectra were generated at 25 °C with a wavelength scan 260 nm to 185 nm, with 0.5 second scanning in a 0.1 cm cell. Peptide concentrations of 40 μM in 10mM sodium phosphate buffer, pH 8.0 (peptides with FAM) or pH 7.4 (peptides without FAM) were used.

Peptidase Concentration Determination

Pronase E enzymes from *Streptomyces griseus* were purchased as a lyophilized powder from Sigma-Aldrich (EC 3.4.24.31). Stocks were made by bringing up 5 mg of enzyme in 15 mL of 10 mM sodium phosphate buffer, 100 mM NaCl pH 7.48 (0.33 mg/mL). 1 ml aliquots were stored in the -80° C freezer for future use. As 7 μL of enzyme stock in 1.3 mL of peptide solution completely digested the **ScramOWOWO** peptide in 15 minutes, this was the volume of enzyme used in all protease studies.

Peptide Degradation by Pronase E

The enzymatic proteolysis procedure was adapted from Cline and Waters.[13c] The reaction was initiated by the addition of 7 μL of pronase E stock solution to 1.293 mL solution of 100 μM peptide in 10 mM sodium phosphate buffer, 100 mM NaCl pH 7.5 at 37°C . 75 μL aliquots were taken at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 720, 1080, 1440 minutes and quenched with 10 μL of acetic acid and 15 μL of buffer generating a total volume of 100 μL. 25 μL of each quenched sample was injected on the analytical RP-HPLC (Waters) and the fraction of peptide remaining at each time point

was determined based on the absorbance of each peptide at 214 nm and/or 280 nm. Peak integrations were done using Empower Pro 2 software. The acetic acid peak was used as an internal standard. The fraction of full-length peptide at each time point was determined by dividing the total peak area of the full-length peptide at each time point by the area of the acetic acid peak. This number was divided by the corresponding value of the zero time point.

Abl Kinase Phosphorylation Assay

Phosphorylation assays were conducted by mixing 4 μL of 10X assay buffer (500 mM Tris pH 7.5, 5 mM $MnCl_2$ and 25 mM $MgCl_2$), 4 μL of 10X DTT (20 mM DTT in water), 4 μL of 10X Abl kinase (3 ng/μL) in 50 mM Tris [pH 7.5], 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 50% (v/v) glycerol, 14 μL of water and 12 μL of 100 μM peptide in a reaction vessel. The reaction was initiated with the addition of 2 μL of 20 mM ATP and the reactions were incubated at 30°C. The reactions were quenched at 90°C for 4 minutes. Samples were stored at -20°C until time of analysis.

Ba/F3 BCR-ABL Lysate Degradation Assay

Ba/F3 BCR-Abl cells cultured in T-25 or T-75 culture flasks combined centrifuged at 0.8 rcf for 2.5 minutes. Media was discarded and the cells were washed twice with 10 ml of 1X PBS (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.75 mM KH_2PO_4, pH 7.4). Supernatant was discarded and the combined pellet was resuspended in 50-200 μL of 1X

PBS. Cells were submerged in liquid nitrogen for 1 min and immediately thawed in a 37°C water bath. The freeze-thaw cycle was repeated 2 times (a total of 3 cycles). Cells were centrifuged at 14,000 rcf at 4°C for 4 minutes. The supernatant was removed and kept at 4°C for the duration of the experiment. The pellet was discarded. Lysates were used within 4 hours of generation. Total protein concentration in the Ba/F3 BCR-Abl lysate was determined by mixing 30 μL of lysate (diluted 1:1000 in PBS) with 10 μL of fluorescamine (3 mg/mL) and allowing them to react for 5 minutes at room temperature in the dark. Immediately after, the fluorescence was measured using M5 Molecular Devices Spectrometer (475 nm) and compared with that of BSA protein standards mixed with fluorescamine. A 200- 400 μL solution was made containing 3 mg/mL final Ba/F3 BCR-Abl lysate concentration and 10 uM final peptide concentration. The peptide/lysate solutions were incubated at 37 °C and aliquots of 20-40 μL were removed at various times points (0, 1, 2, 3, 5, 10, 15, 30 minutes) and quenched with equal amounts of 200 mM HCl. Aliquots were frozen and stored at -20°C until time of analysis.

Capillary Electrophoresis

Detection of peptide phosphorylation *in vitro* and peptide degradation in Ba/F3 BCR-Abl lyastes was achieved by laser-induced detection (LIF, 488 nm) during capillary electrophoresis (ProteomeLab PA800, Beckman Coulter, Fullerton, CA). Fused-silica capillaries (30 or μm 50 μm inner diameter, 360 μm outer diameter, Polymicro Technologies, Phoenix, Az) were used with an effective length of 20 cm and a total length of 30 cm. Capillaries were preconditioned with 1 NaOH for 12 h, DI H_2O for 1 h,

0.1 M HCl for 6 h followed by DI H_2O for 12 h. Between each sample run, a pressure of 20 psi was applied at the capillary inlet allowing the capillaries to be rinsed with 1 NaOH for 2 min, DI H_2O for 2 min, and with electrophoretic buffer for 2 min. Quenched samples from the Abl kinase phosphorylation assay Ba/F3 BCR-Abl lysate degradation assay were separated using 150 mM sodium phosphate, 5 mM SDC, pH 7.3 as the electrophoretic buffer. Samples were loaded into the capillary hydrodynamically by applying a pressure of 0.5 psi at the inlet for 5 sec. A negative voltage was then applied to initiate electrophoresis. Electrophoresis was done for 20 mins with a field strength of 12 kV cm^{-1}. Results were analyzed using 32 Karat, version 8.0 (Beckman Coulter, Fullerman, CA).

Chapter IV

β-HAIRPIN PEPTIDES AS SUBTRATES AND REPORTERS OF UBIQUITIN PROTEASOME SYSTEM ACTIVITY

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A. Background

Degradation of proteins in the cell

Regulation of cellular homeostasis, migration, and division is accomplished via a continuous cycle of protein expression, post-translational modification and degradation.[1] Degradation of misfolded proteins or inactivated proteins is accomplished by two major pathways; proteasomal degradation and lysosomal degradation. Targeting a protein for either pathway usually involves post-translational polyubiquitination at a lysine sidechain of the target protein, though modification at cysteine, serine, or threonine sidechains has been reported.[1] Ubiquitin is a small 76-amino acid (8.5 kDa) globular protein whose attachment and removal from substrate proteins is accomplished by over a thousand enzymes collectively known as the Ubiquitin-Proteasome System (UPS, Figure 4.1).[2]

[1]Amm, I.; Sommer, T.; Wolf, D.H. *Biochem et Biophys Acta***2014**, 1843, 182-96.

[2]Eletr, Z.M.; Wilkinson, K.D. *Biochem et Biophys Acta***2014**, 1843, 114-128.

[3]Melvin, A.T.; Woss, G.S.; Park, J.H.; Waters, M.L.; Allbritton, N.L. *Cell Biochem Biophys*, **2013**, 67, 75-89.

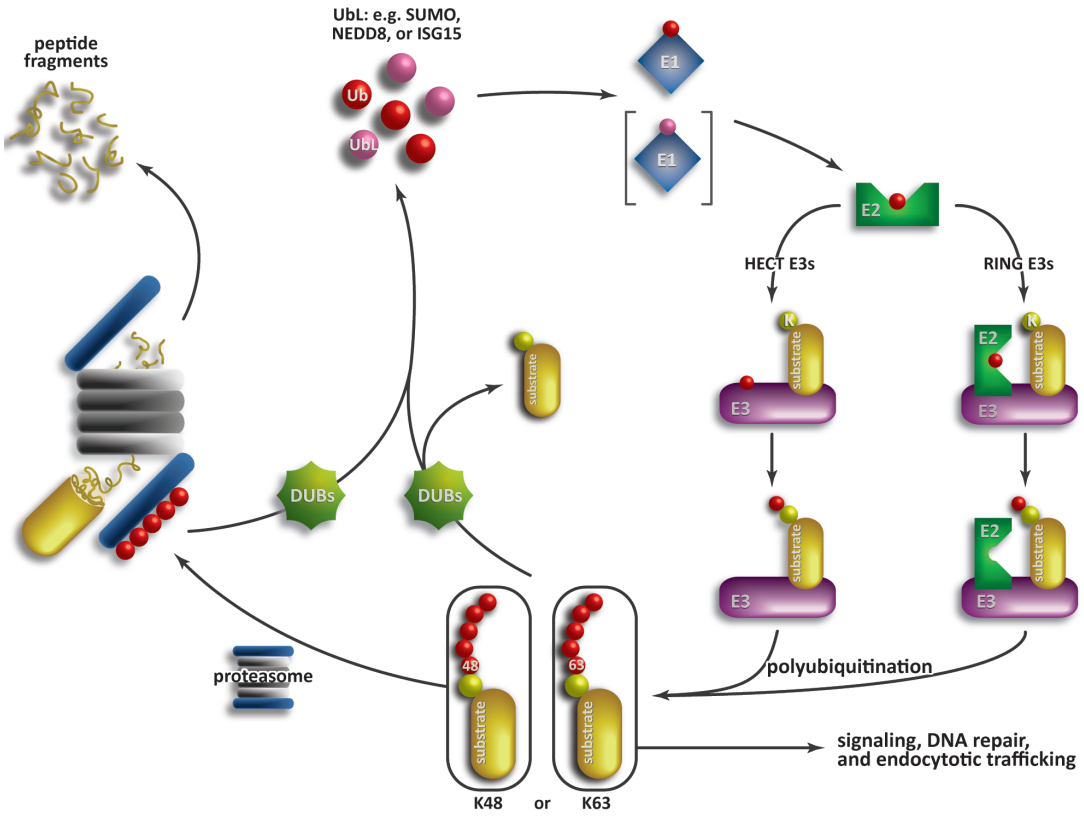

Figure 4.1 The Ubiquitin Proteasome System.[3] The enzyme cascade responsible for the transfer of ubiquitin and ubiquitin-like proteins (UbLs) to substrates targeted for proteasomal degradation. Ubiquitin (or UbL) is activated by an E1 enzyme and transferred to an E2 enzyme. The ubiquitin conjugated E2 enzyme binds to one of two types of E3 ligases (HECT or RING), which mediate the transfer of one or more ubiquitin moieties to the protein substrate. The ubiquitinated protein is trafficked to the proteasome and just before protein degradation, the ubiquitin moieties are removed by DUBs bound to the 19S cap of the proteasome. Ubiquitin is recycled and the protein substrate is hydrolyzed by the proteasome into oligopeptide fragments.

Polyubiquitination occurs through the formation of an isopeptide bond between the C-terminal carboxylate of an incoming ubiquitin and a lysine sidechain of the substrate-conjugated ubiquitin. Ubiquitin has seven Lys residues allowing for linear and branched polyubiquitination and multiple sites of monoubiquitination, each signaling for a different substrate protein fate. A linear chain of four or more ubiquitins, linked at Lys48 on ubiquitin, is the signal most commonly associated with proteasomal degradation.[4]

Three families of enzymes are responsible for transferring ubiquitin onto substrate proteins. The E1 ubiquitin activating enzyme, adenylates the C-terminus of ubiquitin via ATP hydrolysis allowing it to react with an E2 ubiquitin conjugating enzyme forming, a thioester intermediate bond between the E2 and ubiquitin. The final class of enzymes, the E3 ubiquitin ligases, bind to the E2 enzyme and some bind ubiquitin and either serve as a scaffold or directly catalyze the condensation of the C-terminal carboxylate of ubiquitin with Lys ε-amino group of the substrate protein,forming an isopeptide bond. There are two types of E3 ligases, HECT family E3 ligases and RING family E3 ligases which mediate the indirect and direct transfer of ubiquitin respectively.[2] Just before degradation by the proteasome, the ubiquitin chain can be removed via hydrolysis of the isopeptide bond by a family of proteases known as deubiquitinating enzymes (DUBs).[2]The 26S proteasome is a large protein complex made of the 19S regulatory unit and the 20S catalytic core.[4] The regulatory unit (RP) serves as a cap and regulates access of targeted proteins to the catalytic pore.

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[4]Schmidt, M.; Finley, D. *Biochem et Biophys Acta***2014**, 1843, 13-25.

The 20S unit harbors three proteolysis chambers, one with chymotrypsin-like activity (cleaving after hydrophobic residues), one with tryspin-like activity (cleaving after basic residues), and one with caspase-like activity (cleaving after acidic residues).[4] Along with the 26S proteasome, a small population of immunoproteasomes have been reported, which are responsible for digesting proteins into peptide fragments within immune cells for antigen presentation and recognition by neighboring immune cells.[4] Once proteins are digested into oligopeptides by any type of proteasome, the oligopeptides are further digested by cytosolic peptidases into smaller fragments for MHC-class presentation (to initiate and immune response) or into individual amino acids which are recycled for cellular anabolism. It is important to note that the UPS is responsible for far more than protein degradation within the cell. Protein ubiquitination is part cell cycle regulation, embryonic development and regulating immune responses.[5] The UPS machinery also installs other ubiquitin-like proteins (UbLs) such as SUMO (small-ubiquitin-like modifier, 12 kDa) and NEDD8 (6 kDa), onto substrate proteins to dictate a particular cellular fate such as apoptosis or cell cycle progression.[6] These latter functions of the UPS are beyond the scope of this work and will not be discussed further here.

[5]Myung, J.; Kim, K.B.; Crews, C.M. *Med Res Rev*, **2001**, 21, 245-73.

[6](a) Bohren, K.M.; Nadkarni, V.; Song, J.H.; Gabbay, K.H.; Owerbach, D. *J Biol Chem*, **2004**, 279, 27233-8 (b)Gong, L.; Yeh, E.T.H, *J Biol Chem*, **1999**, 274, 12036-42.

Clinical Relevance of UPS Activity

Regulating proteasome expression and function as a way of treating cancer, neurodegenerative diseases, and cardiac disease has become a popular area of drug discovery research[4] The success of the FDA approved drug bortezomib (Velcade®), a reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome in the treatment of refractory multiple myeloma, inspired research and exploration of the UPS that could one day rival provide as many druggable target as that of the kinase/phosphatase proteome.[7] Carfilzomib (Kryprolis), a more potent irreversible inhibitor of the chymotrypsin-like activity of the 26S proteasome, has recently been approved for treatment of bortezomib-resistant multiple myeloma patients.[8] Carfilzomib also inhibits the immunoproteasome giving an added advantage over bortezomib.[8] In neurodegenerative diseases and cardiac disease, increasing proteasome expression and activity rather than inhibition of activity, is the objective of treatment. There are eight known E1 enzymes, approximately forty E2 enzymes, and several hundred identified E3 enzymes in addition to nearly eighty DUBs. The success of proteasome inhibitors has driven the development of drugs that inhibit the catalytic activity of E1, E2 and DUBs enzymes and drugs that disrupt E3 ligase protein-protein interactions.[8] Of particular interest are inhibitors of murine double minute 2 homolog, MDM2 (or HDM2, the human counterpart), the E3 ligases responsible for the ubiquitination of the transcription factor p53.

[7]Cohen, P.; Tcherpakov, M. *Cell***2010**, 143,686-93.

[8]Mattern, M.R.; Wu, J.; Nicholson, B. *Biochem et Biophys Acta*, **2012**, 1823, 2014-21.

Famously, p53 is referred to as the guardian of the genome because it serves as a tumor suppressor by employing cell cycle arrest and apoptosis in the presence of oncogenic protein expression.[9] Functional wild-type p53 is lacking in more than 50% of cancer patients, making restoration of its activity one of the most highly sought after goals in cancer therapeutic research.[9] It is believed that tumor cells target p53 to the proteasome through ubiquitination of p53 by the E3 ligase MDM2 (or HDM2) leading to the degradation of p53 by the proteasome, effectively preventing tumor cell apoptosis. Thalidomide, originally prescribed in the 1950s to treat morning sickness before it was linked to birth defects, is now used to treat leprosy and multiple myeloma. It was recently reported that thalidomide disrupts protein-protein interactions of an E3 ligase complex by binding the protein cereblon, which modulates the substrate specificity of the Cul4A-Roc-DBB1 E3 ligase complex.[10] These are just two of several examples of how cancer therapy regiments are being expanded to include drugs that disrupt UPS activity. Inhibition of DUB activity is also being explored as a way to promote proteasome-mediated proteolysis in diseases where there is low activity. [2] The development of UPS modulators for clinical use necessitates the development of diagnostic tools that can detect activity of the UPS *ex vivo*.

[9] (a) Hoe, K.K.; Verma, C.S.; Lane, D.P. *Nat Rev Drug Discovery*.**2014**, 13, 217-236. (b) Lane, D.P. *Nature***1992**, 358, 15-6.

Detection of UPS activity using peptides synthesized by solid phase peptide synthesis

The expression of UPS proteins can be detected in cell lysates using antibody-based assays. The most commonly employed methods of detecting UPS activity involves genetically engineered substrates that are ubiquitinated within the cells, inducing a change in fluorescence that can be detected.[3] While gene expression products have provided significant insight about regulation of proteasome activity, they are impractical when evaluating UPS activity in patient tumor cells. Kumar et al. synthesized ubiquitinated peptides using solid phase peptide synthesis (SPPS) and native chemical ligation and used these constructs to evaluate DUB activity in vitro.[11] UPS activity has been evaluated using short fluorescently tagged peptide substrates of UPS enzymes also synthesized SPPS.[12] The sequences of the reporter peptides were derived from intracellular proteins whose activity is controlled by ubiquitin-dependent degradation by the 26S proteasome. These peptides contained a degradation signal or degron, which was recognized by E3 ligases as a substrate for ubiquitination.

[10]Ito, T.; Ando, H.; Handa, H., *Cell Mol Life Sci*, **2011**, 68, 1569-79.

[11]Kumar, K.S.A.; Spasser, L.; Ohayon, S.; Erlich, L.A.; Brik A. *Bioconjugate Chem***2011**, 22, 137-43.

[12]Melvin, A.T.;Woss, G.S.,;Park, J.H.; Dumberger, L.D.; Waters, M.L.,; Allbritton, N.L. *PLoS ONE*, **2013**, 8, e78082.

Degrons recognized by the UPS

Degrons are structural motifs on proteins recognized by cytosolic peptidases and UPS-associated degradation complexes that trigger the degradation of that protein.[13] One stipulation is that these sequences must be portable, meaning when attached to any substrate peptide these degrons target the peptide for degradation within the cells. UPS-specific degrons include N-terminal degrons or N-degrons, phosphodegrons, oxygen-dependent degrons, and ER-associated degrons. A N-degron is an free amino terminus bearing a specific amino acid often exposed after a cleavage event, that is recognized and bound by a specific E3 ligase leading to the ubiquitination and degradation of that particular protein.[13] The N-end rule pathway, first characterized by Varshavsky and coworkers, showed that the identity of the N-terminal amino acid determines the lifetime of the protein in the cell. Amino acids such as Arg, Lys, or His residues at the N-terminus of a protein in the cell result in a half-life of ~2 minutes, while newly synthesized proteins have a stabilizing Met residue at the N-terminus.[14] Phosphodegrons are specific amino acid sequences within a protein that when phosphorylated at Ser or Thr residues are subsequently recognized by a specific E3 ligase, such as $SCF^{\beta TrCP}$, to promote ubiquitination.[13] Protein phosphorylation paired with ubiquitination of cytosolic and nuclear proteins are important for cell cycle progression, leading to the activation of cell growth signaling proteins and the degradation of their inhibitory regulators.[13-14]

[13]Ravid, T.; Hochstrasser, M. *Nat Rev Mol Cell Bio*, **2008**, 9, 679-90.

[14] a) Varshavsky,A. *Proc. Natl. Acad. Sci. Biochem*, **1996**, 93, 12142-49 b) Varshavsky, A. *Prot Sci,***2011**, 20, 1298-1345.

Oxygen-dependent degrons are unique in that they are proteins recognized and targeted for degradations in oxygenated cellular conditions.[13] The most common oxygen-dependent degron is the protein hypoxia-inducible factor-1 (HIF1) transcription factor complex, which is active in hypoxic but its HIF-1α subunit is ubiquitinated and degraded by the proteasome after cellular molecular oxygen concentrations increase.[13] Endoplasmic reticulum (ER) degrons are thought to be hydrophobic patches on proteins only exposed when they are misfolded. Such regions are recognized by chaperones in the ER, prompting the export, ubiquitination, and degradation of these proteins.[13] It has been reported that the portable degron itself may not sufficient for degradation by the proteasome.

The aforementioned degrons are sufficient for substrate ubiquitination but conjugated to short peptides would not ensure proteasomal degradation. The peptide or protein must be of certain chain length and lack secondary or tertiary structure C-terminal to the ubiquitination site to be a substrate for proteasome-mediated degradation. Degron-based peptides with these structural features capable of being ubiquitinated by E3 ligases and degraded by the proteasome are called two-component degrons.[16] The N-terminal portion is recognized and ubiquitinated by the corresponding E1, E2, and E3 enzymes while the unstructured C-terminal portion is bound by the 19S cap of the 26S proteasome directed into the 20S catalytic cleft, initiating degradation.

[16](a)Alfassy, O.S; Cohen, I. Reiss, Y.; Tirosh, B.; Ravid, T. *J Biol Chem*, **2013**, 288, 12645-53. (b) Inobe, T.; Fishbain, S.; Prakash, S.; Matouschek, A. *Nat Chem Biol*, **2011**, 7, 161-7.

It is presumed that the N-terminal portion of the two-component degron is deubiquitinated and unfolded (if it has a higher order of structure) and guided into the catalytic cleft as well. Verhoef et al. found that an additional 25 amino acids on the substrate peptide C-terminal to the ubiquitination site was required for proteasomal degradation in both mammalian cells and yeast.[17] Shabek et al. demonstrated that monoubiquitination was sufficient for ubiquitinated substrates shorter than 150 residues but proteasomal degradation of longer substrates required polyubiquitination.[18]

Typically, when investigating modification of degrons by the UPS, Lys residues within the degron sequence are the sites of modification. Here we report that a 12-residue β-hairpin peptide functions as a degron with an Orn rather than Lys as the ubiquitination site. Orn is a noncanonical amino acid lysine analog with one less CH_2 group in its sidechain. We demonstrate that secondary structure of the degron promotes its resistance to degradation in cell lysates. Finally we report preliminary work investigating the E3 ligase(s) responsible for the ubiquitination of these novel degrons. The work presented here will lay the groundwork for the development of *in vivo* UPS activity reporters.

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[17]Verhoef, L.G.G.C.; Heinen, C.; Selivanova, A.; Halff, E.F.; Salomons, F.A.; Dantuma, N.P. *J Fed Am Soc Exp Biol***2009**, 23, 123-33.

[18]Shabek, N.; Herman-Bachinsky, Y.; Buchsbaum, S. Lewinson, O.; Haj-Yahya, H.; Hejjaoui, M.; Lahsuel, H.A.; Sommer, T.; Brik, Cienhanover, A. *Mol Cell***2012**, 48, 87-97.

A. Design and Synthesis

Seven Orn containing peptides were synthesized using SPPS as described earlier. The **OWOWO**β-hairpin peptide served as the parent degron and four versions of the peptide were made where one or more of the ubiquitinatable Orn residues were replaced with an Arg residue to determine the preferential site(s) of ubiquitination . The **ScramOWOWO** peptide, containing an equal number of Orn residues as **OWOWO**, was synthesized as an unstructured control. The **OWOWO-RRRG**peptide was synthesized to increase the protease resistance of the Bonger degron peptide and to serve as a two-component degron.[12,20] The **III-67B** peptide was included as a positive control for the OPM-2 cell lysate degradation assay as it has previously been shown to degrade rapidly in cell lysates.[19] The fluorophore 6-carboxyfluorescein (FAM) was covalently attached to an Orn sidechain next to the turn sequence or to the N-terminus of all peptides investigated for detection. The sequences of all peptides investigated and discussed in this chapter are listed in Table 4.1.

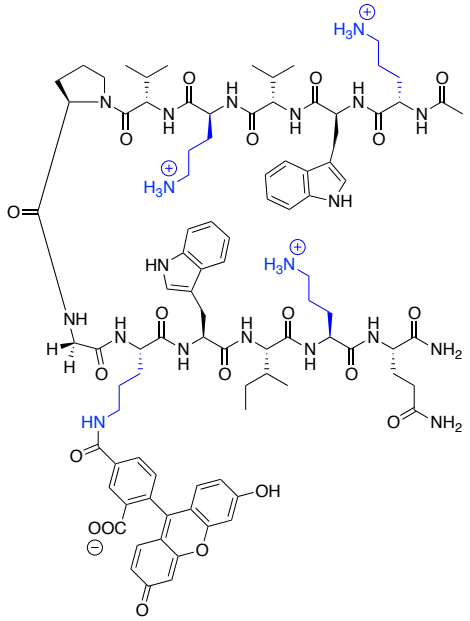
[19]Proctor, A.; Wang, Q.; Lawrence, D. S.; Allbritton, N. L. *Analyst***2012,** 137, 3028–38.

Table 4.1 Ubiquitinatable degrons and positive control peptide[a]

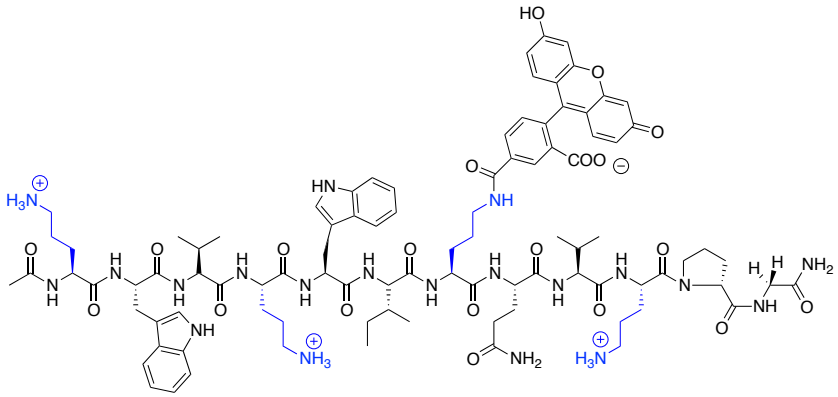
Degron Peptide	Sequence
ScramOWOWO	Ac-**O**WV**O**WIO(FAM)QV**O**pG-NH_2
OWOWO	Ac-**O**WV**O**VpGO(FAM)WI**O**Q- NH_2
OWRWR	Ac-**O**WVRVpGO(FAM)WIRQ- NH_2
RWOWR	Ac-RWV**O**VpGO(FAM)WIRQ- NH_2
RWRWO	Ac-RWVRVpGO(FAM)WI**O**Q- NH_2
RWRWR	Ac-RWVRVpGO(FAM)WIRQ- NH_2
III-67B (Control) [19]	FAM-GGAYAATKKKKA- NH_2
RRRG	FAM-RRRGGGG**K**-NH_2
OWOWO-RRRG [12,20]	Ac-**O**WV**O**VpGO(FAM)WI**O**Q-$(PEG)_2$-RRRGGGG**K**-NH_2

[a]**III-67B**- BCR-Abl substrate peptide known to degrade rapidly in cell lysates. Residues highlighted in blue are potentials sites of ubiquitination. The **III-67B** was not tested in any ubiquitination assays.

a)



b)



c)

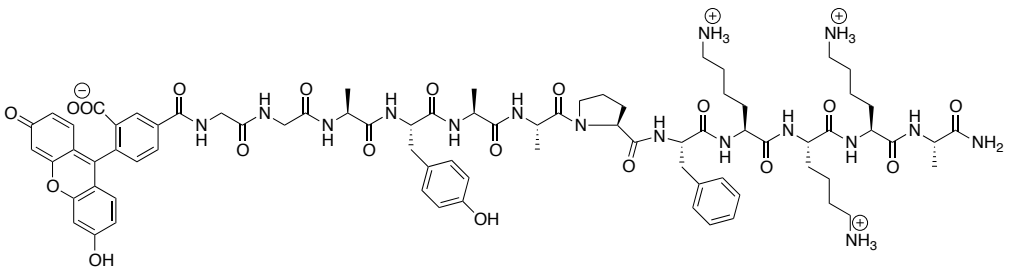


Figure 4.2. Structures of the ornithine-containing degrons. (a) **OWOWO** (b) **ScrOWOWO** (c) **III-67B** (non-degron control peptide that is rapidly degraded in OPM2 lysates). Highlighted in blue are Orn residues sites which are sites to be modified by ubiquitination.

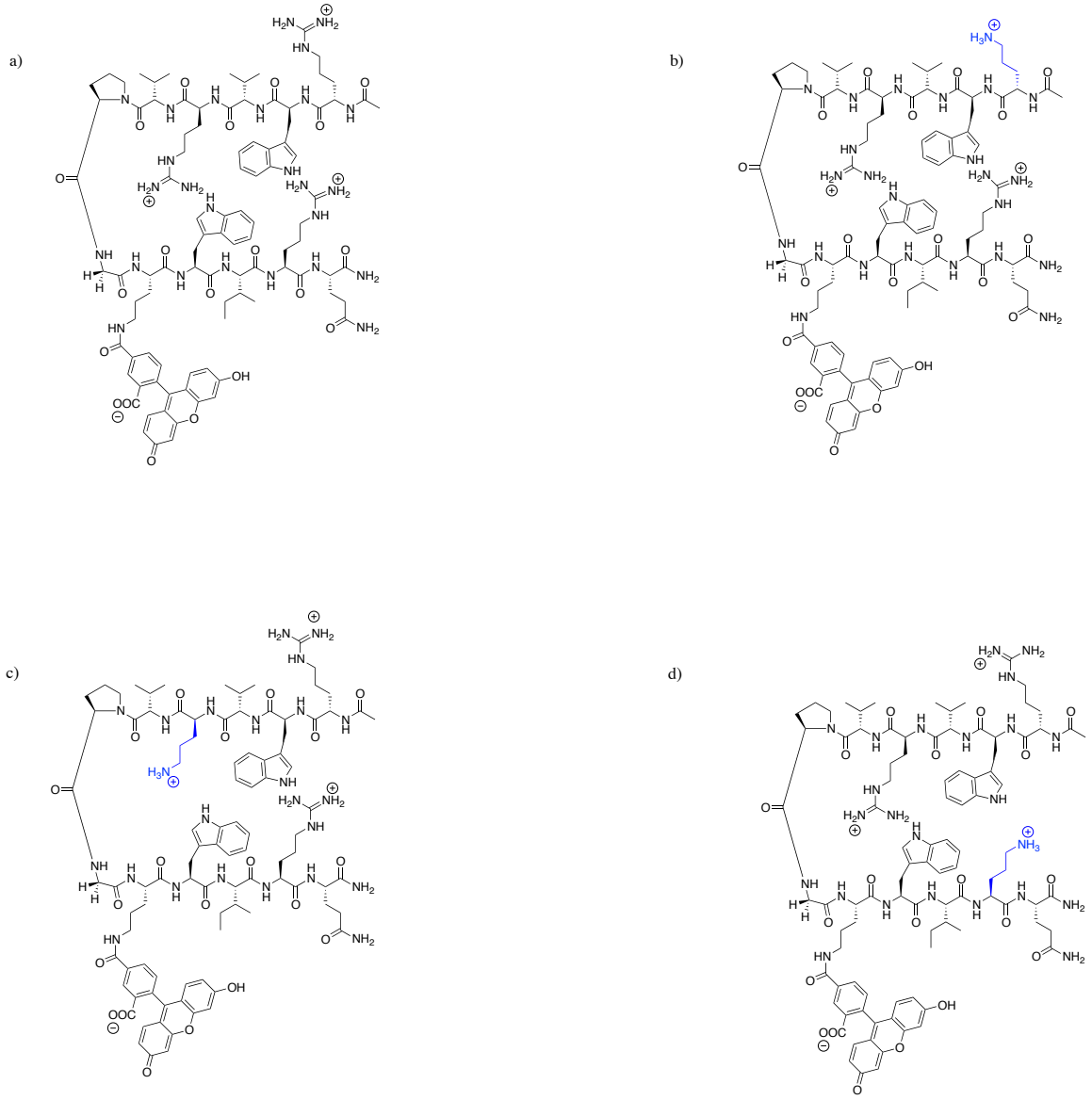


Figure 4.3. Structures of **OWOWO** Arg-substituted degrons investigated for ubiquitination. (a) **RWRWR** (b) **OWRWR** (c) **RWOWR** (d) **RWRWO**. Orn resides are highlighted in blue as sites modifiable by ubiquitination.

B. Results and discussion

i. Ornithine ubiquitination i*n vitro*

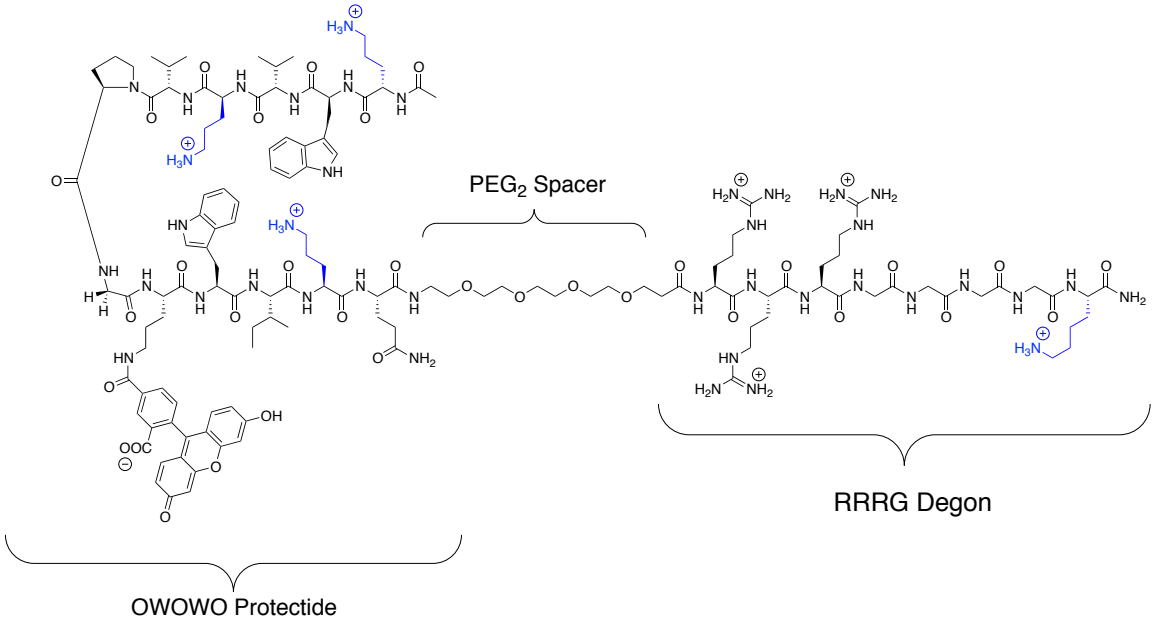


Figure 4.4. Structure of **OWOWO-RRRG**proteasome reporter. Residues highlighted in blue are sites modified by ubiquitination.

The **RRRG** (Table 4.1) degron peptide, reported by Bonger et al. and further characterized by Melvin et al., has been shown to be a rapidily ubiquitinated substrate that adequately targets attached proteins to the proteasome within the cell.[12,20] The **RRRG** degron (Sequence: RRRGGGG**K)** alone was sufficiently ubiquitinated, but was previously demonstrated to be susceptible to hydrolysis by cytosolic proteases.[12] The **OWOWO**β-hairpin protectide, which demonstrated substantial resistance to nonspecific proteases *in vitro*, was covalently attached to **RRRG** to protect the degron from degradation by proteases (Figure 4.4). To determine the viability of the protected degron as a substrate for ubiquitination, the **OWOWO-RRRG** peptide was incubated *in vitro*with HeLa S100 cytosolic lysates as the source of E1 activating enzyme, E2

conjugating enzyme, E3 protein ligase, as well as ATP-ERS (ATP energy regenerating solution), a DUB inhibitor (ubiquitin aldehyde), and methylated ubiquitin (MeUb) which prevents the formation of polyubiquitin chains due to methylation of all seven Ub Lys residue sidechains.[21] The reactions were quenched at 4° C then subsequently incubated with control agarose beads prior to pull down (or immunoprecipitation) with agarose beads conjugated anti-ubiquitin antibodies to isolate only the ubiquitinated substrates (Tandem Ubiquitin Binding Entities or TUBEs, LifeSensors).[22] The concentrated mixtures were then separated by gel electrophoresis and imaged using fluorescence detection (Typhoon Imager, GE LifeSciences at the Lineberger Cancer Institute) due the presence of the FAM fluorophore covalently attached to the β-hairpin peptides. Despite using MeUb, which cannot form polyubiquitin chains, the results revealed polyubiquitination and/or multi-monoubiquitination of the **OWOWO-RRRG** peptide, a degron with only one canonical modification site, a Lys residue at its C-terminus (Figure 4.5, Table 4.1).[21] We hypothesized that Orn, an analog of Lys, could serve as a ubiquitination site for the E3 ligases, resulting in multi-mono-ubiquitination of the peptide.

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[20]Bonger, K.M.; Chen, L.C.; Liu, C.W.; Wandless, T.J. *Nat Chem Biol*, **2011**, 7, 531-7.

[21]Houston, K.M.; Melvin, A.T.; Woss, G.S.; Waters, M.L.; Allbritton, N.L. *Manuscript in preparation*.

[22]Hjerpe, R.; Aillet, F.; Lopitz-Otsoa, F.; Lang, V.; England, P.; Rodriguez, M.S. *Eur Mol Biol Org***2009**, 10, 1250-58.

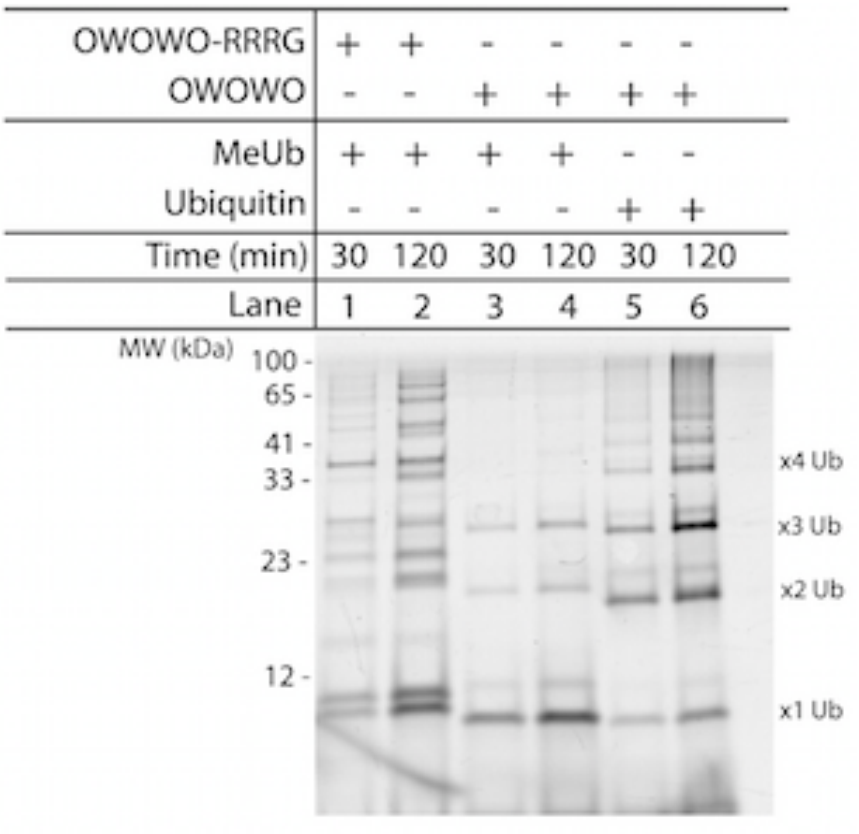

Figure 4.5 The ubiquitination of Orn residues by endogenous UPS enzymes from HeLa S100 lysates. The **OWOWO** and **OWOWO-RRRG** degrons were incubated with HeLa S100 lysates in the presence of exogenous ubiquitin or methylate ubiquitin (MeUb). The modified degrons were isolated using TUBEs, were separated by gel electrophoresis and the results were determined by fluorescence imaging.[21]

To test this hypothesis, **OWOWO** without the degron was incubated with HeLa S100 lysates with wild type ubiquitin or MeUb to evaluate substrate poly vs. multi-monoubiquitination. Multi-ubiquitination was observed in both instances suggesting (Figure 4.5) that Orn does in fact serve as a ubiquitination site for modification by endogenous UPS enzymes, and that **OWOWO** can be ubiquitinated. The results in lanes 3 and 4 of Figure 4.5 suggest that **OWOWO** can be ubiquitinated at multiple sites since MeUb can not form polyubiquitin chains, though polyubiquitination of endogenous ubiquitin cannot ruled out.

ii. Determination of the primary site of ornithine ubiquitination

To determine if there was a preferred ubiquitination site on the **OWOWO** peptide, several peptides were synthesized with all but one of the native Orn residues replaced with an Arg residue (Figure 4.3, Table 4.1). The **RWRWR** peptide, where all of the Orn residues were substituted for Arg residues, served as a negative ubiquitination control. Each of the Arg-substituted peptides, **OWRWR**, **RWOWR**, **RWRWO**, and **RWRWR**, as well as **OWOWO** were incubated with HeLa S100 lysates and either wild type ubiquitin or no Lys ubiquitin (No Lys Ub) where all of the ubiquitin Lys residues were mutated to Arg residues to prevent polyubiquitin chain. Ubiquitin aldehyde, a potent DUB inhibitor, was also added to prevent deubiquitination of the modified reporters. The ubiquitin-modified degrons were isolated using TUBEs, separated by gel electrophoresis and imaged using fluorescence detection. The **OWOWO** peptide displayed multi-ubiquitination in the presence of Ub as seen previously (Figure 4.5). Faint bands indicating multi-site ubiquitination were observed for **OWOWO** in the presence of No Lys ubiquitin but the majority of the peptide appeared to be mono-

ubiquitinated (Figure 4.6). The observation of predominantly mono-ubiquitinated peptide suggests that there is a primary site of modification and two minor modification sites though a small amount of peptide may have been polyubiquitinated by endogenous ubiquitin. The results of the **OWRWR**, **RWOWR**, and **RWRWO** Ub-pull down assays demonstrated that the N-terminal Orn residue is the primary site of both mono-ubiquitination and poly-ubiquitination (Figure 4.6). This was not entirely surprising as N-terminal basic residues are a class of degrons according to the N-end rule proposed by Varshavsky.[12] N-terminal basic residues present on proteins are more destabilizing, linked with intracellular lifetimes of approximately 2 minutes suggesting rapid turnover of such proteins by the proteasome.[12] Faint bands indicative of tri-ubiquitinated product in the presence of No Lys ubiquitin could be due to polyubiquitination by endogenous ubiquitin.

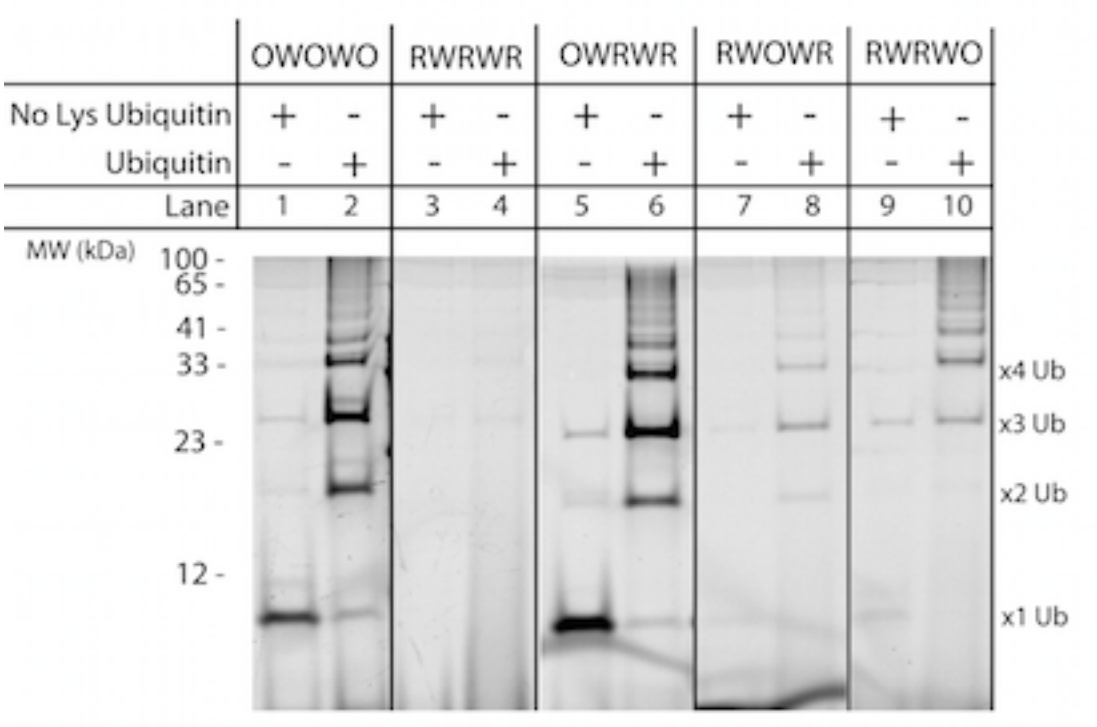

Figure 4.6 Determination of the primary site of Orn ubiquitination on the **OWOWO** peptide. The **OWOWO**, **RWRWR**, **OWRWR**, **RWOWR,** and **RWRWO** peptides were incubated in the presence of HeLa S100 lysates as a source of E1, E2, and E3 and exogenous ubiquitin or No Lys ubiquitin at 37° C. Modified degrons were isolated by immunopreciptiation, separated by gel electrophoresis, imaged by fluorescence detection.[21]

iii. Secondary Structure Determination

To determine whether or not the intrinsic structure of the **OWOWO** and **OWRWR** degron peptides affected the degree of ubiquitination, their secondary structure was characterized by circular dichroism (CD). Additionally, a scrambled version of the **OWOWO** peptide, **ScramOWOWO** was synthesized and investigated by CD as well. The **ScramOWOWO** was designed to be a random coil peptide in order to determine whether or not secondary structure impacted the degree Orn ubiquitination. The CD spectrum of the **ScramOWOWO** peptide suggests that it is unstructured based on the minima near 195 nm. The CD spectrum of the **OWOWO** peptide displayed a β-hairpin conformation by the presence of minima near 205 nm and 215 nm (Figure 4.7).[21] The maximum near 225 nm suggests that there are strong interactions between the indole rings of the cross-strand Trp residues.[23] The **OWRWR** CD spectrum also illustrated β-hairpin character with minima near 205 nm and 215 nm. Exciton coupling between the sidechains of the cross-strand Trp residues was exhibited by the maximum near 225 nm. Though the CD spectra of **OWOWO** and **OWRWR** were nearly identical in terms of shape, there is a large difference in their mean residue ellipticities (MRE). The minimum MRE of **OWRWR** is near 14,000 deg•cm^2•dmol^{-1} while the minimum MRE of the **OWOWO**β-hairpin is near 26,000 deg•cm^2•dmol^{-1}. This suggested that **OWOWO** was more tightly folded than **OWRWR**. This was not expected since Trp-Arg diagonal pairs yield more tightly folded β-hairpins than Trp-Lys pairs, and Trp-Orn cation-π interactions are weaker than Trp-Lys cation-π interaction.[24]

[23] Cochran, A.G.; Skelton, N.J.; Starovasnik, M.A. *Proc Natl Acad Sci***2001**, 98, 5578-83.

Butterfield et al. reported 93% folding (determined by NMR) of a peptide very similar to **RWRWR**, which only differed by two amino acid residues. [24c] Butterfield et al. also showed that the β-hairpin peptide with cross-strand Trp-Lys pair was 96% folded, slightly more tightly folded than the peptide with the cross-strand Trp-Arg pairs. The **OWOWO** and **OWRWR** peptides are similar to those reported by Butterfield et al. in that they have the potential for cross-strand cation-π and thus may behave differently than the β-hairpins with diagonal cation-π interactions reported previously. Investigation by NMR would provide further information about degree of folding of the **OWOWO** and **OWRWR** β-hairpin peptides.

[24] (a)Tatko, C.D., Waters, M.L. *Protein Sci*, **2003**, 12, 2443-2452. (b) [19]Hughes, R.M.; Benshoff, M.L.; Waters, M.L. *Chem-Eur J***2007** 13, 5753-64. (c) Butterfield,S.M.; Sweeney, M.M.; Waters, M.L.; *J Org Chem***2005**, 70, 1105-14.

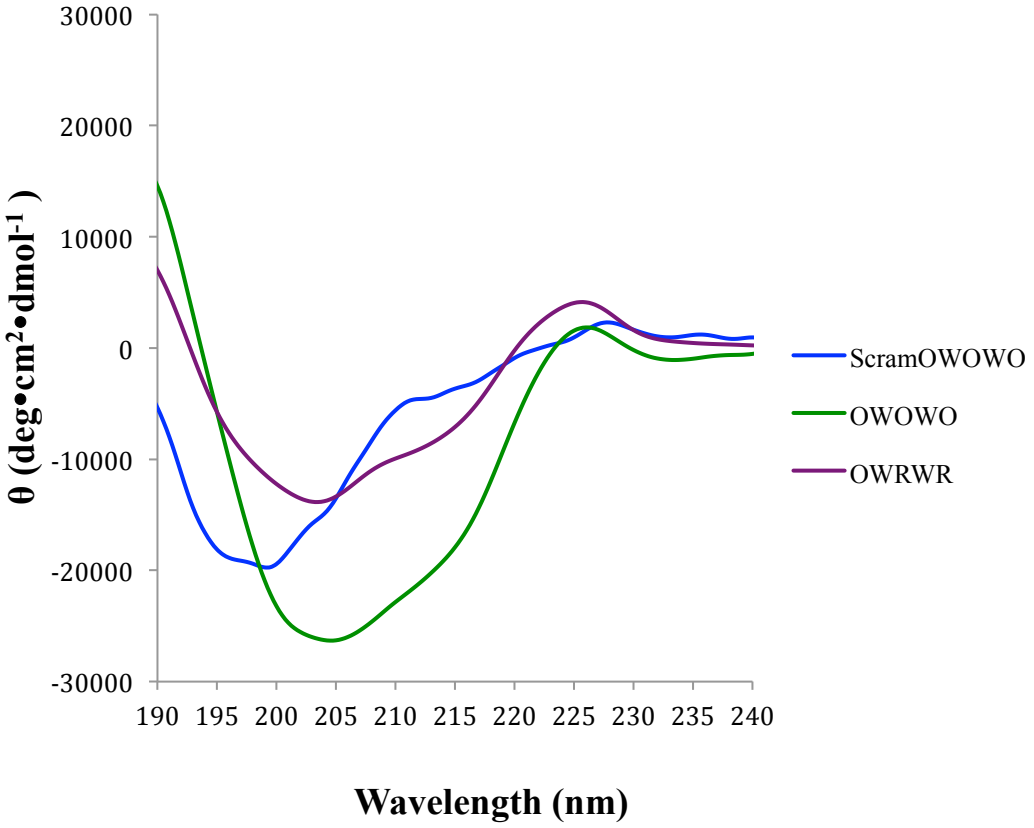

Figure 4.7 Circular dichroism spectra of the **ScramOWOWO**, **OWOWO**, **OWRWR** degron peptides. Experiments were performed using 40 μM peptide in 10 mM sodium phosphate buffer, pH 8 at 25°C. **ScramOWOWO** (blue) was found to be a random coil while **OWOWO** and **OWRWR** (green and purple respectively) both fold into a β-sheet conformation.[21]

vi. Role of Secondary Structure

Several of the degrons used to investigate UPS activity, prior to this work, have been sequences derived from proteins that are natural substrates UPS in vivo.[3,12] These short peptides, isolated from their full-length protein, were presumed to lack secondary structure or the secondary structure was ignored. Here we investigate the role of secondary structure by qualitatively examining the difference in the degree of ubiquitination of the **ScramOWOWO** random coil peptide and the **OWOWO** and **OWRWR**β-hairpin peptides. The **OWOWO** and the **ScramOWOWO** have the same amino acids in their primary sequence, the same number of potential ubiquitination sites, but the amino acids are ordered differently resulting in a difference in secondary structure. The peptides were incubated with OPM-2 cell lysates (multiple myeloma cell line), ubiquitin-aldehyde and added exogenous ubiquitin or No Lys ubiquitin, then isolated using a pull-down assay, and separated by gel electrophoresis. The results shown in Figure 4.8 suggest that regardless of secondary structure, Orn is a substrate of ubiquitination.[21] It is interesting to note that a preference of for the site of ubiquitination may differ depending on whether or not the degron peptide is inherently structured. Further investigation of this would involve mutating the Orn residues on the **ScramOWOWO** peptide individually to Arg residues to elucidate the primary site(s) of ubiquitination.

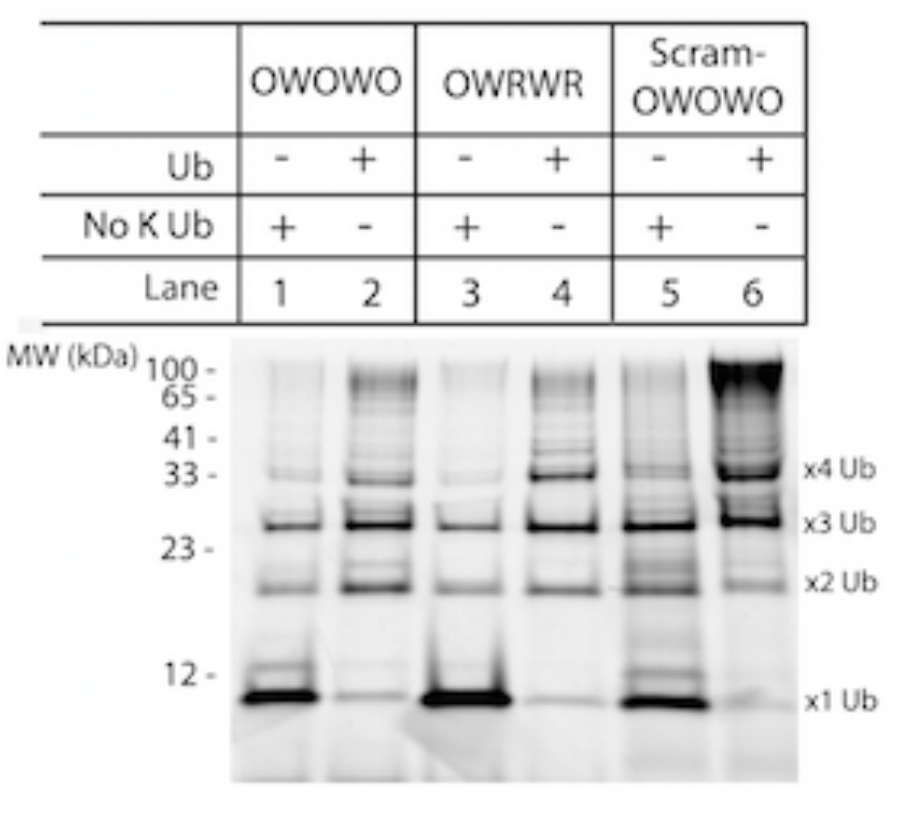

Figure 4.8 Determination of the significance of secondary structure in the ubiquitination of Orn residues. **OWOWO**, **OWRWR** (β-hairpin peptides) and **ScramOWOWO** (random coil peptide) were incubated with OPM-2 lysates as a source of E1, E2, and E3 enzymes and with exogenous ubiquitin or No Lys ubiquitin at 37° C. Modified degrons were isolated by immunopreciptiation, separated by gel electrophoresis, imaged by fluorescence detection.[21]

v. Resistance to proteolytic degradation in OPM-2 cell lysates

One of the major challenges in the development of peptide-based reporters of enzymatic activity is their susceptibility to hydrolysis by cytosolic peptidases. We have shown that resistance to proteolysis can be conferred with a combination of secondary structure and the incorporation of noncanonical amino acids into the reporter primary amino acid sequence. Previously we showed that **OWOWO** possessed an average half-life of 315 minutes when incubated with nonspecific proteases *in vitro* (Chapter II, Figure 2.9, Table 2.2). A β-hairpin peptide with the same number of noncanonical amino acids as **OWRWR** had a half-life of 143 minutes under same conditions (Chapter II, Figure 2.9, Table 2.2). Since many of the proteases within cells are confined within membrane-bound organelles, we expected that β-hairpin peptides would be even more resilient in the cytosol of intact cells.[25]

The **OWOWO**, **OWRWR**, **ScramOWOWO**, and **III-67B** (positive control peptide) were incubated with OPM-2 whole cell lysates at 37° C and were quenched at various times between 0-120 minutes with 200 mM HCl. The percentage of degron peptide hydrolysis by lysate proteases was determined using capillary electrophoresis with laser-induced fluorescence (CE-LIF).

The **III-67B** peptide is an Abl kinase reporter peptide whose degradations has been characterized in multiple cancer cell lines.[19] The **III-67B** peptide is a 12-mer consisting of all canonical amino acids and presumably lacking secondary structure.

[25]Belkhiri, A.; Lytvyn, V.; Guilbault, C.; Bourget, L.; Massie, B.; Nägler, D.K.; Ménard, R. *Anal Biochem*, **2002**, 306, 237-46.

The lack of structure and canonical amino acids made it susceptible to proteolytic degradation by cytosolic peptidase. In previous reports, the **III-67B** peptide was fully degraded by cytosolic proteases in 15 minutes. We were unaware of any previous reports of characterizing the degradation of short peptides in OPM-2 cell lysates, thus **III-67B** served two purposes: 1) to ensure that the OPM-cytosolic protease activity survived the cell lysis conditions and 2) to allow comparison of proteolytic activity in a multiple myeloma cell lysates with the protease activity of other cancer cell line lysates.[19] We used this peptide as a control for the OPM-2 lysate degradation assay only. The ability of the **III-67B** to serve as a substrate for the UPS machinery was not assessed in this study.

The **III-67B** peptide was fully degraded after 30 minutes in OPM-2 cell lysates, with a half-life of 6 minutes (Figure 4.9). We are still optimizing the electrophoretic buffer conditions necessary to separate all of degradation fragments of the **ScramOWOWO**, **OWRWR** and the **OWOWO** peptides. Thus the exact half-lives of these peptides in the presence of the proteases from OPM-2 cell lysates have not yet been determined though. It is expected that the **ScramOWOWO** degron peptide, which lacks secondary structure, but has five noncanonical amino acids in its primary sequence, will be cleaved rapidly but not digested as rapidly as the **III-67B** peptide. Immune cell-specific proteases such as tripeptidyl peptidase II (TPPII), though they are not presumed to be highly specific, have not evolved to cleave after Orn or ^{D}Pro residues which are not present in naturally occurring proteins.[26]

[26]Tomkinson, B.; Lindas, A.C. *Int J Biochem Cell Biol*, **2005**, 37, 1933-7

Given that trypsin, which specifically cleaves after basic amino acids, hydrolyzes Orn peptide bonds much less quickly than lysine amide bonds, the rates of hydrolysis by the aforementioned cytosolic proteases would likely be lower as well.[27] We expect that the combination of secondary structure and noncanonical amino acids will result in increased resistance to degradation by cytosolic peptides. Both **OWRWR** and **OWOWO** demonstrate significant secondary structure, thus we expect that these two β-hairpin peptides will be resistant to hydrolysis for longer than both the **III-67B** and the **ScramOWOWO** peptide. However, the **OWOWO** peptide has more noncanonical amino acids than the **OWRWR** peptide and is therefore expected have the longest half-life. Based on the long half-life of the **OWOWO** peptide in the presence of pronase E, it is possible that the **OWOWO** peptide will remain intact for the 2-hour duration of the OPM-2 lysate degradation assay. We expect that the **OWRWR** and the **OWOWO** peptides will display a resiliency that will prove them suitable for single cell UPS activity analysis for long time periods.

[27]Seely, J.H.; Benoiton, N.L. *Can J Biochem***1970**, 48, 1122-31.

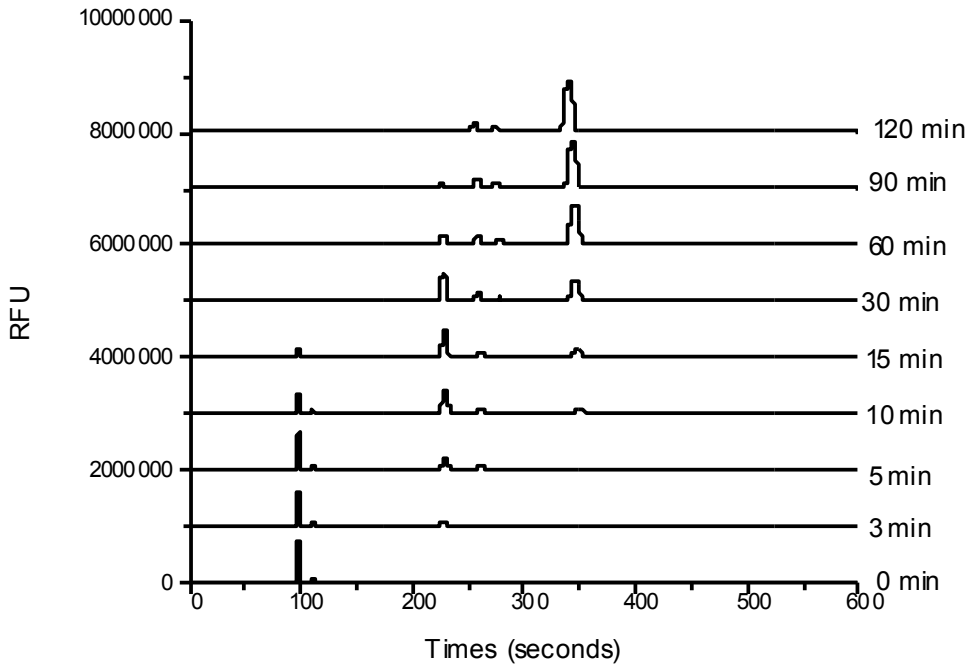

Figure 4.9 Electropherogram of **III-67B** peptide after degradation by proteolytic enzymes present in OPM-2 lysates as determined by CE-LIF. Parent peptide (~100 seconds) was fully degraded after 30 mins. Parent peptide and degradation products were separated in 100 mM Tris, 100 mM Tricine buffer pH 8.1 at 15 kV for 600 seconds (10 minutes) in a 30 μm capillary.

vi. Investigation of possible acting E3 ligase

There are several commercially available inhibitors that specifically target E3 ligases to modulate UPS activity within diseased cells. A few of those inhibitors are in Phase I clinical trials (Serdemetan and Nutlin-3) while others are in clinical use (thalidomide).[8] Though there are hundreds of E3 ligases in the cell, we were limited in our selection of potential targets due the narrow range of inhibitors to evaluate potential E3 ligases that facilitate the ubiquitination of **OWRWR**. We chose to use **OWRWR** to allow us to elucidate any effect of the E3 ligase inhibitors on polyubiquitination. **OWRWR** was incubated in the presence of HeLa S100 lysates, exogenous ubiquitin, and one of six E3 ligase inhibitors (Serdemetan[28a], Nutlin-3[28b], Skpin C1[28c], SMER3[28d], TAME HCl[28e], and thalidomide[10,28f]) shown in Figure 4.10, as well as with ubiquitin aldehyde, a DUB inhibitor, to prevent the deubiquitination of the peptide.[290]

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[28]**(a)**Kojima, K.; Burks, J.K.; Arts, J.; Andreef, M. *Mol Cancer Ther*, **2010**, 9, 2545-57. **(b)** Vassilev, L.T; Vu, B.T.; Graves, B.; Carvajal, D.; Podlaski, F.; Filipovic, Z.; Kong, N.; Kammlott, U.; Lukacs, C.; Klein, C.; Fotouhi, N.; Liu, E.A. *Science*, **2004**, 303, 844-8. **(c)** Wu, L.; Grigoryan, A.V.; Li, Y.; Hao, B.; Pagano, M.; Cardozo, T.J. *Chem Biol*, **2012**, 19, 1515-24. **(d)** Aghajan, A.; Jonai, N.; Flick, K.; Fu, F.; Luo, M.; Cai, X.; Ouni, I.; Pierce, N.; Tang, X.; Lomenick, B;, Damoiseaux, R.; Hao, R.; Del Moral, P.M.; Verma, R., Li, Y., Li, C., Houk, K.N., Jung, M.E., Zheng, N., Huang, L., Deshaies, R.J., Kaiser, P.; Huang, J. *Nat Biotechnol*, **2010**, 28, 738-42. **(e)** Zeng, X.; Sigoillot, F.; Gaur, S.; Choi, S.; Pfaff, K.L.; Oh, D.C.; Hathaway, N.; Dimova, N.; Cuny, G.D.; King, R.W. *Cancer Cell*, **2010**, 18, 382-95. **(f)** Ito, T.;Ando, H.; Suzuki, T.; Ogura,T.; Hotta, K.; Imamura, Y.; Yamaguchi, Y.; Handa, H. *Science*, **2010**, 327, 1345-50.

[29]Hershko, A.; Rose, I.A., *Proc Natl Acad Sci USA*, **1987**, 84, 1829-33.

The reactions were quenched, isolated, and detected using the Ub-pull-down assay described earlier. An E1 activating enzyme inhibitor, PYR-41, was incubated with OWRWR as a negative control and DMSO was added to one reaction mixture instead of an inhibitor to serve as a positive ubiquitination control.[30] The results are shown in Figure 4.11. There is less ubiquitination observed in the presence of PYR-41 than in the presence of DMSO, but there is still some modification of **OWRWR** seen, suggesting that the PYR-41 did not fully inhibit ubiquitination. PYR-41 is not a global E1 inhibitor though it dramatically reduced ubiquitination activation HeLa cells at 50 μM inhibitor concentration. Our assay was done at 30 μM inhibitor concentration (three times the IC_{50}) thus increasing the inhibitor concentrations may remedy the problem. There could have been E1 enzymes present in the lysate already loaded with endogenous ubiquitin, although this concentration was expected to be small. **OWRWR** ubiquitination was seen in the presence of all the E3 ligase inhibitors though polyubiquitin chain formation was greatly reduced in the presence of SMER3, TAME HCl and thalidomide (Figure 4.11, Lanes 4, 5, 6). These experiments were all performed, in triplicate, at inhibitor concentrations three times that of the IC_{50} value for all the enzymes. Experiments are currently underway at five times the IC_{50} to confirm these findings.

[30]Yang, Y.; Kitagaki, J.; Dai, R.M.; Tsai, Y.C.,;Lorick, K.L.;Ludwig, R.L.; Pierre, S. A.,;Jensen, J.P.; Davydov, I.V.; Oberoi, P.;Li, C.C.H.; Kenton, J.H.; Beutler, J.A.,; Vousden, K. H.; Weissman, A.M. *Cancer Res*, **2007**, 67, 9472-81

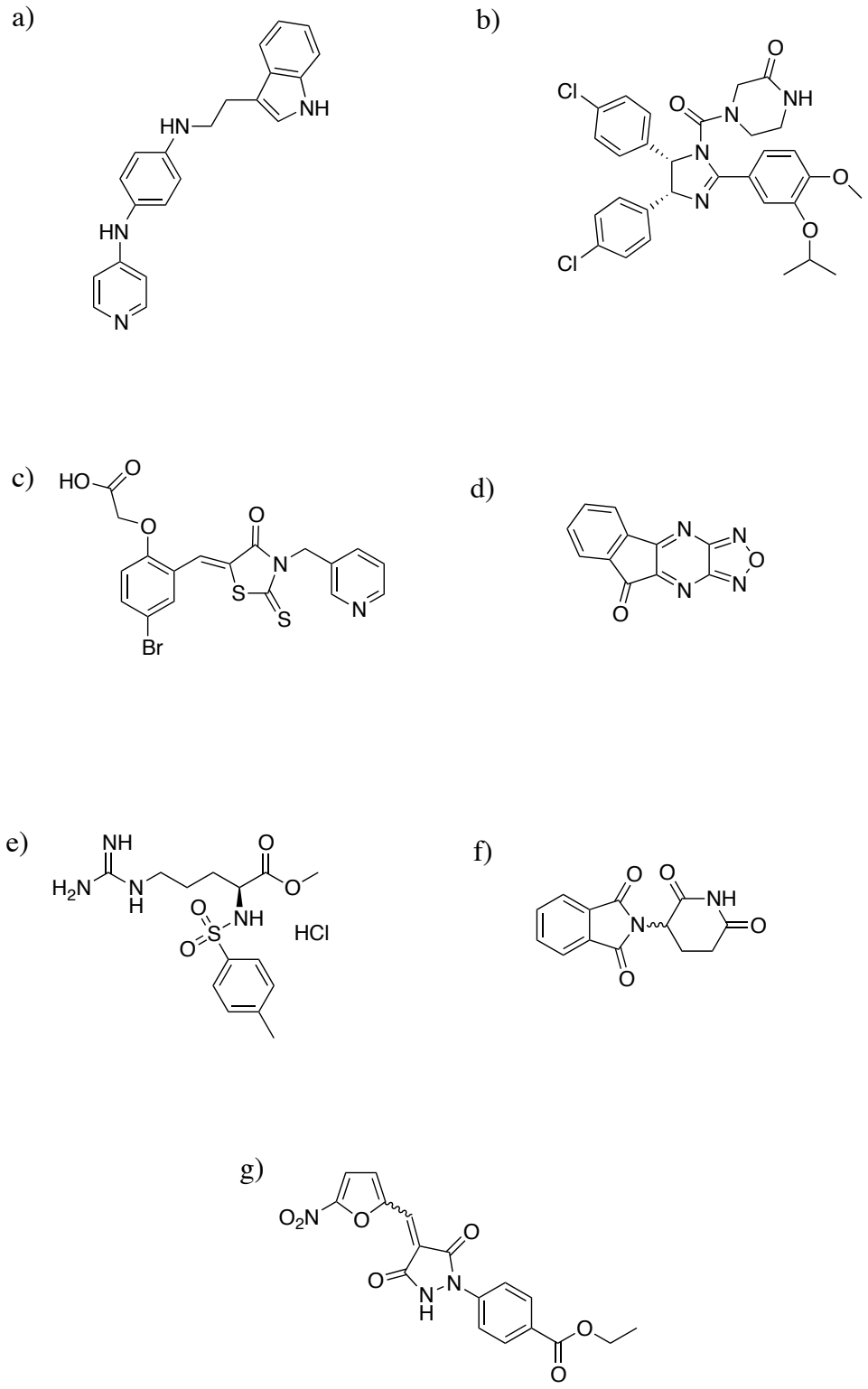

Figure 4.10 Structures of E3 ligase inhibitors and of an E1 conjugating enzyme inhibitor used in the ubiquitination pull-down assay. a) **Serdemetan** (HDM2 inhibitor) b) **Nutlin-3** (MDM2 inhibitor) c) **Skpin C1** (SCF-Skp2 inhibitor)d) **SMER3** (SCF^{Met30} inhibitor) e) **TAME HCl** (APC/C inhibitor) f) **Thalidomide** (Cereblon-DDB1-Cul4A complex inhibitor) g) **PYR-41** (E1 inhibitor).[10,21,28]

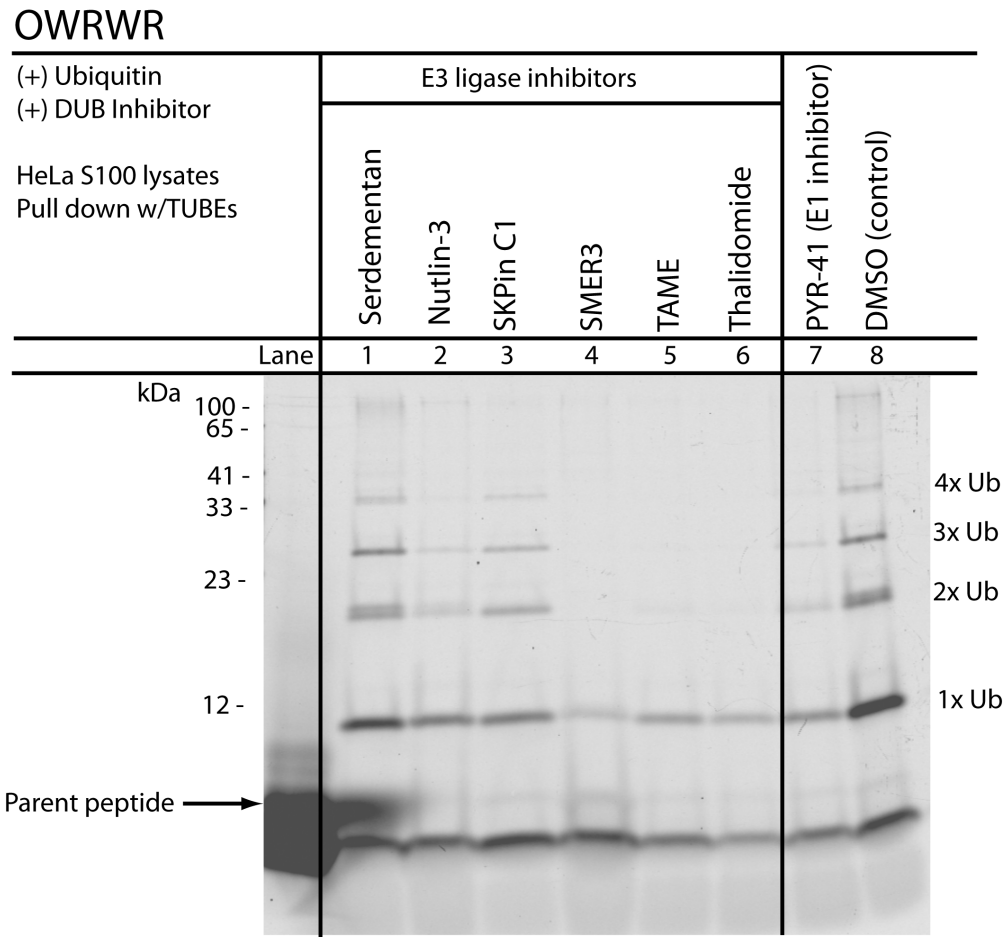

Figure 4.11. E3 Ligase Inhibitor Assay. The **OWRWR** peptide (10 μM) was incubated with HeLa S100 lysates (2 mg/mL) and in the presence of E3 ligase inhibitors (30 μM serdementan, 0.27 μM nutlin-3, 150 μM SKPin C1, 300 μM SMER3, 600 μM TAME, 90 μM thalidomide,or 30 μM PYR-41) for 2-8 hours. Ubiquitinated **OWRWR** peptide was isolated and separated by gel electrophoresis and analyzed by fluorescence imaging.[21]

vii. Cell permeability of Orn-degrons

Measuring cellular ubiquitin-mediated proteasomal activity in patient samples *ex vivo* must be done using a method that is amenable to small sample sizes of less than one thousand diseased cells. Assays utilizing cell lysates and/or antibody detection generally require one million cells or greater to surpass detection limits. An easily detected, cell permeable UPS activity reporter, coupled with sensitive instrumentation, would be ideal for such an application. Arginine-rich peptides, such as the TAT sequence, are known to be cell permeable and are generally believed to be endocytosed by HeLa cells.[31] It was expected that our highly positively charged degron peptides would be cell permeable in HeLa cells as well. Fluorescence microscopy of positively charged β-hairpin peptides incubated with HeLa cells revealed membrane permeability of the peptides but the observance of punctate domains implied endosomal entrapment (data not shown). Though cell permeability is a necessary feature of our degron peptides to be used in assays with small patient sample sizes, endosomal entrapment of the degrons would prevent ubiquitination and detection of modified or degraded reporter. OPM-2 cells however may have little or no transmembrane trafficking by endocytotic mechanisms. Cell permeable Orn-degron reporter peptides that can penetrate OPM-2 cells by direct penetration could serve as model systems for optimizing the detection of UPS activity in multiple myeloma patient cells using single cell CE-LIF.

[31]Zhang, X.; Jin, Y., Plummer, M.R.; Pooyan, S.; Gunaseelan, S.; Sinko, P.J. *Mol Pharm*, **2009**, 3, 836-48.

To determine the cell-permeability of the Orn-degron β-hairpins **OWRWR** and **OWOWO** in OPM-2 cells, each peptide was incubated with OPM-2 cells at different cell densities for 2-4 hours at 37°C. After incubation, the cells were trypsinized to digest any peptide that was present in the extracellular space and on the outside of the cell membranes, and then washed to removed the digested fragments. The cells were then imaged using fluorescence microscopy. No fluorescence was seen inside the cells (data not shown) indicating that OPM-2 peptides were not significantly cell permeable (data not shown).

viii. *Ex vivo* ubiquitination of OWOWO degron peptide

The poor membrane permeability of the **OWOWO** prevented the detection of intracellular ubiquitination *ex vivo* by simply incubating the peptide with the cells unassisted. Other methods for peptide cell permeation include microinjection (of single cells), electroporation, or pinocytosis. Microinjection has been used previously for the beta-bend protectides used as kinase reporters.[32] We chose electroporation because we already had the necessary equipment and reagents. Electroporation of cells is the induction of transient pore formation within the cell plasma membrane by the an applied electric field.[33] OPM-2 cells (5×10^5-2×10^6 per well) were electroporated with 10 μM or 25 μM **OWOWO** and then allowed to recover at 37° C for 4 hours. We also added a DUB inhibitor (PR-619) and a proteasome inhibitor (MG-132) to prevent the deubiquitination or degradation of the **OWOWO** degron peptide. The cells were then lysed and the degree of ubiquitination of the **OWOWO** degron peptide was determined using gel electrophoresis and fluorescence detection (Figure 4.12).

No ubiquitination of **OWOWO** was observed. It is important to note that in previous assays, exogenous ubiquitin was added and the lysates were incubated with ubiquitin antibodies conjugated to beads to isolate modified reporter only, thus enhancing the signal. No such measures were taken with the *ex vivo* cellular assay. Before repeating the assay and following up with a Ub-pull-down assay, we wanted to ensure that the **OWOWO** peptide was being sufficiently loaded using electroporation. The OPM-2 cells were electroporated with 10 μM or 25 μM **OWOWO** peptide and allowed to recovery at 37°C for 30 minutes. The cells were then imaged using fluorescence microscopy (Figure 4.12). Using the most optimal protocol, **OWOWO** appeared to be loaded into the OPM-2 cells though the loading efficiency was not determined. Few cells showed uniformly dispersed fluorescence, while the majority of cells containing fluorescent degron peptide displayed punctate domains of fluorescence characteristic of endosomal entrapment.[34] The lack of detection of ubiquitinated peptide in the *ex vivo* cellular assay may have been due to inefficient loading of the **OWOWO** peptide degron or its localization within endosomes. Despite many attempts to optimize the electroporation protocol, punctate domains were repeatedly observed by fluorescence microscopy.

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[32]Yang,S.; Proctor, A., Cline, L.L.; Houston, K.M.; Waters, M.L., Allbritton, N.L. *Analyst*, **2013,** 138, 4305-11.

[33]Glogauer, M.; Lee, W.; McCulloch, C.A.G. *Exp Cell Res*, **1993**, 208, 232-40.

[34]Richard, J.P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M.J.;Chernomodik, L.V.; Lebleu, B. *J Biol Chem*, **2003**, 278, 585-90.

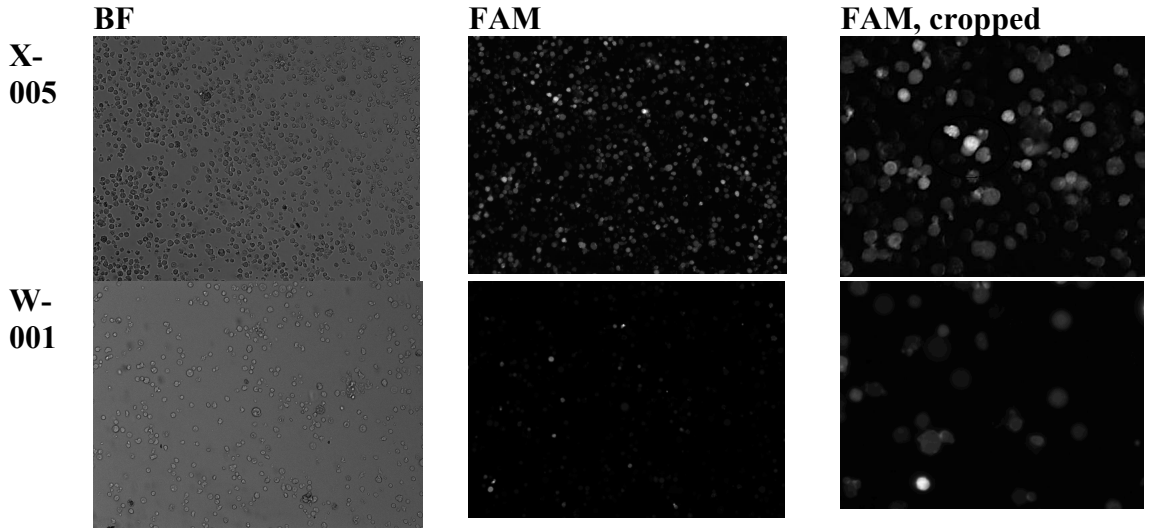

Figure 4.12 Electroporation of OPM-2 cells for the membrane permeation of the **OWOWO** degron peptide imaged by fluorescence microscopy. 10 μM **OWOWO** peptide was used with a cell density of 1.5 x10^7. BF represents bright field images of the cells. FAM represents cells imaged by fluorescence detection. X-005 was the electroporation preset protocol optimized for U266B1 (lyphoblast plasmacytoma, myeloma) cells. W-001 was the preset protocol for U-937 (human histiocytic lymphoma) cells.

The lack of detection of modified **OWOWO** in the *ex vivo* cellular assay could have been due to many impeding factors. Unaided, **OWOWO** may not be able to penetrate OPM-2 plasma membranes efficiently. With the assistance of electroporation, **OWOWO** is loaded into OPM-2 cells but may be trapped in endosomes. Sufficient amounts of the **OWOWO** peptide need to be free within the cytosol, where the UPS machinery is located, to be modified and detected. Though electroporation greatly enhances cellular uptake of otherwise blocked exogenous molecules, electroporation is also associated with increased uptake by endocytosis.[34] It is possible that the majority of the **OWOWO** loaded into the OPM-2 cells was present in endosomes and thus unavailable to serve as a substrate for the UPS machinery in the cytosol.

Another factor to take into consideration is how well **OWOWO** can compete with endogenous substrates of UPS machinery. Though we have shown that ornithine can be ubiquitinated, the rate and catalytic efficiency of ornithine ubiquitination may be lower than that of lysine ubiquitination. Intracellular concentrations of ubiquitin may be too low to modify **OWOWO** enough to be observed by gel electrophoresis-fluorescence detection. Currently work is being done in the Allbritton Group in collaboration with the Waters Group to detect ubiquitination of **OWRWR** degron peptide using CE-LIF.

D. Conclusions

We have successfully synthesized and characterized novel degron peptides that adequately served as reporters of UPS activity *in vitro*. We identified the noncanonical amino acid ornithine as substrate for endogenous UPS proteins in HeLa cell lysates (cervical cancer cell line) and OPM-2 cell lysates (multiple myeloma cell line) regardless of degron peptide secondary structure. We observed poly-ubiquitin chain formation on the OWRWR degron peptide, however the type of linkage (K48 or K63 linked, branched or linear, etc) was not determined. The unstructured and structured orn-degron peptides are expected to show greater resistance to proteolysis by OPM-2 cell lysates than the all-canonical amino acid control peptide. With the continued identification of new E3 ligases (currently >600 known E3s), it is likely that Orn-degron peptides are nonspecific reporters of UPS activity and can be modified by multiple families of E3 ligases. Nonetheless, we demonstrated that the addition of certain E3 ligase inhibitors, namely, SMER3, TAME HCl and Thalidomide, prevent poly-Ub chain formation of **OWRWR** though mono-Ub was seen in the presence of all inhibitors. While this data did not indicate the specificity of our peptide reporter, it does show the potential of the **OWRWR** to be used to determine the effect of thalidomide, SMER-3 and TAME-HCl treatment on patient UPS activity. It would be interesting to determine if there is a difference in ubiquitination of the **OWRWR** peptide in cell lysates treated with both a proteasome inhibitor and an E3 ligase inhibitor such as thalidomide which is commonly used in addition to proteasome inhibitors to treat multiple myeloma patients. Further optimization is required however before the **OWRWR** or **OWOWO** degron peptides can be used as cellular UPS activity reporters *ex vivo*. Inherent OPM-2 cell permeability of

the **OWOWO** and **OWRWR** degron peptides was not detected by fluorescence microscopy, meaning use in single patient cells would require microinjection or further modification to induce cell permeability. The **OWOWO** peptide was successfully loaded into OPM-2 cells by electroporation but the majority of the peptide was likely trapped in endosomes. The concentration of **OWOWO** that was freely diffusing in the cytosols of intact OPM-2 cells was not sufficient enough to compete with endogenous substrates of the UPS proteins to be modified at detectable levels for analysis by gel electrophoresis-fluorescence detection. Given that ornithine ubiquitination may be slower and less efficient than lysine ubiquitination, higher concentrations of reporter effectively loaded non-endosomally may remedy the lack of modified reporter detection. The use of ornithine in peptide-based reporters of UPS activity is still valuable as it confers protease resistance allowing detection of UPS activity for extended periods of time in the absence of protease inhibitors.

E. Ongoing work

Currently, the Allbritton Group in collaboration with the Waters Group is evaluating ubiquitination of the **OWRWR** degron peptide in other cancer cell lines using CE-LIF. The sensitivity of CE-LIF allows for lower limits of detection and the use of fewer cells in the analysis of detection of UPS activity in whole single cells and cell lysates assays. Eventually further modification will be done to promote non-endocytotic cell permeability of **OWRWR**.

F. Experimental

Peptide Synthesis and Purification

Peptides were synthesized using manual or automated standard solid phase peptide synthesis (Thuramed Peptide Synthesizer, CEM Liberty1 Microwave Peptide Synthesizer) using Fmoc protected amino acids on 0.06 mmol of CLEAR Amide resin from Peptide International. Four equivalents of standard amino acids were used each peptide synthesis while 2 equivalents of noncanonical amino acids and fluorophores were used. All peptides were acetylated at the N-terminus by treating the resin with 5% acetic anhydride and 6% 2,6-lutidine in 5 mL of DMF bubbling with N_2 for 40 minutes. 5(6)-Carboxyfluorescein or 6-carboxyfluorescein (FAM) was coupled to the εNH of lysine or the δNH of ornithine in i+4 position of the beta-hairpins or the centrally located amino acids of the scrambled sequences. The sidechains of these amino acids were orthogonally protected with an allyloxycarbonyl group (N-Aloc) or with 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-3-methylbutyl (N-ivDde). Aloc removal done based on the protocol published by Pazos et al.[35] The removal of ivDde from the sidechain of lysine or ornithine was accomplished by treating the peptide resin three times with 20 mL of 2% hydrazine monohydrate in DMF bubbling with N_2 for two minutes each time followed by washing with DMF, MeOH, DCM. Following deprotection, FAM was coupled using 2 equivalents of FAM, 5 equivalents of PyBOP/HOBt and 8 equivalents of DIPEA in 5 mL of DMF and allowed to bubble with N_2 for 4-8 hours. Double coupling was sometimes required.

[35]Pazos, E.; Jiménez-Balsa, A.; Maszareñas, J.L.; Vázquez, M.E. *Chem Sci***2011**, 2, 1984-87

Kaiser testing was done after the capping, deprotection and coupling steps. Peptides without cysteine were cleaved from the resin in 9.5:2.5:2.5 trifluoroacetic acid (TFA), TIPS and water respectively for 3-4 hours. Peptides containing cysteine were cleaved using 9.4:2.5:2.5:1 trifluoroacetic acid (TFA), EDT, water and TIPS respectively for 3-4 hours. The TFA was evaporated and the cleaved peptides were precipitated using cold ethyl ether and extracted with water. Extracted peptides were lyophilized and then purified using RP-HPLC on a Vydac C18 semi-preparative column with a gradient from '0 to 100% B' in 45-120 minutes. Solvent A was 95% water, 5% acetonitrile and 0.1% TFA and Solvent B was made of 95% acetonitrile, 5% water and 0.1% TFA. Purified peptides were lyophilized and their synthesis and purity confirmed by ESI-TOF or MALDI-TOF using dihydroxybenzoic acid matrix.

Peptide Concentration Determination.

UV/Vis was used for the concentration determination of each peptide in 10 mM sodium phosphate buffer (100 mM NaCl, pH 7.48 for protease degradation studies) pH 8 using FAM abosorbance at 492 nm, ε_{492} = 78,000 M^{-1} cm^{-1} for peptides with FAM or through 6M Guanidine hydrochloride denaturation and tryptophan absorbance at 280 nm ε_{280} = 5690 $M^{-1}cm^{-1}$ for those peptides without FAM.

CD Spectroscopy

CD spectroscopy data was collected using an Applied Photophysics Chiroscan Circular Dicroism Spectrophotometer. Spectra were generated at 25 °C with a wavelength scan 260 nm to 185 nm, with 0.5 second scanning in a 0.1 cm cell. Peptide concentrations of 40 μM in 10 mM sodium phosphate buffer, pH 8.02 (peptides with FAM) or pH 7.42 (peptides without FAM) were used.

Cell culture and lysate generation

HeLa S3 cells (ATCC) were maintained in Dulbecco's modified eagle medium (DMEM) with 10% v/v bovine calf serum (HyClone) and maintained in a 37°C, 5% CO_2 environment. OPM-2 cells (a kind gift from Donald McDonnell) were maintained in RPMI 1640 media supplemented with 12% fetal bovine serum (HyClone), 21.8 mM glucose, 8.6 mM HEPES (pH 7.4) and 1.0 mM sodium pyruvate. All media components are from Cellgro unless otherwise noted. Unless otherwise noted all reagents used in following assays are from Sigma-Aldrich. HeLa S100 cytosolic lysates were generated from cells based on the Dignam protocol as previously described.[1] Isolated HeLa S100 cytosolic lysates were then quantified with a Nanodrop 2000 (Thermo Scientific), aliquoted, and stored at -80°C. To generate lysates from OPM-2 cells, ~2.4x 10^7 cells were washed twice with 1X PBS and then resuspended in 200 μL of 1X PBS. Cells were submerged in liquid nitrogen for 1 min and immediately thawed in a 37° C water bath. The freeze-thaw cycle was repeated 3-5 times (a total of 4-6 cycles) followed by centrifugation at 14,000 rcf at 4° C for 15 min. The supernatant was removed and kept at 4° C for the duration of the experiment and the pellet was discarded. Lysates were used

within 4 h of generation. Total protein concentration of the OPM-2 lysate was determined by mixing 30 μL of lysate (diluted 1:1000 in 1X PBS) with 10 μL of fluorescamine (3 mg/mL) followed by a 5 min incubation in the dark at room temperature. Fluorescence was measured with a M5 Molecular Devices Spectrometer (475 nm) and compared with a BSA/fluorescamine protein standard for quantitation purposes.

Measurement of Degron Peptide Degradation in OPM-2 Cell Lysate.

OPM-2 cells cultured in six T-75 culture flasks (with an approximate cell density of 4 x 10^6 celss/flask) combined centrifuged at 0.8 rcf for 2.5 minutes. Media was discarded the cells were washed twice with 10 ml of 1X PBS (137 mM NaCl, 27 mM KCl, 10 mM Na_2HP0_4, 1.75 mM KH_2PO_4, pH 7.4). Supernatant was discarded and the combined pellet was resuspended in 200 μL of 1X PBS. Cells were submerged in liquid nitrogen for 1 min and immediately thawed in a 37° C water bath. The freeze-thaw cycle was repeated 3-5 times (a total of 4-6 cycles). Cells were centrifuged at 14,000 rpm at 4° C for 15 minutes. The supernatant was removed and kept at 4° C for the duration of the experiment. The pellet was discarded. Lysates were used within 4 hours of generation. Total protein concentration in the OPM-2 lysate was determined by mixing 30 μL of lysate (diluted 1:1000 in PBS) with 10 μL of fluorescamine (3 mg/mL) and allowing them to react for 5 minutes at room temperature in the dark. Immediately after, the fluorescence was measured using M5 Molecular Devices Spectrometer (475 nm) and compared with that of BSA protein standards mixed with fluorescamine. A 200- 400 μL

solution was made containing 3 mg/mL final OPM-2 lysate concentration and 10 uM final peptide concentration. The peptide/lysate solutions were incubated at 37 °C and aliquots of 20-40 μL were removed at various times points (1, 3, 5, 10, 15, 30, 60, 90, 120 minutes) and quenched with equal amounts of 200 mM HCl. Aliquots were frozen and stored at -20° C until time of analysis.

Capillary Electrophoresis

Detection of peptide degradation in OPM-2 lyastes was achieved by laser-induced detection (LIF, 488 nm) during capillary electrophoresis(ProteomeLab PA800, Beckman Coulter, Fullerton, CA). Fused-silica capillaries (30 or μm 50 μm inner diameter, 360 μm outer diameter, Polymicro Technologies, Phoenix, Az) were used with an effective length of 20 cm and a total length of 30 cm. Capillaries were preconditioned with 1 NaOH for 12 h, DI H_2O for 1 h, 0.1 M HCl for 6 h followed by DI H_2O for 12 h. Between each sample run, a pressure of 20 psi was applied at the capillary inlet allowing the capillaries to be rinsed with 1 NaOH for 2 min, DI H_2O for 2 min, and with electrophoretic buffer for 2 min. Quenched samples from the OPM-2 lysate degradation assay were diluted in 100 mM Sodium Phosphate Buffer (4 μL sample in 196 μL of buffer). Several electrophoresis buffers were tested. Initially quenched samples were diluted 1:100 in sample buffers that were identical to the electrophoretic buffers. As more electrophoretic buffers were tested to determine optimal separation conditions, 4 μL of each sample was diluted in 196 uL of 100 mM sodium phosphate buffer, pH 8.3, regardless of the electrophoretic buffer. The electrophoretic buffers tested include, 100

mM Tris, 100 mM Tricine Buffer pH 8. 1, 400 μM sodium phosphate buffer, pH 7.3, 10 mM sodium phosphate buffer, pH 8. 3, (30 μm capillary), 100 mM sodium phosphate buffer, pH 8.3, 200 mM sodium phosphate buffer, pH 8.3, 300 mM sodium phosphate buffer, pH 8.3, 400 mM sodium phosphate buffer, pH 8.3, 500 mM sodium phosphate buffer pH 7.3, 150 mM sodium phosphate buffer, 1 mM sodium deoxycholate (SDC), pH 7.3, 150 mM sodium phosphate buffer, 2 mM sodium deoxycholate (SDC), pH 7.3, 150 mM sodium phosphate buffer, 3 mM sodium deoxycholate (SDC), pH 7.3, 150 mM sodium phosphate buffer, 4 mM sodium deoxycholate (SDC), pH 7.3, 150 mM sodium phosphate buffer, 5 mM sodium deoxycholate (SDC), pH 7.3, 300 mM sodium phosphate buffer, 1 mM sodium deoxycholate (SDC), pH 7.3, 300 mM sodium phosphate buffer, 2 mM sodium deoxycholate (SDC), pH 7.3, 300 mM sodium phosphate buffer, 3 mM sodium deoxycholate (SDC), pH 7.3, 300 mM sodium phosphate buffer, 4 mM sodium deoxycholate (SDC), pH 7.3 (50 μm capillary). Sample was loaded into the capillary hydrodynamically by applying a pressure of 0.5 psi at the inlet for 5 sec. A negative voltage was then applied to initiate electrophoresis. Electrophoresis was done for 10 mins with a field strength of 8 kV cm^{-1} (Tris-Tricine buffer), 30 mins with a field strength of 8 kV cm^{-1} (sodium phosphate buffers), or for 60 mins with a field strength of 8 kV cm^{-1} (sodium phosphate buffers during OWRWR sample separations). Results were analyzed using 32 Karat, version 8.0 (Beckman Coulter, Fullerman, CA).

Ubiquitin pull down assay

The ubiquitin pull down assay was carried out at the indicated times at 37° C in a total reaction volume of 100 µL containing assay buffer (10 mM Tris-HCl pH 7.6 and 5 mM $MgCl_2$) with 2 mM DTT, 20 µg/mL ubiquitin aldehyde (Boston Biochem), 400 µg/mL ubiquitin or no Lys ubiquitin (Boston Biochem), 1X ATP energy regenerating solution (ATP-ERS, Boston Biochem), and 4.2 µg of indicated peptide substrate or 10 µM OWRWR degron peptide (E 3 Ligase inhibitor assay). Either 2 mg/mL HeLa S100 cytosolic lysates or 3.2 mg/mL OPM-2 lysates were used as the source of E1, E2, and E3 enzymes. Single inhibitory compounds (all obtained from LifeSensors) were added to the assay at the following concentrations for the duration of the assay: 30 µM serdementan, 0.27 µM nutlin-3, 150 µM SKPin C1, 300 µM SMER3, 600 µM TAME, 90 µM thalidomide,or 30 µM PYR-41. At the end of the indicated times, samples were incubated with Control-Agarose beads (LifeSensors), diluted in TBS-T buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.1% v/v Tween-20), for 60 min on a tube rotator at 4°C. Samples were subsequently centrifuged at 1800xg for 5 min to pellet and remove control beads. The supernatant was transferred to a solution of Agarose-TUBEs (LifeSensors) diluted in TBS-T and incubated overnight on a tube rotator at 4° C. Ubiquitin-bound beads were washed 5X with TBS-T and then the samples were eluted off of the bead with 2X tricine sample buffer, heated for 5 min (>90° C), and then isolated by centrifugation for 5 min at 13000xg. Samples were loaded onto SDS page gels (precast 16.5% Mini PROTEAN Tris-Tricine, Bio-Rad) using 1X tris-tricine running buffer (80 minutes at 120 V) and visualized with a Typhoon Imager 9400 at 600-1000 PMT (GE Healthcare Life Sciences). Gels were quantified using ImageJ (US National Institute of Health) by

comparing sample intensity to unreacted parent peptide intensity, which is not depicted in the gels.

Electroporation

All electroporation of OPM-2 cells was done using a Nucleofector™ manufactured by Lonza. Protocols A-030 (T2 cell line), T-005 (NSO cell line), W-001 (U-937) , X-001 CCRF-CEM cell line), and X-005 (U266B1 cell line) were tried using solution kit C were followed according to the instructions published on the Lonza website. Recovery times were 15 mins-4 h. Cell count was between 1-10 $x10^7$.

Fluorescence Microscopy

Post-electroporation cells were allowed to recover in OPM-2 complete media for 15 min-4 h at 37°C in 5% CO_2. Cells were then washed 3X with 1 X PBS and resuspended in extracellular buffer, pH 7.4. Cells were imaged at room temperature on plastic coverslips incased by rubber o-rings without being immobilized by Cell Tak™. Bright field images were taken with a 3 ms exposure time while fluorescence imaging was done using a GFP filter with 100 ms, 500 ms, and/or 1000 ms exposures.

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