Title
Design and Development of a High Affinity Peptide Ligand for Deoligomerizing Caveolin-1

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Gilliam, Amanda

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Design and Development of a High Affinity Peptide Ligand for Deoligomerizing Caveolin-1

DISSERTATION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by

Amanda Jean Hughes Gilliam

Dissertation Committee:
Professor Gregory Weiss, Chair
Associate Professor Andrej Lupták
Assistant Professor Jennifer Prescher

2015
DEDICATION

For

James Ross Hughes
the greatest man I have ever known
or ever hope to know

“Do you mean to tell me that you’re thinking seriously of building that way, when and if you are an architect?”

“Yes”

“My dear fellow, who will let you?”

“That’s not the point. The point is, who will stop me?”

-Ayn Rand, The Fountainhead
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vii-ix</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>x-xi</td>
</tr>
<tr>
<td>ABSTRACT OF THE DISSERTATION</td>
<td>xii-xiii</td>
</tr>
<tr>
<td>CHAPTER 1: Caveolin-1 as a Target for Research and Therapeutics</td>
<td>1-6</td>
</tr>
<tr>
<td>1.1 A Primer on Caveolin</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Caveolin’s Medical Relevance</td>
<td>3</td>
</tr>
<tr>
<td>1.3 The Challenge of Caveolin</td>
<td>3</td>
</tr>
<tr>
<td>1.4 References</td>
<td>5</td>
</tr>
<tr>
<td>CHAPTER 2: Measuring Dissociation Constants for High Affinity Ligands</td>
<td>7-28</td>
</tr>
<tr>
<td>2.1 Overview</td>
<td>7</td>
</tr>
<tr>
<td>2.2 Method Types</td>
<td>8</td>
</tr>
<tr>
<td>2.3 A Note on Labeling and Immobilization</td>
<td>8</td>
</tr>
<tr>
<td>2.4 Mass-Sensitive Methods</td>
<td>11</td>
</tr>
<tr>
<td>2.5 Movement-Sensitive Methods</td>
<td>15</td>
</tr>
<tr>
<td>2.6 Conformation-Sensitive Methods</td>
<td>18</td>
</tr>
<tr>
<td>2.7 Calorimetric Methods</td>
<td>19</td>
</tr>
<tr>
<td>2.8 Concentration-Sensitive Methods</td>
<td>21</td>
</tr>
<tr>
<td>2.9 Intrinsic Fluorescence</td>
<td>23</td>
</tr>
<tr>
<td>2.10 Conclusions</td>
<td>24</td>
</tr>
<tr>
<td>2.11 References</td>
<td>25</td>
</tr>
</tbody>
</table>
CHAPTER 3: Affinity-Guided Design of Caveolin-1 Ligands for Deoligomerization 29-50

3.1 Introduction 29
3.2 Results and Discussion 30
3.3 Conclusion 41
3.4 Experimental Section 43
3.5 Analytical HPLC traces for SPPS synthesized peptides 48
3.6 References 49

CHAPTER 4: Towards Understanding and Replacing the Disulfide Bridge in WL47 51-65

4.1 The Disulfide Liability 51
4.2 Dimerization is Vital to WL47 Function 51
4.3 Initial Attempts to Replace Disulfide 52
4.4 Conclusion of Initial Attempts to Replace Disulfide 56
4.5 Attempts to Convert Rather than Replace Disulfide 57
4.6 Conclusion of Attempts to Convert Disulfide 60
4.7 Experimental Section 62
4.8 References 65

CHAPTER 5: Conclusions and Future Directions 66-73

5.1 Current Status of WL47 66
5.2 The Dimerization Trap 66
5.3 Cell Penetration 67
5.4 Activity Assays 70
5.5 Conclusion 71
5.6 References 73
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1 Cartoon representation of a caveola.</td>
<td>1</td>
</tr>
<tr>
<td>Figure 3.1 Evolution of caveolin ligands.</td>
<td>30</td>
</tr>
<tr>
<td>Figure 3.2 Initial identification of caveolin interacting region.</td>
<td>31</td>
</tr>
<tr>
<td>Figure 3.3 Identification of key residues.</td>
<td>32</td>
</tr>
<tr>
<td>Figure 3.4 Removal of detrimental amino acids.</td>
<td>33</td>
</tr>
<tr>
<td>Figure 3.5 Disulfide dimerization explored.</td>
<td>34</td>
</tr>
<tr>
<td>Figure 3.6 Unnecessary residues identified for removal.</td>
<td>35</td>
</tr>
<tr>
<td>Figure 3.7 Sequences shuffled.</td>
<td>36</td>
</tr>
<tr>
<td>Figure 3.8 Demonstration of selectivity.</td>
<td>37</td>
</tr>
<tr>
<td>Figure 3.9 Determination of binding affinity.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 3.10 Demonstration of ability to deoligomerize caveolin.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 4.1 Disruption of oligomers.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 4.2 Structures of architectures from initial disulfide replacement study.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 4.3 Screening of branched and thioether-converted architectures.</td>
<td>58</td>
</tr>
<tr>
<td>Figure 5.1 Proposed design of peptide conjugates for testing cell permeability.</td>
<td>68</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.2  Overview of binding assay techniques.</td>
<td>10</td>
</tr>
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</table>
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I must first express my deepest appreciation to my advisor, Professor Gregory A. Weiss. Thank you for taking a chance on me by letting me rotate through your lab, despite my having followed a decidedly non-linear path into the world of Chemistry. I was proud to be your first rotation student from the Medicinal Chemistry and Pharmacology gateway program, and I was and remain honored and humbled to have been a member of your lab. I especially appreciate your patience with my stubborn refusal to switch projects even when it seemed impossible that the caveolin ligand project could ever succeed. I am also incredibly grateful to have been your Teaching Assistant for three quarters: your passion for teaching is infectious and I have learned so much about the kind of teacher I hope to be someday.

I would also like to thank my wonderful committee members, Professors Andrej Lupták and Jennifer Prescher. Andrej has been my champion from the moment I was accepted to the MCP program, and throughout the rocky days of graduate school I always knew that with Andrej on my side things would turn out all right somehow. Jen has been an inspiration to me, as a pioneer in the fledgling field of chemical biology and as a woman in science; on some of my darkest days I would wonder What Would Jen Do? and it would inspire me to push myself harder.
I am also grateful for the help and guidance I have received from Professors James Nowick and Sheryl Tsai, both of whom absolutely ripped me to shreds during my advancement to candidacy, which was terrifying, helpful, and gave me confidence in knowing that I really earned my advancement. Later, James would take me under his wing when I was frantically trying to put together a massive NIH fellowship application, and Sheryl has always taken a kind interest in me and my future.

I was also blessed to have been able to work with a cohort of exceptional colleagues in the Weiss lab. I am grateful for Drs. Agi Hajduczki and Sudipta Majumdar who laid the original groundwork for the caveolin ligand project, and to Josh Smith who has been my protein provider, my partner in crime science, and my rock throughout my time here. I am also grateful for the warmth and wisdom of Drs. Tivoli Olsen and Mark Richardson, and for the help and companionship of Andrew Gansmiller and Kevin Johnston.

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I also cannot possibly overstate how thankful I am for Tim Valentic, who is the kindest, the funniest, most caring and most supportive person alive, and also the best boyfriend anyone could hope to have. Whether I needed to be held while I melted down, or kicked in the butt when I was moping myself into oblivion, or just reminded to have a snack and trust that tomorrow would be better, Tim was there to do all that and more.
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Education and Honors
University of California Irvine, Department of Chemistry, Irvine, CA
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- Bachelor of Arts in Neuroscience, with minors in Chemistry and Theatre, May 2009.
Awards and Honors
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Graduate Student Researcher in Protein Engineering University of California Irvine, Department of Chemistry, Irvine, CA January 2012 – Current
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- Used multiple peptide synthesis schemes and fluorescence affinity assays, including assays involving tissue culture, to design and validate a novel peptide ligand to disrupt oligomerization of the protein caveolin-1.

Glycobiology Research Assistant University of Oklahoma Health Sciences Center, Biochemistry & Molecular Biology Dept, Oklahoma City, OK September 2009 – December 2010
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- Investigated the properties and potential drug delivery applications for glycosaminoglycan synthases and their products using techniques such as chemoenzymatic synthesis and radiation-based activity assays.

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- Used basic cloning, protein expression, protein purification, and protein crystallization while working closely with supervising professor to explore the structural biology of a biofilm-associated protein.

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- Prepared and maintained immortalized human cell lines for research on cellular response to low-dose ionizing radiation.

Publications


Technical Skills
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- Binding assays including isothermal titration calorimetry (ITC) and fluorescence anisotropy (FA)
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• Protein heterologous expression and purification by affinity chromatography
• Polyacrylamide electrophoresis, including SDS PAGE, CN-PAGE, and BN-PAGE
• Plasmid engineering, PCR, Western Blot, ELISA, agarose electrophoresis
• Novel assay design, development, and implementation
• Analytical and preparatory HPLC, reverse phase and size exclusion
• Peptide, carbohydrate, and small molecule TLC
• MALDI-TOF and ESI mass spectroscopy
• Ascending and descending paper chromatography
• Radioisotope usage and safe handling, $^{14}$C and $^{3}$H

Personal Skills
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• Able to think critically and creatively in problem solving situations
• Patient, determined, and persistent
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Teaching Assistant Experience
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Introduction to Chemical Biology CHEM 128 lecture and discussions Winter 2015
Organic Chemistry CHEM 51A/CHEM 51C lecture and discussions Fall 2013/Fall 2012
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Stage Management, Directing, Acting, Wardrobe, Props
• Utilized skills in problem solving, group management and leadership, communication, and organization.

Other Skills
• Proficient in French
• Skilled in graphic design
• Extensive experience working with special needs children and adults
ABSTRACT OF DISSERTATION

Design and Development of a High Affinity Peptide Ligand for Deoligomerizing Caveolin-1

by

Amanda Jean Hughes Gilliam

Doctor of Philosophy in Chemistry

University of California, Irvine, 2015

Professor Gregory Weiss, Chair

The caveolins are a family of human membrane proteins implicated in cancer and other diseases. In Chapter 1, I briefly explain some of the basic biochemistry of the caveolins, and why they are not only a promising target for research and therapeutics but also a particularly challenging target. The novel peptide WL47, the development of which is described in later chapters, is introduced as a caveolin-1 ligand. WL47 will allow new avenues of investigation for this challenging target.

In Chapter 2, I review, compare, and contrast some of the myriad techniques available to quantify binding affinity for high affinity binding interactions such as the interaction between caveolin-1 and WL47. As I discovered in my own attempts to measure the dissociation constant for this interaction, high affinity is a boon for future use of a
ligand probe, but it can be challenging to quantify initially. Because a review of techniques specifically suited to high affinity measurement was not available at the time I needed it, I have written this chapter to fill this gap in the literature.

In Chapter 3, I discuss the development of peptide ligand **WL47** through an iterative process of library design, synthesis, screening, and data analysis. High affinity binding, target selectivity, and activity as an inducer of deoligomerization of oligomeric caveolin-1 is presented. Beyond providing a novel tool for probing the function of caveolin-1 oligomerization, this chapter is also a roadmap for the creation of future peptide ligands through this generalizable, iterative process.

Chapter 4 details the progress that has been made toward understanding the importance of **WL47** dimerization through a disulfide bridge. This chapter also covers initial efforts towards designing a redox-stable version of **WL47** that will remain functional in conditions like those found in the interior of a live cell.

Chapter 5 concludes the dissertation with a discussion of the current state of the caveolin ligand project, including detailed descriptions of the experiments that will be necessary for future researchers to bring this project to its conclusion.
Chapter 1: Caveolin-1 as a Target for Research and Therapeutics

1.1 A Primer on Caveolin

The caveolin proteins are a ubiquitous class of membrane proteins with structural, signaling, and transport roles within the cell. The best known and most studied of these, caveolin-1 (hereafter referred to as “caveolin”), is a 22 KDa protein with a monotopic membrane insertion. Caveolin penetrates only one leaflet of the lipid bilayer and both the N- and C- termini remain on the cytoplasmic side.1-3 These proteins oligomerize to form high molecular weight complexes, which bend the membrane inward to form invaginations, termed “caveolae,” that are 50-100 nm in diameter (Figure 1.1).3-5 The cholesterol- and sphingolipid-rich membrane of these caveolae regions are considered a sub-type of lipid raft.6,7 While these invaginations can mediate endocytosis in a similar manner to clathrin-coated pits, current research emphasizes their role in signaling pathways. Caveolin’s binding partners are manifold, among them cAMP-dependent protein kinase A (PKA), endothelial nitric oxide

Figure 1.1. Cartoon representation of a caveola. Oligomeric caveolin (blue) embedded in the lipid bilayer bends the cell membrane (grey) to form an omega-shaped structure. Cholesterol (yellow) levels are enriched within the membrane of the caveola.
synthase (eNOS), insulin receptors, and the HIV coat protein gp41, among many others. In binding, caveolin both sequesters these signaling molecules and can trap them in either their active or inactive conformations, thereby modulating the associated signaling pathways. In addition to regulation of signaling pathways, caveolin is involved in viral entry into cells. For example, caveolin interacts with the HIV envelope protein gp41 during viral fusion, and a distinct caveolin binding motif has been identified in the sequence of gp41. Caveolin is also highly involved in cholesterol trafficking, and plays a key role in cellular response to mechanical stress.

Oligomerization of caveolin is mediated by two separate domains and undergoes two oligomerization steps that are spatially and temporally distinct within the cell. The first oligomerization step occurs in the endoplasmic reticulum. The number of caveolin monomers in each of these homooligomeric species has not been definitively determined, but different reports suggest a range of 7 to 16 units. Subsequently, these oligomers are transported to the Golgi, where they undergo a second step that forms larger multimers of the pre-assembled oligomers. Evidence suggests that the primary oligomerization step is mediated by the classical caveolin oligomerization domain that overlaps the caveolin scaffolding domain (CSD) on the N-terminal cytoplasmic region, whereas the secondary oligomerization step is mediated by the distal third of the C-terminal cytoplasmic region. Caveolin homooligomers of approximately 200, 400, and 600 kDa have previously been observed using sucrose gradient ultracentrifugation.
1.2 Caveolin’s Medical Relevance

Due to their diverse functions, the overexpression, underexpression, or dysfunction of caveolins can contribute to myriad diseases, including cancer, diabetes, Alzheimer's disease, muscular dystrophy, obesity, asthma, scleroderma and other fibrotic diseases, heart disease and hypertension, and HIV.\textsuperscript{24-29} Focusing first on cancer various approaches suggest that caveolin-1 levels can be upregulated, downregulated, or mutated depending on the type and stage of cancer.\textsuperscript{30} The dominant negative mutation P132L has been identified in 16\% of human cancers and leads to reduced caveolin-1 levels.\textsuperscript{31} Limb-girdle Muscular Dystrophy is similarly caused by dominant negative mutations in which mutated caveolin-3 protein aggregates with wild-type caveolin-3, directing it toward degradation pathways and reducing the overall levels of caveolin-3.\textsuperscript{32,33} Conversely, another form of muscular dystrophy, Duchenne's Muscular Dystrophy, is associated with significant upregulation of caveolin-3 rather than downregulation but nonetheless causes a similar phenotype.\textsuperscript{34} This paradoxical aspect of caveolin – the same types of disease states can be associated with either up- or down-regulation of caveolins – clearly shows that, despite how far the field of caveolin research has come, vast unknowns remain to be addressed.

1.3 The Challenge of Caveolin

Although these and other associated diseases make the caveolins desirable targets for research and drug design, progress has been hampered by a lack of techniques available to probe their structure and functions. Much of what is currently known about caveolin has been derived from studies involving knock-outs, mutagenesis, or extreme cholesterol depletion to reduce caveolin levels; these approaches do not allow for the targeted study of
the wild-type protein under native conditions.\textsuperscript{1-3,5,35} In this dissertation, I will present the peptide ligand \textbf{WL47}, a novel molecular tool targeting caveolin, and demonstrate its ability to selectively disrupt the \textit{in vivo} oligomerization function of the most common and best studied of the caveolins, caveolin-1. Such a tool will facilitate future research and generate a potential lead compound for therapeutics to treat the caveolin-associated diseases described above.
1.4 References


Chapter 2: Measuring Dissociation Constants for High Affinity Ligands

2.1 Overview

In the common parlance of science, higher affinity ligands are generally considered “better” than lower affinity ligands. A high affinity ligand allows greater target binding to be achieved using a lower dosage, which often translates to research tools and pharmaceuticals with lower costs and fewer off-target effects. Once a promising lead compound has been identified as a ligand for a given target, a major goal for the medicinal chemist is to optimize the affinity of the ligand for its target.\textsuperscript{1,2}

Despite improving the performance of drugs and research reagents, high affinity can also make it difficult to accurately measure the thermodynamics and kinetics of the binding interaction. This situation can strand a researcher with a phenomenal ligand with binding too strong to easily quantify.\textsuperscript{3} Some major considerations for any researcher attempting to quantify binding for a ligand with troublingly good affinity will be equipment availability, the characteristics of the ligand and the binding target, and the pros and cons of the various methods for measuring binding affinity (Table 2.1). A 2010 review by Vuignier et. al. on the topic of drug binding to blood plasma proteins provides an excellent overview and critical analysis of many available techniques for ligands with $K_D$ in the mM to $\mu$M range.\textsuperscript{4} Among the methods suitable for this $K_D$ range are ultrafiltration, parallel artificial membrane permeability assays, and affinity capillary electrophoresis.
In the following chapter, I will review some of the techniques used for experimentally measuring the dissociation constant for a high affinity ligand (nM to fM \( K_0 \) range). This review is designed as a guide for researchers seeking the appropriate method for obtaining binding data, and thus the theory and details for each method will not be provided in depth. However, wherever possible, references with such information can guide those interested in greater detail.

### 2.2 Method types

Binding assays fall into several general types based on the way in which they obtain information about the binding interaction. The five most common categories can be characterized as Mass-Sensitive, Conformation-Sensitive, Movement-Sensitive, Calorimetric, or Concentration-Sensitive. These categorizations are more than just taxonomical but can be revealing as to the types of interactions for which they will be most useful in measuring.

### 2.3 A Note on Labeling and Immobilization

Label-free systems are less disruptive to native binding interactions, and thus methods that do not rely on labeling are favored whenever possible.\(^5\) While label-free methods are considered more robust owing to their potential to more closely mimic native interactions, many label-free techniques are hampered by size sensitivity. This means that signal strength is roughly proportional to the size of the ligand being assayed. By using a fluorescent or radiolabeled ligand, the signal strength can become independent of ligand mass.
Many techniques are touted as label-free methods, but use of the term “label-free” should be viewed with caution; immobilization of either binding partner on a solid surface can be thought of as a labeling process writ large. Immobilization of the larger partner is often considered to be a superior tactic to labeling the smaller partner. While this logic holds true in a general sense, each situation will be slightly different and the researcher must determine, possibly experimentally, which method will be least disruptive for their particular interaction to be assayed. For example, a receptor can often be immobilized at a site far from the site of ligand interaction; whereas all parts of a small ligand are generally involved in the binding interaction, and addition of a label to any part of the ligand has a higher probability of being disruptive to native binding interaction. Despite the lower likelihood of disruption, it has been well established that different methods and locations of receptor immobilization can themselves alter the observed interaction. In an experiment by Reddy et. al., significant (p < 0.0001) differences in measured \( K_D \) were seen depending on the location and chemistry used to immobilize antibodies on an SPR chip. Another issue that applies to all methods with receptor immobilization is the possibility of non-specific binding; the mere presence of a solid support for the immobilized receptor also adds a potential surface for ligands to bind, potentially creating data artifacts. Nonetheless, kinetic data, if desired, can often only be obtained using methods that rely on immobilization of one partner. So, the researcher must determine what method best suits his or her needs, with a clear understanding of the methods’ caveats.
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<th>Immobilized or Labeled Target Protein</th>
<th>Labeled Ligand</th>
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<th>Provides Kinetic Data</th>
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<th>Amount of Material Needed</th>
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Table 2.1. Overview of binding assay techniques. All methods listed here have a lower limit of $K_D$ measurement between $10^{-8}$ and $10^{-12}$ M. Actual limits of detection will depend on the individual parameters chosen, including the mass, buffer, temperature, and any fluorophores or immobilization methods.
2.4 Mass-Sensitive Methods

Mass-Sensitive methods include optical and acoustic methods, such as surface plasmon resonance (SPR), quartz crystal microbalance, and several types of interferometry. In these methods, ligands bind to a receptor-functionalized surface to add additional mass and charge. This mass, and often charge, then alters the way in which the surface interacts with a light- or resonance-based signal. For any mass-based method, signal strength will be directly proportional to ligand mass, making these techniques ideal for measuring interactions between two large binding partners, such as protein-protein interactions or investigating small ligands that modulate such interactions in a competition binding mode. On the other hand, these methods are often ill-suited for measuring small-molecule binding interactions. One way around the mass-signal issue is to immobilize the ligand rather than the target receptor. This method has been used successfully but carries the risk of increased perturbation of the native binding interaction for the reasons noted above. Alternatively, a high-mass label can be attached to the ligand to increase its heft, but approach this increases the likelihood of steric hinderance.

Of these, SPR is the best-known and most-utilized method. Monochromatic light directed at the gold-coated underside of a receptor-functionalized SPR chip excites the surface electrons of the gold, causing them to oscillate as a plasmonic wave. The electrical field of this plasmonic wave is sensitive to mass changes on the receptor-coated side of the chip. As the mass on the upper, sensing surface is altered by ligand binding to receptors, the refractive index of the reflecting surface changes. The resulting change in the angle of reflected light provides a measurable signal proportional to the total mass bound to the
sensing surface. A quick perusal of recent literature provides myriad examples of this technique. Of particular interest is recent work by Reddy et. al., mentioned briefly above, wherein malarial antigens Merozoite Surface Protein 2 (MSP2) and Apical Membrane Antigen 1 (AMA1) were immobilized on SPR chips via N-terminal or C-terminal coupling chemistry or non-covalently via His-tag. The affinities of both purified monoclonal antibodies and unpurified serum containing naturally-acquired polyclonal antibodies were measured for these antigens. Measured affinity differed significantly depending on the orientation of the immobilized antigen, emphasizing that future work on the development of anti-malarial antibodies must take care that the target proteins are oriented in such a way that the antibodies evolved against them will be functional in vivo.

One reason why SPR is so favored is because it permits the researcher to obtain kinetic information. As ligand is introduced to the SPR chip via a flow channel, time-resolved binding association ($k_{on}$) can be observed. Analogously, by halting the introduction of additional ligand and allowing a wash-out step of solvent though the flow channel, the time-resolved dissociation ($k_{off}$) can also be observed. SPR is also non-destructive, both the receptor-functionalized chip and the dissolved ligand can often each be reused for subsequent assays. SPR also requires very little receptor and ligand to perform an assay, as the technique is quite sensitive. QCM performs similarly, but uses an acoustic rather than optical signal to measure binding of ligand to a receptor-functionalized piezoelectric quartz crystal; the frequency of the bulk acoustic wave resonance decreases proportionally to the mass, providing a signal analogous to the changing angle of reflected light used in SPR. One example of QCM performed by McMillan et. al. probes the interaction between the protein flavocytochrome $c_3$ and its
native binding partner, the respiratory-chain enzyme CymA. Coupling this technique with several electrochemical assays, the authors present this transient protein-protein interaction as a modulatory mechanism for the electron transport chain of the bacteria S. oneidensis.

Other methods that rely on interaction of light with the functionalized surface include dual-polarization interferometry (DPI) and bio layer interferometry (BLI). In these methods, the thickness of the biological layer adhering to the measurement surface is proportional to the refractive index of the layer, which in turn alters the signal output of either a laser-based evanescent wave or white light reflection, respectively. Thickness of this biological layer is generally linked to mass, although conformational changes to the immobilized receptor can, in certain cases, also change the apparent thickness. This phenomenon can be useful in cases where the binding ligand is of very small mass but produces a significant structural change to the binding partner. Alternatively, some researchers have broadened the scope of the approach by applying it not only to binding induced mass changes but also to pH-associated structural changes to the protein layer. Sheu et. al. investigated C-reactive protein, a blood serum protein associated with inflammation, cardiovascular disease, and metabolic syndrome that also displays remarkable conformational heterogeneity. Sheu et. al. use DPI to demonstrate three distinct oligomeric forms of the protein at three different pH levels, which they believe contributes to the complex and variable functions of C-reactive protein associated with different pathologies. A model experiment by Shah et. al. of E. coli ATP synthase catalytic complex binding the inhibitory subunit ε provides a visualized protocol for BLI. Like SPR,
these interferometric methods also allow for the collection of kinetic information because data is time-resolved.

While kinetic assays provide valuable insight into binding behavior, it is sometimes necessary to sacrifice kinetics in order to obtain any data. The necessarily dilution of reagents when testing high-affinity ligands reduces the signal strength below that of the instrument’s sensitivity. These mass-based methods such as SPR and QCM can alternatively be done with homogeneous mixtures in the form of binding inhibition assays, which makes the signal less reliant on the mass of the interacting partner, also precludes time-resolved data acquisition.6 The details of binding inhibition assays will be further addressed later, in the context of kinetic exclusion assays.

Mass-Sensitive methods that can be performed without immobilization include light-scattering approaches and non-covalent mass spectrometry. Light scattering methods such as concentration gradient dynamic light scattering (CG-DLS,) and concentration gradient multi-angle light scattering (CG-MALS) use size and shape as a proxy for mass by measuring the ways in which light interacts with particles in solution.9,10 Measurement of the intensity and scattering angles of the light allow for computational determination of average particle size. Work by Holm et. al. uses DLS in tandem with calorimetric measurements to investigate the interplay between ligand binding and thermal unfolding of the multimeric high-affinity folate binding protein, a transport and signaling protein, at variable pH.26

Non-covalent mass spectrometry uses direct mass-based measurements of unlabeled and non-immobilized binding partners. Receptor that has been pre-incubated
with ligand is subjected to mass spectroscopy measurements using soft (non-fragmenting) ionization techniques such as electrospray ionization (ESI). The peaks observed correspond to receptor with and without bound ligand. This technique is especially suited for probing binding interactions that display complex stoichiometry because it measures mass directly, independently quantifying the amount of both bound and free receptor, rather than a weighted average. Recent work by Maple et. al. used non-covalent mass spectrometry to investigate the binding interaction between a novel atropisomeric small molecule and antiapoptotic target proteins Bcl-2 and Bcl-xL, with the resulting $K_D$ values showing excellent agreement with complementary data obtained through Conformation-Sensitive and Concentration-Sensitive methods.

Nonetheless, non-covalent mass spectrometry suffers from several drawbacks that have hindered its common usage, such as the dubious correlation of ionized material to solution-phase interaction. In a worst case, ionization can completely destroy the non-covalent interaction. While doggedly pursued in the 1990s, this method has fallen out of favor and is considered more as an adjunct to other more robust methods. Progress continues to be made in this realm, particularly with regard to developing increasingly gentle methods for ionization in aqueous conditions, possibly indicating a renewal of interest by the scientific community.

2.5 Movement-Sensitive Methods

Movement-Sensitive methods such as microscale thermophoresis (MST) and fluorescence anisotropy (FA) are indirect methods of probing mass change. Unlike the directly Mass-Sensitive methods discussed above, signal strength is not proportional to the
mass of the ligand, but is tied to the difference in mass between the ligand and the receptor-ligand complex.\textsuperscript{37} A small ligand can tumble and diffuse swiftly in solution, whereas the larger target receptor moves and rotates considerably slower. The mass difference between the unbound ligand and the bound complex must be significant in order for the difference in movement to be measured by these methods. This criterion is easily met by receptor-ligand interactions involving proteins.

MST uses heat to induce movement in a small sample volume of ligand and target receptor, and the speed of the fluorescently tagged ligand’s movement in and out of the small observed region is proportional to its size.\textsuperscript{38} In this way, the degree of movement can be correlated to the fraction of the labeled fluorophore that is unbound versus bound to the much larger target receptor. A review by Jerabek-Willemsen et. al. emphasizes that thermophoretic-induced movement is associated not only with size but also with solvation and charge.\textsuperscript{38} This makes it a flexible technique for a multitude of binding interactions regardless of mass change. Because changes to thermophoretic movement can be caused by attributes other than size, this method can potentially be performed with a fluorescently tagged target receptor rather than a tagged ligand. As discussed before, modification of the larger partner rather than the smaller partner is often less disruptive to native binding interactions. This modified strategy has been successfully implemented by Khavrutskii et. al. using the convenient GFP-fusion to the protein receptor of interest.\textsuperscript{39} Khavrutskii et. al. created a fusion of GFP to a STAT3 transcription factor protein that was then tested for binding to variable oligonucleotide sequences without the need for purification out of the cell lysate.\textsuperscript{39} Others, such as Yu et. al. have successfully utilized MST to observe the interaction of bacterial lipid extracts with a novel antimicrobial peptide.\textsuperscript{40}
FA, sometimes referred to as fluorescence polarization, similarly uses the mass difference between the bound and the unbound fluorescently labeled ligand to determine the fraction of receptor-ligand complex. The key measurement for this method relies upon the time differential between molecular tumbling in solution and the fluorescence lifetime of the fluorophore – how much the molecule is able to move between excitation and emission. A fluorescently tagged ligand is excited by polarized light. In its free, fast tumbling state, the fluorophore experiences significant movement before re-emission of the photon, allowing the polarization to be lost. This emitted light, when measured through polarization filters parallel or perpendicular to the original polarized excitation light, will have nearly equal signal strength because nearly all of the initial polarization has been lost. Conversely, in the ligand’s bound state, the fluorophore will experience decreased movement due to the larger mass of the ligand-receptor complex. This slower movement reduces the likelihood that the fluorophore will be in a significantly different position when the photon is re-emitted, resulting in more retained polarization. Measurement of emitted light through a polarization filter parallel to the original polarization will have high signal strength, whereas measurement of emitted light through a perpendicular filter will have low signal strength, indicating retention of polarization. A molecule of sufficiently small mass will tumble so quickly that the theoretical signal will be entirely isotropic, which is to say that it exhibits no polarization at all. A fluorescent ligand that is bound to a target receptor of large mass will tumble much more slowly relative to the fluorescence lifetime. The light will be reemitted with more polarization retained. Such a signal with retained polarization is anisotropic. The amount of anisotropy shown by a mixture of ligand and receptor is a weighted average of the anisotropy signal contributed by the receptor-ligand
complex and the anisotropy signal contributed by the unbound ligand, making the degree of anisotropy proportional to the fraction of ligand bound for a given concentration of ligand and receptor.\textsuperscript{42} Knape et. al. have implemented this technique to study binding interactions under various conditions, specifically the interaction of protein kinase A with a pseudosubstrate inhibitor in the presence of either Mg\textsuperscript{2+} or Ca\textsuperscript{2+} ions.\textsuperscript{43} Results demonstrated that Mg\textsuperscript{2+} promotes high affinity binding between protein kinase A and the inhibitor, whereas Ca\textsuperscript{2+} reduced binding by three orders of magnitude.\textsuperscript{43}

2.6 Conformation-Sensitive methods

Conformation-Sensitive methods relate specifically to ligand-induced stabilization of the target receptor. Thermal shift assays, known as ThermoFluor or differential scanning fluorimetry, traditionally use a reporter dye that fluoresces when bound to partially denatured receptor, measuring the thermal denaturation of the receptor.\textsuperscript{44-46} In this method, the binding of the ligand to the receptor of interest generates a shift in thermal stability. The magnitude of change observed with different concentrations of ligand provides a binding curve that can be fit to determine $K_D$. Several recent papers use thermal shift assays as adjuncts to other methods, as they are simple to set up and are higher throughput than many other methods.\textsuperscript{45,47} Circular dichroism (CD) can also be used to assay the dose-dependant structural changes that a ligand causes to the target receptor. CD can be performed with a thermal shift in order to measure the effect of ligand binding on receptor stability without the use of a fluorescent reporter dye.\textsuperscript{48} In some cases, the mere act of binding is enough to cause a detectable level of structural change without the need for thermal denaturation.\textsuperscript{49}
Even more sensitive than CD without denaturation are the several ways in which nuclear magnetic resonance (NMR) can be harnessed to yield binding data.\textsuperscript{50,51} One of these methods is NMR chemical shift titration, in which the NMR signal for the $^{15}$N-labeled target receptor is obtained in the presence of variable amounts of unlabeled ligand observe the changes in receptor chemical shift signals upon ligand binding.\textsuperscript{52} Such experiments are time consuming and can be expensive due to the necessity of producing isotopically labeled target receptor. If the target receptor's structure is well characterized, careful signal interpretation can yield the added benefit of identifying the site of binding by identifying key residues that correspond to those NMR signals showing the greatest signal shift upon ligand binding.\textsuperscript{2,53} Yan et. al. demonstrate this function in their investigation of small molecules targeting the Ral GTPase, a protein associated with tumor growth and metastasis.\textsuperscript{19} Additional, more complex strategies for obtaining binding information by NMR have been addressed elsewhere.\textsuperscript{54-56}

2.7 Calorimetric Methods

Isothermal titration calorimetry (ITC) is currently considered the “gold standard” for binding affinity measurement.\textsuperscript{2} As a calorimetric method, ITC measures the enthalpy, or heat change, inherent to molecular interactions.\textsuperscript{57-60} A sample cell containing a known concentration of one binding partner is treated with multiple microinjections of a solution of the other binding partner at a known concentration. Meanwhile a reference cell containing only water or buffer is kept within the same adiabatic shield but not subjected to injections. The power used by the machine to keep the two cells at identical temperatures is proportional to the change in heat caused by binding of the two partners.
This power use is charted over time as more of the second binding partner is titrated in. Because this heat change is inherent, it is unnecessary to label, immobilize, or separate out any components of the binding interaction, making it capable of measuring binding in the most physiological environment possible.\textsuperscript{5,57} A simple model measurement by Duff et. al. of \textit{E. coli} chromosomal dihydrofolate reductase protein binding its NADPH cofactor provides a convenient visualized protocol for ITC.\textsuperscript{61} Speltz et. al. use ITC to measure affinity for each of three tricopeptide repeat interactions, two of which are designed to be orthogonal to the original pair; by comparing affinities, the researchers also demonstrated low cross-reactivity between the three pairs.\textsuperscript{62} For ligands with very high binding affinity, the necessarily low concentration of ligand results in only small heat changes, potentially below the limit of detection for the ITC apparatus.\textsuperscript{2}

A more sensitive option is differential scanning calorimetry (DSC), effectively a combination of ITC and a thermal shift assay.\textsuperscript{63-65} By measuring the difference in energy required to maintain equal temperature between a reference cell and the sample cell over a range of temperatures, the partial molar heat capacity ($C_p$) of the material in the sample cell can be determined. Analysis of the trend in $C_p$ across the temperature gradient reveals information about the stability of the sample material.\textsuperscript{5,64} By performing the same experiment at multiple ligand concentrations and a constant receptor concentration, the difference in thermostability between free receptor and bound receptor can be observed in the same fashion as the previously discussed thermal shift assay. This data can be mathematically deconvoluted to provide details of the binding interaction. Maric et. al. use DSC in combination with ITC and thermal shift assays, effectively using both the whole (DSC) as well as the sum of its parts (ITC and Thermofluor) to fully characterize the
modulatory effects of gephyrin-mediated multivalency on the affinity of a glycine receptor for its ligand.\textsuperscript{47}

\subsection*{2.8 Concentration-Sensitive Methods}

Kinetic exclusion assays, or KinExA, are particularly suited to measuring the binding affinity of antibodies for a target protein. KinExA is, in effect, a binding inhibition assay;\textsuperscript{6} the ligand and receptor are allowed to interact freely in solution before being pulled through a resin bed on which a separate population of receptor is immobilized.\textsuperscript{3,66} Because traversal of the resin is done very quickly, the sudden increase in effective concentration should not disrupt the prior equilibrium but will simply capture the free population of ligand as it binds to the receptor within the resin bed.\textsuperscript{3} Subsequent introduction of a fluorophore-conjugated antibody directed at the ligand allows fluorescence-based quantification of the amount of free ligand that existed in solution prior to resin capture. The ligand capture step also concentrates the free ligand. Combined with the post-equilibrium labeling step, this enhances signal strength, making the method more sensitive. On the other hand, fluorophore-conjugated antibodies will not be available for novel ligands. While, pre-labeling of the ligand with a fluorescent or radioisotope tag might solve this problem, it would also render this method no longer label-free. KinExA is most effective for assaying the binding of large antibodies rather than peptide or small molecule ligands.\textsuperscript{66} Drake et. al. directly compared KinExA with SPR for characterization of a model antigen/antibody system, noting that the more commonly used SPR technique gave variable results depending on the choice of chip for antibody immobilization.\textsuperscript{67} While the
model system provides no biologically relevant data, this work specifically demonstrates
the potential benefits of immobilization- and label-free methods such as KinExA.

The Hummel-Dreyer method is a classical technique that requires no dedicated
equipment. In brief, a small amount of the receptor is injected onto a column already pre-
equilibrated with a known concentration of ligand. Equilibrium is maintained because the
receptor is continually pushed into regions containing the known concentration of ligand,
leaving a reduced ligand concentration in its wake due to the amount of ligand it has taken
up and bound.\textsuperscript{68} The measured baseline absorbance represents the absorbance of the pre-
equilibrated concentration of ligand, with signal peak representing the absorbance of the
target receptor with ligand bound, in addition to the basal level of ligand concentration.
The subsequent signal trough represents the ligand concentration depletion caused by the
ligand being bound and swept away by the receptor as it travels through the column. If the
target receptor is spectroscopically silent at the chosen wavelength, the area under the
curve for both spike and dip will be equal. This method was used elegantly by Alzweiri and
Al-Hiari to obtain both the affinity and stoichiometry of interaction between carbonic
anhydrase III and the inhibitor vanillic acid upon discovering that the standard
colorimetric test for carbonic anhydrase inhibition gave flawed results due to pH
sensitivity; the Hummel-Dreyer method was not only more reliable but also provided more
quantitative data.\textsuperscript{69}

The Hummel-Dreyer method assumes that bound and unbound target will migrate
at identical rates; this assumption can generally be assumed valid unless the ligand is very
large or if the ligand has a disruptive effect on target self-interaction.\textsuperscript{70} Traditionally
performed using size-exclusion chromatography, ion exchange columns and electrophoretic methods have also been successfully adapted to the technique.\textsuperscript{71} This method is generally considered label-free, as many ligands will have characteristic absorption, which can be differentiated from target signal. In cases such as protein-peptide binding pairs, however, it may be beneficial for the ligand to bear a fluorescent or radiolabel which can be read using an inline detector. Especially when very low concentrations of ligand are used, a fluorescent or radiolabel will allow for much more sensitive detection.

Equilibrium dialysis (ED) is the least expensive and easiest of all methods described in this review, and consists of allowing target on one side of a dialysis membrane come to equilibrium with ligand that is freely diffusible across the membrane.\textsuperscript{72,73} After equilibrium is reached, ligand concentration is measured for aliquots from either side of the membrane. As with the Hummel-Dreyer method, ED is label-free but can nonetheless benefit from ligand labeling in cases where difficulty arises in differentiating ligand signal from target signal. Due to its simplicity and its long history of use, ED is often used as the standard reference method to which other methods are compared.\textsuperscript{4,30,74}

2.9 Intrinsic fluorescence

One final strategy for obtaining binding information does not fall squarely into any of the previous categories. Fluorescence intensity spectroscopy relies on the fact that the fluorescent output of many fluorophores is linked to the attributes of its microenvironment. Although not all interactions involving fluorophores demonstrate this useful property, ligand bearing such a fluorophore may exhibit enhanced or reduced signal
once bound to the target receptor.\textsuperscript{41,75} Because this change in signal strength is proportional to the amount of ligand bound, it is proportional to the fractional saturation. In some cases, quenching of intrinsic tryptophan fluorescence of the target receptor can even be used by the same method, obviating the need for labels.\textsuperscript{76} Dolgosheina et. al. made elegant use of this technique in order to determine the affinity of their novel fluorescence turn-on aptamer RNA Mango which was, of course, designed specifically to have increased fluorescence upon binding its target.\textsuperscript{77} The authors promote RNA Mango as an efficient new tool to track spatial and temporal expression of RNAs \textit{in vivo}, even for targets with limited copy number.\textsuperscript{77}

\textbf{2.10 Conclusions}

In conclusion, while high affinity binding interactions present a particular challenge to obtaining binding interaction data, there is a wealth of ways available to probe and quantify binding. By thinking critically and carefully about the attributes of the binding interaction in question, keeping in mind the degree of mass change, the pros and cons of labeling and immobilization, and equipment availability, a researcher should have little trouble drawing up a plan of action and choosing one or more techniques to pursue.
2.11 References


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Chapter 3: Affinity-Guided Design of Caveolin-1 Ligands for Deoligomerization

3.1 Introduction

As discussed in Chapter 1, caveolin research has been hampered by a lack of molecular tools available to selectively probe its function. Such a tool is now extant in the form of WL47, which may ultimately hold additional promise as a peptide drug for caveolin-associated diseases. This chapter describes the extensive iterative process of peptide library design, synthesis, screening, and data analysis, using a strategy that includes strategic deletions, substitutions, and sequence shuffling. By this method, we have produced a peptide ligand with 7.5-fold greater affinity than the previously published caveolin ligand while also decreasing sequence length by 80%. Beyond the concrete benefit of a novel tool for caveolin research, the development of WL47 provides a generalizable roadmap for producing small, high-affinity peptide ligands to any target.

Previous research in the Weiss laboratory used the known interaction between caveolin and gp41 as a starting point for generating a ligand for caveolin.\textsuperscript{1} The FDA-approved drug T20 (Fuzeon), a 36 amino acid peptide derived from gp41, blocks HIV viral fusion with CD4+ T-cells.\textsuperscript{1-3} The T20 sequence was mutated extensively in a phage-displayed library for screening and selections targeting caveolin residues (1-104). From this library, 36-mer sequences were isolated with dissociation constants ($K_D$) for caveolin of 155 nM or better. This phage-based, molecular evolution represented a 1000-fold improvement in $K_D$ relative to the original T20 ligand.\textsuperscript{1}
We have undertaken further design refinement of this T20-derived caveolin ligand, called “ligand 4” in a previous publication but here referred to as ligand 1. Iterative cycles of synthesis and assay guided the design to yield an 80% reduction in length with 7.5-fold higher affinity (Figure 3.1). By synthesizing and screening carefully designed peptide library arrays, we also identified key residues, minimized ligand size, and optimized the sequence (Figures 3.2-3.6). We demonstrate that this ligand, WL47, has selectivity and high affinity for its target (Figure 3.7-3.8), and can disrupt caveolin oligomers (Figure 3.9).

3.2 Results And Discussion

Development of ligand WL47 proceeded in three stages (Figure 3.1). First, regions of the starting ligand 1 contributing to the recognition of caveolin were identified. Second, this functional region was trimmed though mutagenesis and screening to eliminate non-essential residues. Third, the remaining key residues were shuffled to identify the most promising arrangement.

**Figure 3.1.** Evolution of caveolin ligands. Sequence alignment showing the progression of peptide ligands from the native gp41 segment sequence to T20, to ligands 1, 2, and 3, and ultimately to ligand WL47, which binds caveolin with 7500-fold higher affinity despite a 80% decrease in length compared to T20. Residues mutated from the original T20 sequence are in red. Numbers above the partial sequence of gp41 indicate the residue numbers from the HIV protein.
First, truncation libraries identified key regions of the starting T20-derived parent ligand, 1 (Figure 3.2). Such information could reduce the length of the ligand required for binding to caveolin. All 22 possible 15-mer sequences within the 36-mer peptide were synthesized and screened as C-terminal adducts to cellulose. This technique, termed SPOT synthesis, allows rapid synthesis and screening of peptides on a positionally addressable array.4 Caveolin (1-104) bearing a fluorescent rhodamine tag was incubated with the peptide array and the degree of fluorescence measured for each library member revealed its relative binding affinity. This experiment identified the C-terminal region of ligand 1 as contributing at least 98% of the observed binding to caveolin. Furthermore, eliminating the non-essential region at the ligand N-terminus reduced ligand size from a 36- to a 15-mer peptide.

**Figure 3.2.** Initial identification of caveolin interacting region. The sequence of ligand 1 was truncated to generate 22 unique peptide sequences of 15 amino acids each. Only two truncation sequences, consisting of residues 21-35 and residues 22-36, respectively, bound caveolin more than 2-fold above the level of the negative control. The negative control was a scrambled sequence of the randomly selected 11th truncation. An additional, blank negative control had a linker region but lacked a peptide. The sequence corresponding to residues 22-36 (indicated by asterisk) was chosen as the template for subsequent libraries and designated ligand 2.
Figure 3.3. Identification of key residues. \textbf{a}) In the library of similar and dissimilar substitutions of amino acids in ligand 2, ligand binding was retained for Lys and His substituents but was abolished by neutral Gln or Ala substituents. At Lys13, ligand binding was eliminated for all substituents except Arg. We conclude that both these residues contribute to binding primarily via positive charge. \textbf{b}) For Glu1, ligand binding was retained for negatively charged Asp and neutral Gln, and was slightly elevated for Ala. For Glu4 and Glu6, ligand binding increased moderately for neutral Gln and Ala. Ligand binding was reduced by substitution with negatively charged Asp, and all Lys substitutions at these three sites increased ligand binding. These trends suggest that these Glu are hindering ligand binding.

Additional trimming and mutagenesis next honed the caveolin ligand. With the 15-mer peptide ligand 2 as a template, a subsequent library featured similar and dissimilar substitutions at each position. This approach defines sidechain contributions, if any, to binding. Screening every member of a chemically synthesized library circumvents the problem of survivorship bias, which is inherent to molecular evolution approaches; only the most successful library members are analyzed following selections. By including every library member in the data set, the results from those members with poor binding affinity can still contribute to a deeper understanding of the functionalities that control caveolin ligand affinity.

This substitution library uncovered peptides with clear preferences for amino acid sidechains in specific positions. Library members with Lys or His substituted for Arg7
retained apparent binding affinity to caveolin (1-104), but substitution with the neutral sidechains of Gln or Ala at this site generated a peptide with reduced apparent affinity for caveolin. Substitution for Lys13 had similar but more drastic changes, with Arg substitution retaining complete binding activity and His, Gln, or Ala substitution abolishing binding. Taken together, this data demonstrates the importance of the two positively charged Lys and Arg sidechains (Figure 3.3a).

Furthermore, the three negatively charged Glu residues clustered at the N-terminus of ligand 2 adversely affected binding. Ala substitution at Glu1, Glu4, or Glu6 resulted in peptides with an unexpected increase in apparent binding affinity (Figure 3.3b). Removing six residues at the N-terminus, including these three negatively charged Glu, produced a 9-mer peptide with an approximately 3-fold increase in apparent binding affinity. Other

![Fluorescence Intensity Relative to Positive Control](image)

**Figure 3.4.** Removal of detrimental amino acids. Truncation of the six N-terminal residues, which included all three Glu, without removing any of the positively charged residues, yielded a peptide (indicated by asterisk) designated ligand 3 that became the template for subsequent library design. All libraries include a scrambled ligand 2 sequence as a negative control.
truncations were less successful. For example, further truncation of the N-terminus eliminated the positively charged Arg7, and produced a sharp reduction in binding (Figure 3.4). This result reemphasizes the importance of the positively charged sidechains. In summary, this SPOT-synthesized library truncated six residues to yield a 9-mer peptide, ligand 3, with increased apparent affinity for caveolin.

Reduced ligand length made it feasible to synthesize a library with more substitutions. Each position of ligand 3 was systematically varied to further examine tolerance to substitution and sidechain requirements for binding. A library was synthesized by SPOT synthesis to include 19 amino acid substitutions at each of the nine individual sites remaining of ligand 3, yielding 171 unique sequences in addition to unsubstituted ligand 3 as a positive control. The most striking data from this library was the dramatic

![Graph A](image1.png)

**Figure 3.5.** Disulfide dimerization explored. **a)** In the library of all amino acid substitutions for every site in ligand 3, all substituents except Leu and Tyr reduce ligand binding when replacing Cys14. This suggests that ligand binding is dependent on dimerization. **b)** The necessity of ligand dimerization by disulfide bond was confirmed by screening a SPOT array containing WL47 under reducing and non-reducing conditions. The green section represents the amount of binding observed under reducing conditions as a fraction of the total binding observed under standard non-reducing conditions. The teal section is the calculated difference in ligand binding between reducing and non-reducing conditions and represents the amount of overall ligand binding that is disulfide dependent. Approximately one third of overall WL47 ligand binding to caveolin is dependent on ligand dimerization.
drop in apparent binding affinity for nearly all substitutions at the C-terminal Cys residue (Figure 3.5a). This observation strongly suggested that ligand binding to caveolin requires dimerization by a disulfide bond mediated by the Cys sidechain. SPOT arrays with the final peptide sequence under reducing conditions later confirmed this observation, as total binding was diminished by 63% compared to non-reducing conditions (Figure 3.5b).

The initial library data also identified residues not contributing significantly to ligand function. Such information guided trimming the ligand to its minimum length. For example, substitution of Phe15 with Ala failed to alter ligand binding ability, making Phe15 a candidate for elimination (Figure 3.6a). The removal of Phe was especially desirable for this small peptide in which every side chain is likely surface-exposed; aromatic sidechains can contribute to nonspecific binding to other targets.\(^5\) Similarly and unexpectedly, mutation of Gly 9 to Ala or Phe results in no apparent reduction in binding affinity (Figure 3.6a). Gly cannot contribute to ligand binding through sidechain contacts, but often allows greater flexibility for a ligand. Here, replacement of Gly 9 with the slightly less flexible Ala showed no reduction in ligand binding. Replacement of Gly 9 with the bulky and

![Figure 3.6](image_url). Unnecessary residues identified for removal. In the library of similar and dissimilar substitutions of amino acids in ligand 2, Ala substitution at either the Gly9 and Phe15 residues was shown to have no significant effect on ligand binding, which suggested that neither residue was essential for ligand binding. Replacement of Gly9 with Phe also did not reduce ligand binding and suggested flexibility is unnecessary at that site. The increase in binding observed when Phe replaces Gly9 is likely due to nonspecific hydrophobic interactions. Same negative control as Figure 3.3.
significantly less flexible Phe also showed no reduction, only a minor, perhaps nonspecific, increase in apparent binding affinity. This data confirms that any flexibility contributed by Gly at this site is superfluous, and the residue can be removed.

Until this point, unnecessary amino acids were easily removed by simple truncation. For example, Phe 15 could be clipped from the N-terminus and leave essential residues untouched. However, direct removal of an internal residue such as Gly 9 could disrupt the spacing of amino acids in the ligand. Furthermore, the evolution and library screening to this point identified key sidechain functionalities. With this in mind, a library was synthesized wherein Gly and Phe were removed and the remaining residues shuffled using the GenScript Scrambled Library Peptide Library Design Tool. A total of 17 shuffled sequences were randomly chosen for synthesis and screening. As expected, simple removal of Gly and Phe without otherwise altering the sequence resulted in a 26% reduction in

![Graph A](image-url)

**Figure 3.7.** Sequences shuffled. **a)** Relative to ligand 3 (blue bar), direct removal of Gly9 and Phe15 from the sequence decreased binding (teal bar). When the sequence lacking Gly and Phe is shuffled, however, many library members retain or increase binding ability. The 7-mer shuffled sequence (indicated by asterisk) with the highest binding was designated **WL47** and used for subsequent studies. **b)** All shuffled sequences for this library were generated using the GenScript Scrambled Library Peptide Library Design Tool.
apparent binding affinity relative to ligand 3. However, a sequence seven residues in length and with 2.6-fold increased binding relative to ligand 3 was isolated from this library (Figure 3.6b). This sequence, referred to as WL47, was chosen as the lead compound for further analysis.

A parallel SPOT screening assay next examined the specificity of WL47 for caveolin and other potential binding partners. In this assay, rhodamine labeled caveolin (1-104) and three other rhodamine labeled proteins chosen for their solubility, ubiquity, and known nonspecific binding properties were each incubated with identical SPOT arrays on which ligand 2, ligand 3, and WL47 had been synthesized along with blank SPOTs bearing only the dual β-Ala linker. These sheets were washed, blocked, and their fluorescence quantified as described above. WL47 bound caveolin (1-104) with significantly stronger binding than the other proteins (Figure 3.8). Furthermore, ligand 3 shows an increase in caveolin (1-104) binding relative to ligand 2, and WL47 has increased binding relative to ligand 3,

Figure 3.8. Demonstration of selectivity. Caveolin (1-104) and several control proteins were labeled with rhodamine and screened against duplicate SPOT sheets containing ligand 2, ligand 3, and WL47 to demonstrate the selectivity of the ligand for the caveolin target. Bovine serum albumin, casein, and hen egg white lysozyme all bond WL47 with reduced affinity relative to caveolin (1-104) as measured by fluorescence. Blank SPOTs containing only the double β-Ala linker were included as negative controls, and signal from these SPOTS for each replicate sheet were subtracted from the corresponding signal for 2, 3 and WL47 SPOTs on each sheet.
confirming that the iterative process used to develop **WL47** is effective at creating ligands with enhanced target binding. Conversely, little change in binding to control proteins is seen across the three ligand generations, affirming that specific rather than nonspecific binding has been enhanced during ligand evolution.

The $K_D$ of the binding interaction between caveolin (1-104) and **WL47** was determined using the measured increase in fluorescence anisotropy of Mantyl-**WL47** upon binding to its target. Slightly smaller than Phe, the N-Methylanthranyl “Mantyl” fluorophore offers minimal bulk to reduce potential binding disruption. This fluorophore was installed to create Mantyl-**WL47**. Varying concentrations of caveolin (1-104) ranging from 0 to 586 nM were incubated with 12 nM Mantyl-**WL47** dimers. Data were fit to the following equation:

$$
\frac{[LR]}{[L]} = \frac{([R]+[L]+K_0)-([R]+[L]+K_0)^2+4*[R]*[L])^{0.5}}{2*[L]} 
$$

(1)

Where $[L]$ is the total concentration of Mantyl-**WL47**, $[R]$ is the total concentration of caveolin (1-104), and $[LR]$ is the concentration of Mantyl-**WL47** bound to caveolin (1-104). While considerably more complex than other common binding equations, equation 1 does not ignore binding site depletion, which is crucial for obtaining an accurate model when the ligand concentration is within an order of magnitude of the final $K_D$ value. By this method, a $K_D$ of roughly 23 nM was determined; binding affinity range from 44 to 3 nM with 95% confidence (Figure 3.9a). A Hill plot of the same data set yields a Hill coefficient ($n_H$) of 2. Based on the two site binding model, this coefficient indicates complete positive cooperativity, which is to say that upon binding one molecule of **WL47**, a second molecule
Figure 3.9. Determination of binding affinity. a) Fluorescence anisotropy was measured for Mantyl-WL47 dimer incubated with the indicated concentrations of caveolin (1-104). Using equation 1, a best fit binding curve was fit to the raw experimental data using a weighted method of best fit squares, and assuming each WL47 dimer functions as a single ligand. The $K_D$ for this binding interaction was calculated to 23 nM. b) A Hill plot of the same data yields a Hill coefficient ($n_H$) of 2. The coefficient is equal to the number of WL47 binding sites on caveolin (1-104), which indicates complete cooperativity such that the binding of one WL47 unit triggers the instantaneous binding of a second unit. This supports our decision to treat a WL47 dimer as a single ligand in $K_D$ calculations.

binds the second site virtually instantaneously. This phenomenon supports our decision to perform $K_D$ calculations with dimerized WL47 considered a single ligand (Figure 3.9b).

Demonstrating WL47 activity requires in vitro caveolin oligomers and a method for measuring the degree of oligomerization. A full-length, soluble variant of caveolin that spontaneously oligomerizes to form caveolin nanoparticles was used to examine deoligomerization by WL47. WL47 effectively disrupts these nanoparticles in a dose-dependent fashion (Figure 3.10). Oligomerization and deoligomerization are a well-known aspect of caveolin activity and caveolae formation. The overlapping nature of the
oligomerization and scaffolding domains suggests the regulatory function of caveolin relies upon its oligomeric state, as shown previously through oligomer complementation.12

As the first validated synthetic ligand for caveolin, WL47 contributes to the ongoing debate over the existence and identity of a specific caveolin binding motif that mediates the interaction between caveolin and its many binding partners. Research in 1997 by Couet et. al. proposed that this binding motif consisted of a characteristic spacing of multiple aromatic amino acids as determined by examining the sequencing results of multiple phage display libraries that had been subject to selections against caveolin (1-101).13 In subsequent research, many (but not all) caveolin binding proteins were found to contain similar sequences. However, a 2012 paper by Byrne et. al. used statistical methods to investigate the incidence of such characteristic aromatic spacing in caveolin binding proteins and in the proteome at large and found no statistical enrichment of these aromatic sequences in caveolin binding proteins.14 The WL47 sequence does not have multiple aromatic residues and thus does not conform to the aromatic richness and spacing of the

Figure 3.10. Demonstration of ability to deoligomerize caveolin. A full length, soluble variant of caveolin forms nanoparticles in solution. Incubation of these nanoparticles with WL47 at varying concentrations followed by DLS measurements shows that WL47 can induce deoligomerization in a dose-dependent manner.
CBM motif, suggesting that whether this motif mediates caveolin binding for some proteins or not, it is clearly not the only way in which caveolin may be targeted. Since caveolin has many binding partners, the interaction between CBMs and the caveolin scaffolding domain likely evolved as a panoply of low affinity interactions such that caveolin could maintain its multiple partners. As a high affinity ligand, WL47 would not be expected to conform to this pre-existing pattern. Notably, the phage display process produced a higher affinity binder with fewer aromatic residues, and subsequent optimization resulted in the removal of an additional aromatic residue.

3.3 Conclusion

The caveolin ligand WL47 is 80% smaller in length and boasts 7500-fold greater affinity than the original T20 parent sequence, while also demonstrating selectivity and deoligomerization activity. The ligand was developed through a generalizable peptide ligand optimization process involving iterative library design, synthesis, and screening. WL47 provides a new tool for research that will allow investigators to probe the functions of caveolin with unprecedented selectivity.

Beyond providing a new tool, the development of WL47 demonstrates the ways in which small molecule medicinal chemistry principles can be successfully applied to the creation of peptide ligands. Recent novel peptide ligands have been derived exclusively from direct mimicry of existing sequences from known proteins. Conversely, the creation of novel small molecule drugs has involved greater experimentation and rational design. The application of these varied strategies ultimately allowed us to progress from our lead compound T20, which had an interaction with caveolin too weak to be successfully
measured by ITC, to a ligand sequence that binds caveolin with a $K_0$ of 23 nM as measured by fluorescence anisotropy experiments.
3.4 Experimental Section

Materials. Fmoc-protected amino acids for peptide synthesis were obtained from multiple suppliers, including NovaBiochem, ChemImpex, Anaspec, and Aroz Tech. All other reagents and materials were obtained through Sigma Aldrich or Fisher Scientific unless otherwise noted.

Protein expression and purification. Caveolin (1-104) and full-length, soluble caveolin variant proteins were expressed and purified as described previously.¹

Synthesis and screening of SPOT arrays. Peptides were synthesized as C-terminal adducts to cellulose as described in Hilpert et. al.¹⁸ All sequences were synthesized and screened in duplicate. Purified caveolin (1-104) was fluorescently labeled by adding a 15-molar excess of NHS-Rhodamine to protein in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4). The mixture was allowed to react 2 h, protected from light. Excess unreacted NHS-Rhodamine was either removed by overnight dialysis using 3.5 kDa molecular weight cut-off Slide-a-Lyzer mini dialysis cups in PBS at 4 °C, or was removed using desalting column according to standard procedures. The completed peptide array was blocked with 0.2% bovine serum albumin fraction V (BSA) in PBS. The sheet was then washed five times with PBS containing 0.05% Tween20 detergent (PBST), then incubated with 0.2% BSA in PBS containing the dialyzed fluorescently-labeled
caveolin (1-104) for at least 2 h. After incubation, the sheet was washed four times with PBST and once with PBS containing no detergent. Sheets were dried flat overnight prior to fluorescence imaging. Fluorescent imaging was performed using a GE Typhoon imager with the following fluorescence settings: Green (533 nm) excitation frequency, 580 nm BP 30 emission frequency window, normal sensitivity, 200 microns/px at 300 V laser strength. The resulting image files were analyzed by ImageQuantTL (.gel format) or ImageJ (.tiff format) to obtain quantitative data. The methodology for selectivity screening was identical except rhodamine tagging was performed separately for caveolin (1-104), bovine serum albumin (BSA), casein, and hen egg white lysozyme (HEWL). These reactions were performed using the same NHS rhodamine stock solution and treated identically. Replicate SPOT sheets carrying synthesized ligand 2, ligand 3, and WL47 and blanks containing only the dual β-Ala linker were each treated with one labeled protein under the above conditions and quantified as described previously. The fluorescence signal for the linker-only blanks for each protein screen were subtracted from the corresponding ligand 2, ligand 3, and WL47 signals before plotting and analysis.

**Synthesis and purification of WL47.** The WL47 peptide was synthesized by SPPS on a 0.1 mmol scale in 5-mL disposable syringe-type polypropylene reaction vessels (obtained from Torviq) with 70 micron polypropylene frit and Luer lock tip, using procedures adapted from Kirin et. al.19 These vessels were outfitted with BD 16 gauge 1-inch needles for drawing up and expelling reagents. The following significant modifications were used: Swelling of resin and all coupling steps were performed in N-Methyl-2-pyrrolidone (NMP).
The deprotection step was performed twice per cycle. Coupling steps were performed using HBTU and without DIPEA. Reaction progress was monitored continuously using bromophenol blue (0.01% final concentration) as described in Krchnak et al., with transition of reaction mixture color from deep blue to yellow indicating completion of coupling. Coupling reactions were allowed to continue 10 min additional after visual monitoring of color change indicated reaction completion. After lyophilization, crude peptide product was purified by preparative HPLC using standard methods. Product presence was confirmed by MALDI-TOF and product purity confirmed by analytical HPLC.

**Fluorescence anisotropy binding affinity assay.** A 15 µl volume containing a range of caveolin (1-104) concentrations was combined with a stock solution of Mantyl-WL47 in PBS with 0.25% sarkosyl detergent (w/v) to yield 11 samples containing 24 nM Mantyl-WL47 (12 nM dimerized Mantyl-WL47) and caveolin (1-104) in a range of 0 to 585.98 nM. Fluorescence data was collected for each of these samples on a Cary Eclipse Spectrofluorometer at an excitation wavelength of 340 nm and emission range of 400 to 500 nm with Savitzky-Golay smoothing at filter size 15. Intensity (I) measurements were taken with four polarization filter conditions: vertical excitation with vertical emission (I_{VV}), vertical excitation with horizontal emission (I_{VH}), horizontal excitation with vertical emission (I_{HV}), and horizontal excitation with horizontal emission (I_{HH}). Anisotropy (r) was calculated by the following standard anisotropy equations:

$$ r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} $$

(2)
and

\[ G = \frac{I_{HV}}{I_{HH}} \]  

(3)

The emission range in conjunction with the indicated smoothing yielded 87 distinct subsets of anisotropy data from 407-493 nm. The greatest magnitude of anisotropy was observed at wavelength 417. This data set was converted to fractional ligand saturation by first calculating the percent change in binding by dividing each value in the subset by the minimum value for the same subset and subtracting 1, then converting these values to fractional saturation by dividing each percent change value by the maximum percent change value for each subset. This data set was fit to equation 1 using a weighted method of least squares that more heavily weights those data points with smaller variance. \( R^2 \) for the fit equation was 0.94. The \( K_0 \) was calculated to be 23 nM, within a potential range of 44 to 3 nM with 95% confidence. A Hill plot was constructed using the same data set, with \( \log(Y/(1-Y)) \) on the vertical axis and \( \log(X) \) on the horizontal axis, where \( Y \) is the fractional saturation and \( X \) is the concentration in nM of caveolin (1-104). The slope of the curve at \( \log(Y/(1-Y)) = 0 \) provides the Hill coefficient, \( n_H \). This coefficient is an indicator of binding cooperativity for binding at multiple sites, with values < 1 indicating negative cooperativity and values > 1 indicating positive coopertivity up to a limit of \( n_H = z \) where \( z \) is the number of binding sites. The \( n_H \) for this binding interaction is 2 with 2 binding sites, indicating complete cooperativity. In this case, binding of two molecules of \textbf{WL47} happens simultaneously as a single binding event, and so we can assume that dimerized \textbf{WL47} functions as a single ligand.
**Deoligomerization shown by Dynamic Light Scattering.** A Malvern Zetasizer ZS Nano DLS instrument was used in backscatter mode to determine particle size of oligomers composed of full-length, soluble caveolin variant. Readings were obtained for oligomers in PBS with or without **WL47** under both reducing (5 mM TCEP) and non-reducing conditions. The General Purpose (non-negative least squares analysis) distribution analysis algorithm provided with the Zetasizer software was used to automatically calculate particle sizes and distribution. Average diameters for each condition were plotted along with the average plus or minus half the calculated polydispersity width in order to represent the distribution of observed diameters.
3.5 Analytical HPLC traces for SPPS synthesized peptides

Purity of **WL47** demonstrated by analytical reversed-phase HPLC. Sample was injected onto a C18 column and eluted via water-methanol gradient. Major peak at 15.432 corresponds to Mantyl-**WL47**. Calcd \( m/z \) 922.17, found \( m/z \) 922.44 by MALDI-TOF.

Purity of Mantyl-**WL47** demonstrated by analytical reversed-phase HPLC. Sample was injected onto a C18 column and eluted via water-methanol gradient. Major peak at 16.264 corresponds to Mantyl-**WL47**. Minor peak at 15.143 represents minor quantities of **WL47** that did not undergo mantylation. Calcd \( m/z \) 1055.32, found \( m/z \) 1055.47 by MALDI-TOF.
3.6 References


(14) Byrne, D. P.; Dart, C.; Rigden, D. J. PLOS ONE 2012, 7.


Chapter 4: Towards Understanding and Replacing the Disulfide Bridge in WL47

4.1 The Disulfide Liability

The importance of disulfide-mediated dimerization for WL47’s ability to bind caveolin is presented in Chapter 3. In this chapter, this importance is further exemplified by demonstrating that activity as well as binding is dependent on WL47 dimerization. WL47 has clearly already demonstrated its utility for in vitro experiments; however if WL47 is to become a truly viable tool for disrupting caveolin oligomers inside live cells, this disulfide bridge becomes a liability. The reducing environment of the cell will reduce the disulfide, monomerizing the WL47 dimer and thus disturbing its binding and deoligomerization activity. In order to solve this problem, alternative methods of dimerization have been explored.

4.2 Dimerization is Vital to WL47 Function

As shown by DLS, in the presence of a reducing agent, incubation of the caveolin variant with WL47 does not disrupt oligomerization (Figure 4.1). This is consistent with our original data showing that optimal WL47 binding depends on disulfide-mediated dimerization. Analysis of WL47 as a dimerized ligand has allowed for more critical analysis of previous work, and suggests that both sites of a two-site system must be filled in order for deoligomerization of caveolin to occur. For example, ITC data from Majumdar et. al. suggested a two-site binding model through negative cooperativity between the ligand and caveolin.¹ For dimerized WL47, one half of the dimer interacting with the first binding site
could bring the second half of the ligand close enough to the second binding site to force binding via increased effective concentration, overcoming the previously observed negative cooperativity. Based on the large positive change in entropy upon binding of the second ligand (ΔS2), Majumdar et al. also observed that binding to the second site must be entropically driven, likely by the “disruption of caveolin oligomers upon binding.”¹ This matches our observation that deoligomerization only occurs when **WL47** is dimerized such that the second binding site is filled. Thus, data from the dimerized **WL47** ligand reinforces and clarifies the conclusions drawn from previous research on ligand 1.

### 4.3 Initial Attempts to Replace Disulfide

Preliminary decisions for disulfide alternative strategies were dominated by ease of installation. In short, only those modifications that were readily adaptable to SPOT synthesis were screened. Two architectures containing a Gly₃ linker were designed in the hope that the original dimerization geometry could be mimicked using a single peptide chain. The terms “retro inverso” and “retro” indicate sequences that have been synthesized in reverse using D amino acids or synthesized in reversed with naturally occurring L amino acids.
acids, respectively.\textsuperscript{2,3} Synthesizing the reversed second half with D amino acids in true retro inverso fashion is canonically more likely to successfully mimic the original sequence. We also decided to synthesize the same overall design as retro but not inverso, using all L amino acids in the hope that we could get a similarly effective structure without resorting to the more expensive D monomers. With an available supply of BM(PEG)\textsubscript{2}, a crosslinker with a 14.7 Å spacer arm, it also seemed prudent to attempt a direct crosslinking reaction to mimic the direct link created by the native disulfide bond. For a negative control, Cys were alkylated to prevent any disulfide bond formation.

In addition to unaltered \textbf{WL47} as a positive control (Figure 4.2a, Structure “A’’), architectures chosen for testing included a sequence containing \textbf{WL47} without Cys (\textbf{WL47ΔC}) followed by Gly\textsubscript{3} and a retro inverso sequence of \textbf{WL47ΔC} (Figure 4.2b, Structure “B’’), a sequence containing \textbf{WL47ΔC} followed by Gly\textsubscript{3} and a retro sequence of \textbf{WL47ΔC} (Figure 4.2c, Structure “C’’), \textbf{WL47} treated with BM(PEG)\textsubscript{2} (1,8-bismaleimido-diethylene glycol) to crosslink Cys to Cys (Figure 4.2d, Structure “D’’), and a control compound of \textbf{WL47} with Cys alkylated by iodoacetamide in order to block the formation of disulfide bonds entirely (Figure 4.2e, Structure “E’’).

The SPOT array is convenient for this screening because it allows concurrent synthesis and then direct comparison of all architectures on a single positionally addressable array.\textsuperscript{4} On the other hand, the fact that all sequences are anchored to the cellulose support at the C-terminus also prevents the peptides from easily moving in relation to one another, effectively creating partial pseudo-dimerization through a scaffolding effect. The outcome is similar to having tables nailed to the floor so that you are
Figure 4.2. Structures of architectures from initial disulfide replacement study. a) Structure “A”, positive control, two WL47 chains dimerized by disulfide. b) Structure “B”, WL47ΔC and retro inverso WL47ΔC with Gly3 linker, synthesized as a single chain. c) Structure “C”, WL47ΔC and retro WL47ΔC with Gly3 linker, synthesized as a single chain. d) Structure “D”, two WL47 chains dimerized by BM(PEG)2 crosslinker. e) Structure “E”, negative control, WL47 with Cys blocked by alkylation. f) None of these preliminary attempts to replace the disulfide dimerization of WL47 showed binding close to that of the positive control (A). If successful, the two single chain architectures (B,C) should have demonstrated double the signal of the positive control, and the BM(PEG)2 architecture (D) should have shown signal equal to the positive control, but none of these was successful. The signal for the negative control (E) is overlaid onto the signal for the positive control (A) to demonstrate the degree of binding that is independent of the disulfide
unable to move individual pieces closer to or further from other tables. This effect must be carefully considered when conducting analysis of the resulting data, as it may lead scaffold-bound monomeric peptides to bind the target better than monomeric peptides in solution. Additionally, we assume that each spot contains approximately the same number of synthesized peptide chains as the spot next to it.\textsuperscript{5} For the single chain architectures B and C, if we assume that the C-terminal \textbf{WL47ΔC} sequence and the N-terminal reverso or retro inverso \textbf{WL47ΔC} sequences allow each single chain to count as one dimer, but for A it takes two peptide chains to create a dimer. Because of this we should theoretically see double the amount of signal if B and C are successful. Data from this binding assay suggests that this is not the case, as the signal for A far outstrips the signal for all other attempted architectures. Far from producing double the signal A, B and C failed to even match the positive control, suggesting that this linker system is not an effective disulfide mimic or that the geometry of the sidechains and/or backbone is incorrect (Figure 4.2f).

The signal for negative control E represents the degree of binding that can be achieved without dimerization. With the signal strength for E overlaid on A, we can see that approximately 2/3 of the binding activity for A can be attributed directly to its disulfide dimerization. The idea that both the \textbf{WL47ΔC} and the retro inverso \textbf{WL47ΔC} sequences within B have some level of binding activity is supported by the fact that B had approximately double the signal of E. Structure C also has higher signal than that of E but less than that of B. This supports the idea that A is more successfully mimicked by retro inverso architecture than it is by retro architecture. While B is overall clearly not a promising replacement for A, the effectiveness of retro inverso structure may become
useful and relevant at a later stage in development and must be kept in mind for future work.

The BM(PEG)\textsubscript{2} crosslinked architecture C did not demonstrate any significant binding increase over that of negative control E (Figure 4.2f). With a homo bifunctional crosslinker such as BM(PEG)\textsubscript{2}, there is always a risk that the linker will react with a single peptide chain without then subsequently linking to a second chain. Such a situation would easily explain why the signal for C does not significantly surpass that of E; rather than crosslinking Cys from one chain to another, each Cys would instead be effectively blocked in much the same manner as the alkylation of E. This situation is especially likely if the crosslinking reagent is used in excess during the reaction. In anticipation of such a potential issue, the reaction was carried out with stoichiometric amount of crosslinker relative to the potential dimers to be formed. Although this does not guarantee that all possible dimers are formed, it does statistically minimize the likelihood that all peptides will be capped rather than crosslinked. Assuming that at least some crosslinking was successful and C was formed, we would expect to see an increase in signal over E if this crosslinking effectively emulated the disulfide linkage. We see in this case that the signal is not significantly different from that of the negative control, and so we must conclude that the geometry of the dimers formed by this crosslinking method does not generate a suitable replacement for disulfide dimerization.

4.4 Conclusion of Initial Attempts to Replace Disulfide

This initial screening, while not revealing an easy, effective disulfide replacement, has provided significant information with which to design subsequent architectures. First
and foremost, it definitively demonstrates what a significant impact the disulfide dimerization has on target binding ability. Secondly, it suggests that crosslinking using BM(PEG)$_2$ is a wholly ineffective method of disulfide replacement. Finally, while neither B nor C is a suitable substitute for A, the fact that the addition of a second WL47-mimicking sequence to the chain generates some increase in binding indicates that both retro inverso WL47 and simply retro WL47 sequence produce some WL47-like binding activity resembling the monomeric peptide. The greater signal shown by B as opposed to C, and observation that the retro-inverso-containing chain B has approximately twice the observed binding of the monomeric negative control E, indicates not only that retro inverso WL47 is a better WL47 mimic than is retro WL47, but also that monomeric retro-inverso WL47 would be as effective a binder as monomeric WL47. This revelation about retro inverso architecture is not unexpected, as conversion of peptides to retro inverso peptides for the purposes of creating more proteolytically stable peptide mimics is a well known phenomenon.$^{2,6}$ Confirming its effectiveness for this particular sequence may prove useful in future architecture development.$^7$

4.5 Attempts to Convert Rather than Replace Disulfide

We next explored the idea of converting existing disulfide bonds to a more stable analogue rather than attempting to replace it outright. Using a thioether to mimic disulfides in peptides has been used since at least 1997, with mixed levels of success.$^8$ Rather than attempt to install a thioether-containing monomer during the course of peptide synthesis, a strategy that would require that we obtain multiple additional building blocks with
**Figure 4.3.** Screening of branched and thioether-converted architectures. a) Structure “A”, *WL47* with intact disulfide bridge as positive control. b) Structure “X”, *WL47* after conversion of disulfide to thioether. c) Structure “Y”, dual *WL47* sequences constructed off a Lys branch point, with intact disulfide bridge. d) Structure “Z”, dual *WL47* sequences constructed off a Lys branch point, after conversion of disulfide bridge to thioether. e) Screening data for these structures is enigmatic; while unbranched (A) and branched (C) disulfide-containing structures show comparable levels of binding, the same constructs subjected to the thioether conversion process show radically different levels of binding. While thioether conversion causes a significant drop in binding for unbranched *WL47* (B), the level of binding for the branched peptide after thioether conversion (D) shows approximately double the amount of binding compared to either disulfide containing peptide (A,C).
expensive and carefully selected orthogonal protecting groups, we opted to follow the simple post-synthesis base-assisted conversion strategy exemplified by Pulka-Ziach et al. After synthesizing desired peptides by SPOT synthesis, the cellulose-bound peptide array was oxidized overnight with aqueous DMSO to ensure all possible disulfides were in place. Subsequently, one half of the array was treated with aqueous 0.5 M NaOH at 40 °C for at least 6.5 hours to generate thioethers from disulfides. After thoroughly rinsing and drying this treated half of the array, the two halves, treated and untreated, were screened with fluorescently tagged caveolin (1-104) as described in the previous chapter.

In addition to testing thioethers, we also designed, synthesized, and tested an architecture consisting of two WL47 sequences branching off of a shared chain at the cellulose-attached C-terminus. To accomplish this, we used Fmoc-Lys(Fmoc), a commercially available Lys building block that, upon conventional Fmoc deprotection, provides two reactive amines, the N-terminus as well as the R-group, upon which further amino acids can be coupled. We hoped that either dimerization at the C-terminus would be sufficient to mimic disulfide dimerization at the N-terminus, or that the combination of connectivity at the C-terminus in concert with a thioether connection at the N-terminus would provide a robust cyclic peptide structure. Bearing in mind the doubling effect that had to be accounted for in the data analysis of our previous screening, we decided to partially cap any spots where we intended to synthesize branched peptides by using a mixture of β-Ala linker and N-acetyl-β-Ala during formation of the linker region between the cellulose and the peptide sequence. This effectively allows for a controlled, partial capping step that ensures that only approximately half of the potential sites for chain growth were available for further coupling.
Screening of positive control **WL47** (Figure 4.3a, Structure “A”), **WL47** after thioether conversion (Figure 4.3b, Structure “X”), branched dual **WL47** (Figure 4.3c, Structure “Y”), and branched dual **WL47** after thioether conversion (Figure 4.3d, Structure “Z”) provided intriguing results (Figure 4.3e). While there was no significant difference in binding between A and Y, the same structures after being treated to the thioether conversion protocol (X and Z) showed strikingly different levels of binding. On its own, the decrease in binding for X might suggest that the prolonged treatment with base could have generated epimerization that could reduce the ability of the peptide sequences to bind regardless of dimerization. However, the near doubling of binding for Z refutes this theory, as epimerization related binding reduction should have affected both architectures. One possible explanation for this result is that the removal of one sulfur from the bridge causes a contraction that brings the two halves of the dimer together more closely in a way that is geometrically unfavorable to binding. This same effect might be mitigated in the branched architecture due to the increased conformational constraints imposed by the cyclic shape.

**4.6 Conclusion of Attempts to Convert Disulfide**

Analysis would be improved with knowledge of the degree of binding seen for structures **A, X, Y, and Z** under reducing conditions, and the experiment was originally designed to include the screening of a matched set of cellulose arrays under such conditions. It was is clear from a visual inspection that the arrays screened in the normal way resembled previously screened arrays, but that arrays screened under reducing conditions did not appear similar to previously screened arrays, even previous arrays screened under reducing conditions. Unfortunately this unforeseen and as-of-yet unsolved
problem with the screening for the reducing conditions prevented the acquisition of meaningful data from those arrays. We cannot conclusively rule out the possibility that whatever defect occurred during this experiment did not also somehow affect the data shown in Figure 4.3. In light of this, all conclusions drawn from this data are considered tentative until this experiment is successfully repeated and further work, described in Chapter 5, is completed.
4.7 Experimental Section

**Materials.** Fmoc-protected amino acids for peptide synthesis were obtained from multiple suppliers, including NovaBiochem, ChemImpex, Anaspec, and Aroz Tech. All other reagents and materials obtained through Sigma Aldrich or Fisher Scientific unless otherwise noted.

**Protein expression and purification.** Caveolin (1-104) and full-length, soluble caveolin variant proteins were expressed and purified as described previously.¹

**Synthesis and screening of SPOT arrays.** Peptides were synthesized as C-terminal adducts to cellulose as described in Hilpert et. al.¹¹ All sequences were synthesized and screened in duplicate. Purified caveolin (1-104) was fluorescently labeled by adding a 15-molar excess of NHS-Rhodamine to protein in phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4). The mixture was allowed to react 2 h, protected from light. Excess unreacted NHS-Rhodamine was either removed by overnight dialysis using 3.5 kDa molecular weight cut-off Slide-a-Lyzer mini dialysis cups in PBS at 4 °C, or was removed using desalting column according to standard procedures. The completed peptide array was blocked with 0.2% bovine serum albumin fraction V (BSA) in PBS. The sheet was then washed five times with PBS containing 0.05% Tween20 detergent (PBST), then incubated with 0.2% BSA in PBS containing the dialyzed fluorescently-labeled caveolin (1-104) for at least 2 h. After incubation, the sheet was washed four times with
PBST and once with PBS containing no detergent. Sheets were dried flat overnight prior to fluorescence imaging. Fluorescent imaging was performed using a GE Typhoon imager with the following fluorescence settings: Green (533 nm) excitation frequency, 580 nm BP 30 emission frequency window, normal sensitivity, 200 microns/px at 300 V laser strength. The resulting image files were analyzed by ImageQuantTL (.gel format) or ImageJ (.tiff format) to obtain quantitative data.

**Synthesis and purification of WL47.** The WL47 peptide was synthesized by SPPS on a 0.1 mmol scale in 5-mL disposable syringe-type polypropylene reaction vessels (obtained from Torviq) with 70 micron polypropylene frit and Luer lock tip, using procedures adapted from Kirin et. al. These vessels were outfitted with BD 16 gauge 1-inch needles for drawing up and expelling reagents. The following significant modifications were used: Swelling of resin and all coupling steps were performed in N-Methyl-2-pyrrolidone (NMP). The deprotection step was performed twice per cycle. Coupling steps were performed using HBTU and without DIPEA. Reaction progress was monitored continuously using bromophenol blue (0.01% final concentration) as described in Krchnak et al., with transition of reaction mixture color from deep blue to yellow indicating completion of coupling. Coupling reactions were allowed to continue 10 min additional after visual monitoring of color change indicated reaction completion. After lyophilization, crude peptide product was purified by preparative HPLC using standard methods. Product presence was confirmed by MALDI-TOF and product purity confirmed by analytical HPLC (shown in Chapter 3 section 3.4).
**Deoligomerization shown by Dynamic Light Scattering.** A Malvern Zetasizer ZS Nano DLS instrument was used in backscatter mode to determine particle size of oligomers composed of full-length, soluble caveolin variant. Readings were obtained for oligomers in PBS with or without **WL47** under both reducing (5 mM TCEP) and non-reducing conditions. The General Purpose (non-negative least squares analysis) distribution analysis algorithm provided with the Zetasizer software was used to automatically calculate particle sizes and distribution. Average diameters for each condition were plotted along with the average plus or minus half the calculated polydispersity width in order to represent the distribution of observed diameters.
4.8 References


Chapter 5: Conclusions and Future Directions

5.1 Current status of WL47

The dimerized peptide WL47 is a first-in-class tool for probing the activity of caveolin. In dimeric form, this new caveolin ligand has an impressive 23 nM $K_D$, and it demonstrates clear activity as disruptor of oligomeric caveolin structures. In conjunction with the full-length caveolin variant concurrently developed by Joshua Smith, WL47 reopens the field of caveolin research by finally allowing experiments to be efficiently conducted in vitro. Nonetheless, further honing and experimentation remains to be done both to optimize the utility of WL47 and also to harness that utility for medically relevant research in cell culture.

5.2 The Dimerization Trap

As discussed in Chapter 4, the benefits of this new tool are hampered by its sensitivity to reducing environments, such as the environment within a live cell. While a possible solution to this problem has emerged, a branched dual-WL47 structure with a thioether bridge, the experiment in which it was revealed suffered problems that make it difficult to be fully confident in the data. More experiments with additional controls will need to be conducted before this monomeric mimic of a WL47 dimer can be conclusively deemed successful. While there is every hope that this design will solve the dimerization problem, alternative architectures have already been considered for testing, such as a dicarba bridge mimic of a disulfide, with or without a branch point.
Any potential structure proposed as the solution to the disulfide problem must be compared to the original disulfide-bridged WL47 dimer. Experiments should include repetition of the fluorescence anisotropy binding assay for both the new structure and the original WL47. The hypothetical new variant, henceforth referred to as “neoWL47”, should display equal or better affinity for caveolin (1-104). Similarly, in vitro deoligomerization activity should be assayed by DLS as was done previously, under both reducing and non-reducing conditions. A successful assay should show that both WL47 and neoWL47 are able to deoligomerize caveolin under non-reducing conditions, but that only neoWL47 should be able to do the same under reducing conditions.

5.3 Cell Penetration

Once a suitable redox-stable neoWL47 has been established, it will be necessary to adapt it for effective cell penetration. As stated in Chapter 1, caveolin is a monotopic membrane protein with both the N- and C-termini facing inward toward the cytoplasm. Because of this, live cell studies using neoWL47 will only be viable if the peptide can be effectively imported into the cytoplasm though the cell membrane.

The field of cell penetrating peptides (CPPs) continues to grow and the absolute attributes necessary for a peptide to penetrate the cell membrane is still not entirely known, but high levels of positively charged residues have been associated with many CPPs. For this reason, it is possible that the positively charged neoWL47 may have intrinsic cell penetrating ability, so first experiments would examine this possibility. However, because it is truly unlikely that neoWL47 would have this natural ability, we also
Figure 5.1 Proposed design of peptide conjugates for testing cell permeability. The proposed redox-stable WL47 variant with a branched chain and thioether bridge could be synthesized with an initial azide-bearing amino acid installed prior to the Lys branch point. Several cell penetrating peptides such as poly-arginine\(^3\), Xentry\(^4\), and TAT\(^5\) would be synthesized separately with an alkyne-bearing acid installed at the N-terminus and a Lys(DABCYL)-Cys tag at the C-terminus. The redox-stable WL47 variant could be conjugated to any of these CPPs via azide-alkyne cycloaddition. The fluorophore EDANS could be treated with 2-iminothiolane to functionalize it with a free sulphydryl. This sulphydryl-bearing EDANS would be attached to the peptide conjugate by oxidative formation of a disulfide bond. Such a disulfide bond would be stable outside cells but would become reduced inside a cell, liberating the EDANS fluorophore which, now unquenched by DABCYL, would provide a fluorescent signal to indicate successful traversal of the cell membrane by the peptide.\(^6\)
propose designing a set of neo\textbf{WL47} variants with a range of known CPPs attached. Figure 5.1 illustrates a potential construction of such CPP-linked variants, as well as the inclusion of a Lys-DABCYL quencher and disulfide attachment of EDANS fluorophore at a free Cys such that, upon successful cell penetration, reduction of the disulfide would free the fluorophore (Figure 5.1). The free EDANS, no longer quenched by DABCYL, would provide a fluorescent signal indicating successful cell entry. This experimental strategy has yet to be attempted or optimized, thus subsequent researchers on this project are advised to regard this plan as advisory rather than prescriptive.

Using neo\textbf{WL47} with an attached fluorophore such as rhodamine, we would also visualize the colocalization with caveolin utilizing a wild-type caveolin construct fused to eGFP fluorescent protein. HEK293 cells, which do not express significant levels of endogenous caveolin, will be transfected to express the fluorescently tagged caveolin. When treated with the cell-penetrating and fluorescently tagged neo\textbf{WL47} construct, colocalization of the caveolin and peptide will be visible by fluorescence microscopy. Additionally, using a non-fluorescent cell-penetrating neo\textbf{WL47} construct, the effect of neo\textbf{WL47} on caveolin oligomerization will be observed and quantified in collaboration with Dr. Michelle Digman using number and molecular brightness (N&B) methodology pioneered by her laboratory. This method uses raster image correlation spectroscopy (RICS) along with specialized software and algorithms to precisely quantify the movement and aggregation of fluorescently tagged molecules in live cells. We would expect to see activity in the form of caveolin oligomer disruption and an increase in the number of free monomers. Should unforeseen problems arise with the N&B method, some oligomerization
data could also be obtained using homo-FRET and fluorescence anisotropy measurements, but this method is less reliable and less quantitative than N&B.\textsuperscript{8}

\textbf{5.4 Activity Assays}

With a stable, cell-penetrating version of \textbf{WL47 (neoWL47)} in hand, the logical next step is to confirm that it retains its activity in live cells. Caveolin influences early cellular response to mechanical stress.\textsuperscript{9-12} Oligomers disassemble when cells are subjected to stretching forces, flattening the caveolae invaginations, which provides additional membrane area to allow the cell to quickly adapt to the mechanical stress. \textsuperscript{9} This gives the cell a small grace period between when the stress is implemented and when the more complex cellular stress responses are able to compensate.\textsuperscript{13} In one experiment by Sinha et. al, mouse lung endothelial cells (MLEC) with either wild-type caveolin expression or null caveolin expression were subjected to hypo-osmotic stress and stained with Trypan Blue, a stain specific for dead cells, to determine the level of membrane tension induced rupture; MLEC without caveolin were significantly more likely to rupture under hypo-osmotic stress than were MLEC expressing caveolin.\textsuperscript{9}

In order to test the activity of \textbf{neoWL47} we will use this same aspect of caveolin to see the results of deoligomerization in action. Caveolin expressing cells such as NIH 3T3 cells will be treated with \textbf{neoWL47} to break up caveolin oligomers, which in turn will flatten caveolae. Although this will initially change the membrane tension of the cell, we will wait for the homeostatic return to normal tension. At this point, cells will be treated with 20x PBS, 1x PBS, or pure water to induce different levels of osmotic stress, along with propidium iodide (PI), a DNA intercalating dye that cannot pass through cell membranes.
Because PI fluoresces weakly when unbound but strongly when interacting with DNA, the fluorescent signal will indicate the level free floating DNA and thus the level of cell rupture. DiO dye (3,3'-Dioctadecyloxycarbocyanine Perchlorate), a membrane-specific dye, will be used as a counter stain, making it possible to normalize PI signal to the total number of cells. Approaching the assay this way, rather than using Trypan blue as was done in the Sinha paper, allows for high-throughput ligand activity readout using a fluorescence plate reader as opposed to more laborious cell counting by microscope. Using this approach, we would expect to see increased rupture of neoWL47-treated cells under hypo-osmotic conditions because the lack of caveolae due to caveolin deoligomerization will not buffer the change in membrane tension.

Among other physiological responses that may be assayed are formation of caveolae, activity of endogenous eNOS, and frequency of caveolae-mediated endocytosis events, although assays for these have yet to be planned out. Since this peptide and its derivatives have the potential to be used as a lead compound for therapeutics, it could also be beneficial to begin investigating the dose needed for activity (EC50) and any toxicity it may show toward the cell lines (LD50) to begin to understand some pharmacodynamic aspects of neoWL47.

5.5 Conclusion

In conclusion, WL47 represents a step forward for the field of caveolin research, but more fine-tuning is required before its true potential can be realized. Stabilization of the dimer, determination or design of cell permeability, and demonstration of binding and
activity *in vitro* and in live cells will ensure that the next version of *WL47* will be facile to use and easily available to researchers in the caveolin field.
5.6. References


