DEVELOPING A PROTEIN-BASED ASSAY FOR IDENTIFYING HRSV ENTRY INHIBITORS AND KNOWLEDGE-BASED APPROACHES TO DESIGN PEPTIDOMIMETICS

A DISSERTATION
SUBMITTED TO THE DEPARTMENT
OF CHEMICAL AND SYSTEMS BIOLOGY
AND THE COMMITTEE ON GRADUATE STUDIES OF STANFORD UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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Abstract

Biotherapeutics have regained their reputation as drug candidates in the drug market due to their remarkable specificity; however, the broader clinical use of biotherapeutics is often challenged by poor pharmacological properties. Therefore, there is enormous interest in developing peptidomimetics as alternative therapeutic options.

Inspired by the first successful example of peptide-based antivirals, Fuzeon®, similar strategies to develop antivirals have been applied to many other viruses that share class I fusion protein-mediated viral fusion. Using human Respiratory Syncytial Virus (hRSV) as a model system, the second chapter of this dissertation demonstrates our successful effort to develop a protein-based assay using a 5-Helix Bundle (5HB) fluorescence polarization (FP) as a screening platform for hRSV fusion inhibitors. The remaining chapters in this thesis all utilize the 5HB-based FP assay to evaluate the potential of short peptides and their peptoid-based peptidomimetics as antivirals to control hRSV infections.

The third chapter proposes that NMEGylation, an alternative to PEGylation that uses a covalent attachment of an oligo-N-methoxyethylglycine (NMEG) chain, may enhance the bioavailability of short therapeutic peptides. The incorporation of optimized numbers of NMEG monomers along with a glycine linker increases the solubility and serum stability greatly, suggesting that NMEGylation may open a new opportunity to use peptoids as modifiers of therapeutically attractive peptides and proteins.
The fourth chapter demonstrates how our novel approach combining alanine, proline, and sarcosine scans can be useful to determine peptoid-replaceable peptide residues and further proves the usefulness of the combined scan strategy using the C_{20} peptide as a parent peptide. Furthermore, two different methods to promote the α-helical conformation of C_{20} analogues by structurally constraining the parent C_{20} peptide using “hydrocarbon-stapling” or “clicking” are described. We report a constrained C_{20} analogue with improved binding affinity, and discuss our structural investigations of the constrained C_{20} analogues using CD spectroscopy.

The studies in the fifth chapter show that phage display can be used for identifying novel hRSV entry inhibitors that are not derived from the original sequence of the hRSV fusion protein F like the peptides described in chapters 2, 3, and 4. We report two 12-mer peptides with a low micromolar binding affinity to the 5HB, and ongoing efforts are aimed towards better understanding the interaction of the 12-mer peptides with the 5HB by co-crystallizing the peptides and 5HB.

Finally, the dissertation concludes with a sixth chapter that summarizes the current status of each research chapter. In addition, future prospects, new directions, and potential applications of the findings in this dissertation are presented in this chapter.
Acknowledgments

Graduate school has been a long but indeed incredible journey to me. I feel so blessed to have such great friends and colleagues who have helped me so much to get through many ups and downs that I have faced during all those years in graduate school. I have learned how to be a good scientist and colleague, and most importantly, a human being from them. Without these helps, I know I could not be here to describe how thankful I am.

I am grateful to acknowledge that my advisors, Professors Annelise E. Barron and Theodore S. Jardetzky who have pushed me to become a better and more independent scientist. I will always keep in mind invaluable training and experience they allowed me to have throughout these years. I also want to show my deep gratitude to the committee members: Professors Tom Wandless, James Chen and Jennifer Cochran, who greatly supported and guided my research project.

I feel deeply indebted to Dr. Modi Wetzler as my scientific mentor as well as a wonderful friend. On the top of kindness and smartness, he is an inspiring and very thoughtful scientist. Drs. Jiwon Seo and James Broering from the Barron group and Dr. Beth Wurzberg from the Jardetzky group also have been great colleagues and friends. With their help, countless mistakes that I have made could be easily converted into valuable knowledge without too much pain. I have learned so much from them, and can only hope to be a mentor like them to someone sooner or later.

I would like to thank my former and current lab-mates from both the Barron and Jardetzky labs who have become my friends and family. With their friendship and support, my life in graduate school could be enjoyable after all.
The most importantly, I am deeply thankful to my mom, dad, and sister for their constant and unconditional love and support. I could come this far because I always knew that my family would be standing by me no matter what happens.

And lastly, YOU! You are my inspiration!
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Introduction

Synthetic peptide therapeutics: Inspiration from Fuzeon

Even with the knowledge gained after tens of billions of dollars spent on small molecule drug discovery, there is a rather disappointing success rate of 3% for reaching preclinical development and 7% for successful launching in the market from clinical trials.\(^1,2\) Consequently, synthetic peptides have regained their standing as potential drug candidates and the biotherapeutic market has been rapidly growing. Clinically-available synthetic peptides have proven their undeniable merits as therapeutics including strong binding affinity, high specificity and selectivity, minimized and often predictable drug-drug interaction profiles and lower toxicity.\(^3,4\) Because of these attractive attributes of peptides as drug candidates, there are approximately 700 peptides in the development stage worldwide including around 270 peptides in clinical phases and about 400 in advanced preclinical phases.\(^3,5\) Additionally, more than 60 synthetic peptides have already reached the market with sales reaching US $40 billion.\(^3,4\)

Among the FDA-approved peptide-based drugs, Fuzeon® (36-amino acid-long, enfuvirtide, Trimeris/Roche), a widely known anti-HIV agent, has been particularly inspiring to us because it represents the first case of a peptide medicine derived from a viral protein, HIV-1 gp41.\(^6-8\) Due to its low nanomolar antiviral activity and the urgent need to develop HIV treatments, Fuzeon® (T-20 before FDA approval)
was granted fast track status for FDA-approval and became the first viral entry inhibitor targeting HIV-1 gp41-mediated viral fusion.\textsuperscript{8-10} Since the product launch in 2003, worldwide sales of Fuzeon® have reached US $170 million in 2008, even with a slight sales drop compared to 2007.\textsuperscript{11} The comparatively low sales of Fuzeon® (‘‘blockbuster’’ drugs typically have annual sales of > US $1 billion) reflects its use as a drug of last resort for resistant HIV-1 infections due to its high cost and injection delivery method. In any case, the great success of Fuzeon® as a new class of antiviral agents has made class I viral fusion proteins, which HIV-1 gp41 belongs to, attractive antiviral targets.

\textbf{(A) Paramyxovirus F}  
\centering  
\includegraphics[width=0.8\textwidth]{paramyxovirus_f.png}  

\textbf{(B) HIV-1 gp41}  
\centering  
\includegraphics[width=0.8\textwidth]{hiv-1_gp41.png}  

\textit{Figure 1-1. Schematic diagram of class I viral fusion proteins} (A) The paramyxovirus fusion protein (F) and (B) HIV glycoprotein (gp) are shown as representatives of class I viral fusion proteins. Fusion peptide (FP) and transmembrane domains (TM) are located adjacent to two heptad repeat regions (HRA and HRB). Images are adapted from Lamb et al. (2007) Curr. Opin. Struc. Biol. 17, 427

\textit{Class I viral fusion proteins}  

Class I viral fusion proteins are frequently found in many virus families including paramixoviridae, e.g., parainfluenza virus 5 (PIV5) and human respiratory syncytial virus (hRSV); retroviridae, e.g., HIV-1; coronaviridae, e.g., severe acute respiratory syndrome coronavirus (SARS-coV), and orthomyxoviridae, e.g., influenza
virus\textsuperscript{12} and share similar structural and functional features. As shown in Fig. 1-1, these fusion proteins are first synthesized as intact precursor proteins and then cleaved by furin-like proteases, yielding two subunits and subsequently forming a trimeric conformation. Two heptad repeat regions (HRA and HRB) are located adjacent to a fusion peptide (FP) and a transmembrane domain (TM) and contain a strong coiled-coil motif. Class I viral fusion proteins are believed to be a primary driving force for the membrane merger between virus and host cell through the formation of an extremely stable 6-helix bundle (6HB). 3-D structures of these proteins determined by X-ray crystallography reveal remarkable structural similarity to each other, where the three HRA helices assemble into an inner core, while the three HRB helices pack against this trimeric inner core in an anti-parallel manner, eventually forming the 6HB (Fig.1-2).

\textbf{(A) hRSV F}

\textbf{(B) HIV-1 gp41}

\textit{Figure 1-2.} \textbf{6-helix bundle structures of class I viral fusion proteins} Core structures of (A) hRSV fusion protein F and (B) HIV-1 gp41 are illustrated from the corresponding X-ray crystal structures, showing striking similarities.
Class I viral fusion protein-mediated membrane merger

Viral entry is the most essential step during the infectious cycle, and is an attractive drug target because it occurs extracellularly, providing relatively easy access to antivirals. Based on pre- and post-fusion structures of PIV5 and HPIV3, respectively, determined by the Jardetzky laboratory, a mechanism of paramyxovirus fusion protein F-mediated membrane merger has been proposed (Fig. 1-3). Upon activation by viral attachment, the F protein assembles into a pre-fusion form, then an intermediate conformation and finally undergoes a dramatic conformational change to a highly stable 6HB as shown in Fig. 1-3. Since Fuzeon® has proved the therapeutic value in blocking this 6HB formation to prevent viral fusion and further viral infection, disruption of the 6HB assembly by peptides derived from viral fusion proteins has been a successful strategy for antiviral drug discovery, yielding several promising viral entry inhibitors currently in clinical trials against viruses that share class I viral fusion proteins such as hRSV and SARS-coV.

Virus

Host cell

Figure 1-3. Proposed model of class I viral fusion protein-mediated virus entry, based on structural studies by X-ray crystallography (Image adapted from Yin et al. (2006) Nature 439, 38) HRA and HRB domains are in green and blue, respectively. Intermediate stage is believed to last several minutes, thereby becoming an attractive antiviral target. Fuzeon® and other peptide antivirals target this stage to prevent 6HB formation and further viral entry.
Shortcomings of peptide therapeutics: A lesson from Fuzeon

Challenges in using synthetic peptides in clinics still remain because of high manufacturing cost and poor biophysical properties, including rapid renal clearance, low solubility, and limited thermal stability.\textsuperscript{20-22} In general, peptides require a certain length to form proper secondary structures such as $\alpha$-helix or $\beta$-turn in aqueous solution and once peptides adapt these distinctive structures, they become functional and less sensitive to proteolytic degradation. Short and therefore unstructured peptides (15–25 aa in length) are highly vulnerable to proteases, resulting in short half-lives (2–5 min).\textsuperscript{23,24} On the other hand, longer peptides can be more structured with relatively low susceptibility to proteases, but more complicated synthesis steps are required for longer peptides, which greatly increase manufacturing costs. In addition, the variable solubility, limited stability, low cell-permeability, and high immunogenicity of peptides still restrict their development and the usage in clinic.

For example, Fuzeon®️, a 36 amino acid-long peptide, requires 106 chemical synthesis steps, resulting in a high cost of production and treatment (~$30,000/year/patient in the United States).\textsuperscript{25,26} Additionally, due to low bioavailability, Fuzeon®️ cannot be orally delivered and needs to be administered twice daily via injection with high dose (90 mg/day), leading to potential side effects as well as poor patient compliance.\textsuperscript{6,27} Even with such a high efficacy as an anti-HIV agent, Fuzeon®️’s high cost and complex administration have restricted its overall growth worldwide as previously discussed.
Recombinant proteins to overcome challenges in biotherapeutics

To circumvent the limitations of synthetic peptides, most of the top 100 FDA-approved biotherapeutics are large or highly structured, accounting for their enhanced chemical, physical and thermal stability. Similarly, instead of using small helical protein segments derived from class I viral fusion proteins, larger recombinant proteins containing multiple protein fragments have been designed and investigated as viral fusion inhibitors. As the first example of this approach, the 5-Helix was created by alternately linking three HRA helices and two HRB helices from the HIV-1 gp41 with short peptide linkers (Fig. 1-4A). This recombinantly engineered protein construct shows extreme thermal stability with high α-helical content. The 5-Helix was initially created as an HIV entry inhibitor; however, it was also tested as a target for screening small molecule anti-HIV-1 agents.

In the case of hRSV, multimerized HRA and HRB helices (HR121 and HR212) were designed from hRSV F protein and tested as anti-hRSV agents (Fig. 1-4B), showing nanomolar activity in cell-cell fusion assay. This exact strategy has also been applied to HIV-1 gp41 by the same research group, highlighting that these multimerized protein inhibitors have great potential to be successful antiviral agents with high antiviral activity. Although these recombinant proteins seem to overcome peptides’ intrinsically poor biophysical properties, there are still unavoidable problems that need to be solved. The molecular sizes of proteins are generally orders of magnitude larger than traditional small molecule drugs, complicating drug delivery and necessitating delicate storage condition with poor shelf-life that make proteins less attractive as pharmaceuticals.
**General approaches to design peptidomimetics**

Recent revolutionary advances in biotechnology have yielded significant success in prolonging the half-lives of synthetic peptides, often utilizing a new class of therapeutically attractive molecules called peptidomimetics. Peptidomimetics can imitate the desired elements of peptides and alter undesirable properties such as proteolytic susceptibility, while more importantly retaining the biological functionality.
of the peptides of interest. The traditional strategy to design therapeutic peptidomimetics starts with biologically active peptides that bind to the target molecules. Once either biologically-derived or synthetically-generated peptides of interest are chosen, these peptides can serve as a template to design peptidomimetics.

Typically, after critical residues for biological functions of peptides are identified by the conventional methods such as truncation and deletion studies and an alanine scan, the resulting optimized peptide sequences can be further refined with the replacement of each amino acid by D-amino acids or unnatural amino acids to define conformation parameters (e.g., chirality, α-helicity, and H-bonds). These parameters allow us to better understand crucial conformations of the biological active core in target peptides, presumably providing the possibility of the modification in the peptide structure to improve the peptides’ pharmacokinetic properties, reduce the degree of proteolytic degradation, and thereby extend the half-lives.

One representative example of such peptide modifications is structurally rigidifying peptides by creating chemical linkages between residues (e.g., stapled peptides, bicyclic peptides, and chemical crosslinking). Since short peptides are generally unstructured, the entropy loss upon binding to target proteins reduces affinity. To minimize this entropy penalty, efforts to introduce conformational constraints to peptides have resulted in promising therapeutic peptidomimetics with substantially enhanced biological activity for the desired target. These peptidomimetics are highly protease-resistant, and have reduced immunogenecity as well as improved bioavailability compared to the parent peptides. As the market for
peptide-based drugs has greatly expanded, peptidomimetics research has also grown using non-peptidic chemicals, or peptidic molecules to mimic structural feature of peptides while biological activities are retained or even enhanced.

**Peptidomimetic folding oligomers: Foldamers**

In addition to peptide mimics that greatly alter the backbone structure such as azapeptides and oligoureas (Fig. 1-5), peptidomimetic folding oligomers, known as foldamers, have received considerable attention because this class of peptidomimetics exhibits more versatile secondary structural elements including both α-helices and β-turns. Foldamers are composed of simple unnatural building blocks as shown in dotted boxes.

![Figure 1-5. Sequence-specific peptidomimetic oligomers](image)

(A repeating unit marked in dotted boxes)
boxes in Fig. 1-5, which greatly increase biostability while effectively mimicking protein conformations and interactions.\textsuperscript{48,49} Moreover, foldamers can be efficiently synthesized with chemically diverse monomers in a cost-effective manner and can be designed to be more water soluble, biocompatible and conformationally even more stable, depending on the specific monomers incorporated.\textsuperscript{50} Among the many types of foldamers, helical foldamers including $\beta$-peptides,\textsuperscript{51-53} $\gamma$-peptides,\textsuperscript{54,55} and $N$-substituted glycines (peptoids)\textsuperscript{56-58} are relatively well characterized and their applications as therapeutic candidates are being actively investigated (Fig. 1-5).

**Peptoid biomimicry**

Peptoids are non-natural, but easily synthesized and sequence-specific oligomers of $N$-substituted glycine. Peptoids are structural isomers of peptides where the side chains are attached at nitrogen atoms instead of carbon atoms (Fig. 1-5F and 1-6). This isomerism leads to several important consequences: there are no hydrogen bond donors in peptoids, and the backbone is achiral and more flexible. However, by incorporating selected $\alpha$-chiral bulky side chains, we can create the conformation of the polyproline type I-like (PPI) helical structure,\textsuperscript{59,60} with controlled handedness. An efficient “submonomer” approach using primary amines as side chains with an automated solid-phase synthesis (Fig. 1-7)\textsuperscript{61,62} has spurred a number of research groups to use peptoids in their research. These efforts have expanded the scope of
peptoid research from the fundamental understanding of the intermolecular interactions in a simple helical mimicking structure to the potential therapeutic applications of peptoids in a highly structured molecular environment.\textsuperscript{35,50,58,60}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Diagram.png}
\caption{Submonomer approach for peptoid synthesis}
\end{figure}

To better understand peptoids as biomimetic material, the relationship between peptoid secondary structures and side chains,\textsuperscript{57,63} and structural analyses on linear (e.g., thread-loop\textsuperscript{64}, β-hairpin-like peptoid,\textsuperscript{65} and PPI-helix\textsuperscript{59,66-68}) and cyclic peptoids\textsuperscript{69,70} have been extensively investigated using CD, NMR and X-ray crystallography, yielding valuable profound knowledge on peptoids (Fig. 1-8).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Images.png}
\caption{Secondary structures of Peptoids NMR structure of (A) a peptoid threaded loop, (B) a β-hairpin mimicking peptoid, and (C) X-ray crystal structure of a cyclic peptoid, highlighting the peptoid backbone in green.}
\end{figure}
Furthermore, the importance of tertiary structures in highly structured proteins should be considered, because specific functions of individual proteins are mostly determined by their tertiary structures. Therefore, the tertiary structure of peptoids has also been investigated by studying the bundling of amphiphilic peptoids\textsuperscript{71} and the self-assembly of peptoid-based helical bundles for mimicking protein helical bundles including Zn-binding metalloproteins (Fig. 1-9).\textsuperscript{72,73}

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{figure19.png}
\caption{Figure 1-9. Example of mimicking protein functions using peptoid helical bundles Two peptoid helical bundles showed nanomolar dissociation constants for zinc, suggesting that peptoids can create well-folded structures with protein-like functions.}
\end{figure}
\end{center}

\textbf{Challenges in transforming therapeutic peptides to peptoids}

Even with these recent accomplishments in peptoid research, there are very few successful studies on developing peptoids as binding partners (e.g., antagonist or agonist) targeting therapeutically important protein-protein interactions. A simple way to convert biologically active peptides to peptoid analogues can be the substitution of
each amino acid in target peptide sequence by peptoid monomers with identical side-chains,\textsuperscript{74,75} or structurally similar side-chains.\textsuperscript{76,77} In addition, since proline is the only naturally existing $N$-substituted amino acid, proline in target peptide sequences can be replaced by peptoid monomers and this strategy has yielded some exciting results including peptoid-based SH3 ligands,\textsuperscript{78,79} somatostatin analogues,\textsuperscript{80} plant peptide hormone analogues,\textsuperscript{81} and antimicrobial peptide analogues.\textsuperscript{82}

Instead of substituting individual peptide residues with peptoid monomers, several peptoids have been designed based on the secondary structures of the parent peptides to mimic the structures and consequently the biological function of bioactive peptides, including HDM2-p53 interaction inhibitors,\textsuperscript{83} lung surfactant protein mimics,\textsuperscript{84-86} and antimicrobial peptoids\textsuperscript{87-89}. To imitate glycoproteins and their important functions in cell-cell recognition, glycopeptoids have been successfully created, however, their biological activity remains to be tested.\textsuperscript{90}

Since biological function is exquisitely affected by even minor structural details, transforming peptides into peptoids is not straightforward. Peptoid incorporation into a peptide backbone causes shifting of side-chains and removes H-bond donors. This might result in an altered conformation, which will deteriorate the biological activity of peptoid analogues, because it is often crucial for each amino acid in a peptide/protein to have precise spacing and geometry for protein-protein interactions. The structural flexibility of peptoids due to rapid $cis/trans$
isomerization at amide bonds can also be problematic (Fig. 1-10). This flexible backbone structure of peptoids can lead to a large entropy penalty upon peptoid binding to the target protein, and possibly cause low binding affinity for peptoid therapeutics. Nevertheless, the simplicity of the synthesis, a diverse set of commercially readily available primary amines as monomers, and the great chemical versatility (e.g., side-chain cyclized helical peptoids9) provide almost infinite opportunity to explore peptoids as attractive druggable molecules. Therefore, we believe that there is a great need for developing a generally applicable methodology to guide the incorporation of peptoid residues into therapeutic peptides, thereby allowing us to create better peptoid-based bioactive therapeutics.

**Overview of the thesis**

To address urgent needs in developing an appropriate assay system to identify antivirals targeting viral entry, hRSV fusion protein F has been a main subject in this thesis as a representative of class I fusion protein-mediated viral entry. We designed a novel assay platform for screening potential hRSV entry inhibitors and then screened possible peptides derived from the hRSV F protein and from phage displayed peptide libraries. We also developed new peptidomimetic approaches to improve the desired properties of therapeutic peptides.

In **Chapter 2**, we established and validated a protein-based fluorescence polarization (FP) assay as a screening platform to specifically identify hRSV entry inhibitors. We hypothesized that a 5-helix bundle (5HB), an engineered hRSV F protein, can provide a well-defined binding site for potential antiviral candidates
targeting hRSV F and proved our system by carrying out peptide truncation studies, suggesting C_{20} peptide (a 20 amino acid-long peptide) can be a potential anti-hRSV agent with a low micromolar binding affinity. In addition, we examined small molecule fusion inhibitors using the 5HB-based system, as the mechanism of action of these compounds has yet to be fully understood and our assay could provide a tool to address this. This 5HB-based FP assay was then utilized in all subsequent work.

In Chapter 3, using the C_{20} peptide as a parent molecule, we explored NMEGylation as a simple method to enhance the bioavailability of relatively short peptides, which are otherwise less attractive as biotherapeutics. The studies reported in Chapter 4 are a part of our effort to develop a generally applicable method to convert therapeutic peptides into peptoid-based peptidomimetics. The knowledge gained here is used to generate structurally constrained peptomeric hRSV inhibitors with expectation of enhanced α-helicity and thus increased binding affinity. In Chapter 5, we expand our scope of searching anti-hRSV agents to 12-mer peptides derived from phage-displayed peptide libraries. In addition to exploring the binding activity of the 12-mer peptides to the 5HB using our FP assays, we made an effort to structurally examine the interaction between 12-mer peptides and the 5HB by X-ray crystallography. Finally, the conclusions and further implications of this research are described in Chapter 6. Consequently we believe that our results in this thesis will provide new insights into the development of antivirals targeting hRSV fusion, expecting that our strategy can be applied to other viruses sharing similar viral fusion mechanism. Also, we anticipate that the method proposed here will provide a simple
and easily applicable method for the design of bioactive peptoid-based peptidomimetics.
References


40. Harrison, R.S., Shepherd, N.E., Hoang, H.N., Ruiz-Gomez, G., Hill, T.A., Driver, R.W., Desai, V.S., Young, P.R., Abbenante, G. & Fairlie, D.P.


Chapter One: Introduction


Chapter Two

Design and evaluation of a structure-guided screening platform for peptide-based hRSV entry inhibitors

Human Respiratory Syncytial Virus (hRSV)

hRSV is a negative-sense single-stranded RNA virus and belongs to the Pneumovirus genus of the Paramyxoviridae family. Among its many glycoproteins, which are critical in viral functions including reproduction, the attachment protein (G) and the fusion protein (F) are major players in the infection and pathogenesis of hRSV, and are thus relatively well studied. The G protein is known to be required for attachment of the virus to respiratory epithelial cells, whereas the F protein directly mediates the viral fusion and entry, inducing the formation of the characteristic syncytium, that is a large multi-nucleated mass of cytoplasm.1

The F protein is synthesized as a precursor F0, and is then processed by proteolytic cleavage, subsequently yielding two subunits, F1 and F2. This step is

![Figure 2-1. Schematic diagram of hRSV fusion (F) protein](image)

F1 and F2 are formed after proteolytic cleavage (arrow) of the precursor protein (F0) by furine-like protease. The fusion peptide (FP) and transmembrane domain (TM) are indicated. Adjacent to FP and TM, heptad repeat regions, HRA and HRB are shown.

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* This chapter is adapted from the following publication:
required for activation. As a strong indication of its importance, the sequence of the F protein is highly conserved among *Paramyxoviridae* family. As shown in Fig. 2-1, two hydrophobic heptad repeats (HRA and HRB) are located adjacent to the fusion peptide (FP) and transmembrane domain (TM) with approximately 250 residues of intervening sequence between them. The HRA and HRB domains contain a sequence motif suggesting α-helical coiled-coil structure.

Viral entry into the host cells is an early but crucial event to the hRSV infectious cycle. Upon attachment via the protein G, hRSV F protein facilitates membrane fusion with the host cells at neutral pH, similar to other paramyxoviruses such as measles virus. A more detailed mechanism has been proposed based on pre- and post-fusion structures of the F protein as discussed earlier in Chapter 1.

![Figure 2-2](image)

**Figure 2-2. X-ray crystal structure of hRSV 6HB** (A) a side and (B) top view of the hRSV F 6HB are shown. HRA and HRB are color-coded in green and blue, respectively. HRA helices form a trimeric innercore and HRB helices pack against the innercore in anti-parallel manner, completing the 6HB assembly. Figures were generated by Pymol software.
Chapter Two: Design and evaluation of a structure-guided screening platform for peptide-based hRSV entry inhibitors

The hRSV F protein-mediated membrane merger is particularly interesting, because it occurs at the cell surface, so the process is accessible to inhibition by antivirals without requiring cell permeability. It thus has been recognized as an attractive therapeutic target, yielding numerous peptidic or small molecule fusion inhibitors.\textsuperscript{4-16} In addition, X-ray structural studies have shown that the HRB regions pack in hydrophobic grooves formed on the surface of the HRA trimer core (Fig. 2-2) showing striking similarity to HIV-1 gp41 core structure (Fig. 1-2).\textsuperscript{17} In the case of HIV-1 gp41, synthetic peptides corresponding to HRA or HRB can effectively inhibit HIV infection.\textsuperscript{18,19} Fuzeon\textsuperscript{®} (enfuvirtide, Roche), a drug currently approved in the U.S. for use against HIV infections, uses this mechanism of inhibition. Collectively, all these features strongly support the hypothesis that targeting the F protein and the viral fusion process would be effective in identifying hRSV entry inhibitors.

Urgent need to develop hRSV treatment

Despite attempts to develop safe, cost-effective treatments to control hRSV-associated illness,\textsuperscript{4,5} hRSV remains the leading pathogen causing lower respiratory tract infections mainly in infants and young children, and a severe public health problem worldwide. hRSV infections cause more than 140,000 pediatric hospitalizations\textsuperscript{20} and 2,000 deaths in the United States alone with costs of $356 – 585 million annually.\textsuperscript{1,21-24} Most children are infected with hRSV at least once before the age of 2, and recurrence is very common.\textsuperscript{25} hRSV has also been an increasingly recognized cause of high morbidity and mortality in immunosuppressed patients and the elderly. In case of the elderly, hRSV infections have been reported as significant
cause for many complications such as pneumonia, resulting in up to 10,000 deaths annually.\textsuperscript{26,27} However, there are no treatment options available to specifically treat hRSV infections and related complications except for supportive care such as oxygen supply.

\textit{Current hRSV treatment options}

Antiviral drug discovery to control hRSV infections has mainly relied on the screening of chemical libraries or natural products using common virology assays and animal models, yielding limited success.\textsuperscript{5,28,29} To date, a nucleoside analog, Ribavirin, is the only clinically approved antiviral agent to treat hRSV infections by interfering with the RNA metabolism required for viral replication. However, due to its controversial efficacy, potential cytotoxicity and severe side effects, its clinical usage is tightly restricted. As discussed earlier in this chapter, the membrane merger between virus and the host cells is the key step that enables the viral genome to enter and initiate the infectious cycle of hRSV. Because this event happens extracellularly, it is a very attractive therapeutic target to develop antivirals against hRSV infections, resulting in many small molecule drug candidates including BTA9881, BMS433771, TMC353121, VP14637, and RFI641 (see small molecule fusion inhibitors section below). These candidates were identified with tissue-cell culture based assays and their mechanism of action is poorly understood except that the compounds presumably interrupt the formation of 6HB during viral fusion by binding in the hydrophobic pocket (Fig. 2-3). However, these efforts have resulted in a high failure rate and none are in advanced stages of clinical trails.\textsuperscript{30,31} With treatment options limited to measures
such as supportive care, development of safe and specific agents against hRSV is essential.  

![Figure 2-3. A key interaction between HRA and HRB helices and the hydrophobic pocket formed by neighboring HRA helices in the 6HB assembly](image)

(A) Two phenylalanine residues of the hRSV F HRB domain marked in red play a crucial role in the interaction with the hRSV F HRA helices shown in green by packing into (B) the hydrophobic pocket shown in red dotted are formed by HRA helices. Figures were generated by PyMol software.

**Current options for hRSV prevention**

Because of the limited treatment options available, prevention strategies are highly desirable. However, vaccine development faces several obstacles: the need to immunize very young infants who may not respond adequately to vaccination and the existence of antigenically different hRSV strains (A and B). A humanized monoclonal antibody, Synagis® (palivizumab, MedImmune) targeting a conserved neutralizing epitope on the hRSV F protein successfully inhibits viral fusion and was FDA-approved in 1998. Since then, it has been used essentially as the only option for
hRSV prevention efforts; however, its usage is restricted to high-risk children (babies born at less than 36 weeks or who have heart or lung problems). Because Synagis® needs to be administered throughout the flu outbreak season (November to March), the cost has become a limiting factor for those who want the treatment.

Numax™ (Motavizumab, Medi-524, MedImmune) is a 2nd generation humanized monoclonal antibody. It is directly derived from Synagis® with only a 13 amino acid difference, but is over 20-fold more potent than Synagis® in *in vitro* microneutralization assays. However, as of September 20, 2010, its FDA approval is on hold and the FDA has requested more data from an additional trial of the drug to support a satisfactory risk/benefit profile for the prophylaxis indication.

**Development of screening methods for hRSV fusion inhibitors**

To our knowledge, there is no established, simple, non-cell-based method to screen potential antivirals specifically targeting the hRSV F protein. Previously, the 5-Helix of the HIV-1 fusion protein gp41 was shown to be a viral entry inhibitor, as well as a suitable target for screening small molecule libraries in a high-throughput format. This suggests that similar approaches would be applicable to other viruses, like hRSV, that rely on class I viral fusion proteins. We thus created a 5-Helix Bundle (5HB), variant hRSV F protein, and developed a competitive fluorescence polarization (FP) based assay using the 5HB as a target protein and a fluorescently labeled peptide as a tracer. To validate that the competitive FP-based 5HB assay can provide a reliable screening platform, a series of *N-* and *C-* terminally truncated peptides derived from HRB domain of the hRSV F protein were synthesized and tested. Thus, we
demonstrated a simple, fast, and low-cost *in vitro* fluorescence polarization assay that can be readily applied to libraries of peptides, peptidomimetics, or small molecules to rapidly screen potential hRSV fusion inhibitors (Scheme 2-1).

**Scheme 2-1.** Strategy designed and developed in this study to identify hRSV fusion inhibitors targeting specifically hRSV F protein. In the right panel (shaded), the 5HB serves as a screening platform for identifying potential hRSV fusion inhibitors. Once inhibitor candidates are selected, these inhibitors can be further studied in tissue-cell culture-based assay. Ideally we expect that the inhibitor candidates identified from the 5HB-based assay will prevent the 6HB formation of hRSV F, thereby blocking viral fusion and entry.
5-Helix Bundle construct design, expression, purification and secondary structure analysis

A 5-Helix construct of HIV-1 fusion protein gp41 has been tested as a fusion inhibitor\textsuperscript{34} and used as a target protein for screening small molecule libraries.\textsuperscript{35} However, analogous constructs for hRSV F have only been tested as fusion inhibitors or vaccine candidates.\textsuperscript{16,36} Therefore, we designed a 5-Helix Bundle (5HB) construct to specifically mimic the 6HB that forms during hRSV infection of the host cell. The 5HB was generated by connecting three HRA and two HRB helices in an alternating sequence using short peptide linkers (Fig. 2-4A). The absence of the 3\textsuperscript{rd} HRB helix in the 5HB would create a large open binding site for potential fusion inhibitors. Soluble 5HB was expressed in BL21 (DE3) cells and purified by metal-affinity chromatography (Fig. 2-4B).

![Diagram](image)

**Figure 2-4.** An illustration of hRSV 5-Helix Bundle (5HB) with a resulting SDS-PAGE of 5HB purification (A) the designed 5HB and its single-chain polypeptide sequence are shown. A binding site for the missing 3\textsuperscript{rd} HRB and potential fusion inhibitors is shown in dotted line. (B) Purified 5HB samples were analyzed by SDS-PAGE on a 12\% non-reducing gel (M; molecular marker, FT; flow through, W; wash, and E; elution fractions).
Since the 5HB is composed of 5 difference helices, it is very crucial to confirm that this protein construct is well-folded. Therefore, the secondary structure of the 5HB was assessed by Circular Dichroism (CD) spectroscopy (Fig. 2-5A). As expected, the 5HB presented very intense $\alpha$-helical features, showing two strong negative peaks at 208 and 220 nm along with a strong positive peak at around 190 nm. The helical content was calculated using Dichroweb,$^{37,38}$ resulting in approximately 90% $\alpha$-helicity. The thermal stability of the 5HB was also examined in the temperature range from 0 to 85 °C and we did not observe any sign of thermal denaturation of the 5HB, indicating that the secondary structure of the 5HB is highly stable (Fig. 2-5B). This is consistent with observation for the HIV-1 gp41 5-Helix.$^{34}$

Figure 2-5. Secondary structure analysis and thermal stability of 5HB by CD spectroscopy (A) CD spectrum of 5HB in 10 mM PBS at pH 7.4. (B) The melting curve of 5HB obtained from the ellipticity measurements at 222 nm between 0 and 85 °C. ([$\Theta$]: per residue molar ellipticity measured in degrees · cm$^2$ · dmol$^{-1}$ · residue$^{-1}$)
Validation of 5HB as an hRSV F protein 6HB mimic by ELISA assays

To validate the functionality of the 5HB as a screening platform, we carried out ELISA assays on the 5HB using a previously described procedure. To do so, a full length of C-peptide (C49) derived from hRSV F HRB domain containing 49 amino acids was prepared (Fig. 2-6). Because this peptide is the missing outer helix, it should bind to the 5HB tightly forming a stable 6HB, and thus proving that the 5HB provides a high affinity binding site as anticipated. Since the C49 was too short to express in E.coli, it was expressed as a thioredoxin (Trx) fusion protein (Trx-C49). Binding of Trx-C49 to the 5HB was monitored with increasing concentration of the 5HB. To monitor the binding of Trx-C49 to the 5HB quantitatively, an ELISA assay against the S-tag on the Trx-C49 was performed, confirming that Trx-C49 tightly binds to the 5HB with a nanomolar affinity (Kd = 13.7 nM) (Fig. 2-7A).

![Figure 2-6. A schematic diagram of Trx-C49 with a resulting SDS-PAGE of purified Trx-C49](image)

(A) Thioredoxin Hlis9 S-tag C49

(B) M E

Figure 2-6. A schematic diagram of Trx-C49 with a resulting SDS-PAGE of purified Trx-C49 (A) An illustration of the designed Trx-C49 and its single-chain polypeptide sequence are shown (B) Purified Trx-C49 samples were analyzed by SDS-PAGE on a 12% non-reducing gel (M; molecular marker, and E; elution fractions). Calculated molecular weight of Trx-C49 is 24587.5 Da.
**Figure 2-7. Indirect and competitive ELISA assays** (A) The binding of Trx-C49 to the 5HB was observed as the concentration of Trx-C49 increased. (B) The competitive capability of Fl-C35 over Trx-C49 was tested using 7.8 nM of the 5HB, 4 nM of Trx-C49 and Fl-C35 concentrations ranging from 0 to 10 µM.

**Saturation binding FP measurement**

Fluorescence polarization (FP) has been widely used for a direct, nearly instantaneous spectroscopic measurement of molecular interactions such as protein-protein, DNA-protein, and small molecule-protein interactions. FP is also more straightforward (i.e., fewer steps) and less expensive (e.g., no antibodies) than other methods such as ELISA. To develop a reliable FP assay, the binding affinity as well as specificity of the probe to the target protein should be high. Previously, it has been shown that a series of 35-amino acid-long peptides from the conserved HRB domain within hRSV F protein could block syncytium formation with EC_{50} values in the range of 0.015 – 0.25 µM. Therefore, we decided to use T-108 (35mer: YDPLVFPSEFDASISQVNEKINQSLAFIRKSDEL) as a probe for developing a FP assay for the following reasons: First, the low EC_{50} value of 0.051 µM suggests that T-
108 should bind tightly to the 5HB; second, T-108 contains two phenylalanine residues (F^{483} and F^{488}) located at the N-terminus of the HRB region, which engage a deep hydrophobic pocket located at the C-terminus of the HRA helices, that is believed to be a good antiviral drug target (Fig. 2-3).^{10,11,48} This T-108 peptide was labeled with fluorescein at its N-terminus (Fl-C_{35}) to sever as a probe in FP.

![Figure 2-8. Binding titration curve of Fl-C_{35} and its binding stability to the 5HB](image)

The fluorescence polarization response of Fl-C_{35} binding to the 5HB was monitored as the concentration of the 5HB increased. The experiment was performed using 5 nM Fl-C_{35} and the 5HB concentration ranged from 0 to 500 nM. (B) The stability of Fl-C_{35} binding to the 5HB was monitored over a 24 hr period using 5 nM of Fl-C_{35} in the presence of increasing amount of the 5HB.

To validate this peptide as a probe, we need to demonstrate that it can effectively compete with the 6\textsuperscript{th} helix of the F protein, as well as tightly and stably bind the 5HB at low concentration. Using the ELISA test with Trx-C_{49}, we were able to show that Fl-C_{35} could displace the 6\textsuperscript{th} helix from the 5HB construct with a comparative IC_{50} value of \sim 40 nM (Fig. 2-7B). To determine the binding affinity of Fl-C_{35}, we used a fixed concentration of 5 nM Fl-C_{35} and monitored the FP response
of Fl-C35 with increasing concentrations of the 5HB, as shown in Fig. 2-8A. The FP results were consistent with high affinity binding (K_d = 21 nM). The stability of the FP assay using Fl-C35 and the 5HB is also important for its potential use for a high-throughput screening format. We therefore tested the stability of Fl-C35 binding to the 5HB by incubating the plate at room temperature over 24 hrs (Fig. 2-8B). The resulting binding curves show that this assay is highly stable and robust, which will allow us to carry out large-scale tests at room temperature.

**Specificity of Fl-C35 binding to the 5HB**

The specificity of Fl-C35 binding to the 5HB was cross-tested against a system derived from the Epstein-Barr virus (EBV), a member of the human herpesviruses. EBV requires a number of envelope glycoproteins for membrane fusion with the host cells. Particularly, the interaction of the EBV gp42 and the gH/gL complex are crucial in viral entry into B cells. The Jardetzky laboratory has reported that the EBV gp42-derived FITC-30mer specifically binds to EBV gH/gL protein with a low nanomolar K_d using a FP assay. To confirm the specificity of both the hRSV 5HB and the Fl-C35 probe, we tested the EBV gp42-derived FITC-30mer against the hRSV 5HB (Fig. 2-9A) and our hRSV-derived Fl-C35 against the EBV gH/gL complex under the same conditions (Fig. 2-9B). There was no evidence of nonspecific binding in these controls, indicating the interaction between Fl-C35 and the 5HB is specific and can provide a solid basis for developing a competitive FP-based 5HB assay.
Figure 2-9. Specificity and selectivity of Fl-C$_{35}$ to the 5HB (A) 5 nM of Fl-C$_{35}$ in the presence of increasing amount of the Epstein-Barr virus (EBV) gH/gL, a fusion protein that leads the EBV infection was tested. (B) 5 nM of EBV gp42 FITC-30mer with a wide range of the 5HB in concentration was monitored.

Competitive FP assays

Based on the $K_d$ value observed in the saturation binding FP assay, we established a competitive FP assay to evaluate potential inhibitors based on their ability to displace the Fl-C$_{35}$ (probe) from the 5HB (target). We first tested unlabelled C$_{35}$ against Fl-C$_{35}$ in the presence of the 5HB (Fig. 2-10A). Unlabelled C$_{35}$ peptide blocked the increase in polarization value with an IC$_{50}$ of 38 nM, competing with Fl-C$_{35}$ over the binding site on the 5HB. To demonstrate the effectiveness of the 5HB-screening platform as a tool capable of evaluating molecules with different binding affinities, we investigated a series of short peptides. Manufacturing longer bioactive peptides can be problematic due to high cost, but shorter unstructured peptides can easily lose their efficacy, and these contradictory criteria greatly affect for the design of peptide therapeutics.
Table 2-1. Peptides derived from the HRB domain of hRSV F protein and their sequences and binding affinities to the 5HB

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence (amino to carboxy)</th>
<th>% Inh.(^a)</th>
<th>IC(_{50}) ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRB</td>
<td>NFDPLVFPDEFDASISQVNEKINQSLAFIRKSDELLHNAGKSTN</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T-108</td>
<td>YDPFLVFPDEFDASISQVNEKINQSLAFIRKSDEL</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>C(_{35})</td>
<td>YDPFLVFPDEFDASISQVNEKINQSLAFIRKSDEL</td>
<td>100</td>
<td>0.038</td>
</tr>
<tr>
<td>C(_{30})</td>
<td>VFPSDEFDASISQVNEKINQSLAFIRKSDE</td>
<td>100</td>
<td>6.80</td>
</tr>
<tr>
<td>C(_{20})</td>
<td>ISQVNEKINQSLAFIRKSDEL</td>
<td>&gt; 90</td>
<td>14.92</td>
</tr>
<tr>
<td>C(_{17})</td>
<td>VNEKINQSLAFIRKSDEL</td>
<td>&lt; 50</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>C(_{13})</td>
<td>INQSLAFIRKSDE</td>
<td>NM(^d)</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>C(_{10})</td>
<td>SLAFIRKSDE</td>
<td>NM</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>N(_{15})</td>
<td>VFPSDEFDASISQVN</td>
<td>&lt; 50</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

\(^a\) % Inhibition of each peptide at the concentration of 100 \(\mu\)M was calculated.
\(^b\) HRB sequence from hRSV F protein
\(^c\) For T-108, the reported value of EC\(_{50}\) from Lambert et al. is used instead of an IC\(_{50}\) value, crude peptide T-108 was analyzed for its ability to prevent cytopathologic effect (CPE) in infectivity assays with hRSV.
\(^d\) NM: Not measurable

We therefore prepared a series of truncated peptides derived from the HRB domain of hRSV F protein (Table 2-1) and investigated their ability to compete against Fl-C\(_{35}\) using our competitive 5HB-based FP assay. The truncated peptides tested in this study mostly do not have the two phenylalanines that bind to the hydrophobic pocket (Fig. 2-3) that has been the focus of small molecule drug discovery effort.\(^4,48,51\) However, in previous work by Lambert et al., multiple 35-mer peptides that lacked those phenylalanines still exhibited significant antiviral activity.\(^6\) Notably, our hope was that shorter peptides with even modest binding affinity could act synergistically with small molecule drugs that would target the hydrophobic
binding pocket. The range of truncation peptide length described here addresses intermediate peptide lengths (< 35 amino acid-long), so we believe our study can provide an evidence that the entire hydrophobic groove on the C-terminus of the neighboring HRA helices would be available as a potential drug target for developing specific hRSV fusion inhibitors. The results summarized in Fig. 2-10B and Table 2-1 provide quantitative observations of the binding activity of shorter peptides (< 35 amino acid-long).

Figure 2-10. Competitive FP assays of unlabeled C_{35} and N- and C-terminally truncated peptides (A) Unlabeled C_{35} were competed with 5 nM Fl-C_{35} in the presence of 20 nM 5HB and its ability to displace Fl-C_{35} was monitored by FP. (B) Various concentrations of peptides were competed with 5 nM Fl-C_{35} in the presence of 20 nM 5HB.

**Suitability as a high-throughput screening system**

Previous pharmaceutical lead compounds were identified using week-long tissue cell cultures using live virus. Since the FP assay system could reduce costs by at least an order of magnitude, it could enable much broader efforts to develop effective
hRSV treatments. As discussed above, the 5HB-baed FP assays are stable at room temperature over 24 hrs (Fig. 2-8B). In addition, the reproducibility of measurements for free and unbound Fl-C\textsubscript{35} controls were examined (Fig. 2-11) for calculating the Z’ factor, a measure of the quality and robustness of an assay without test compounds.\textsuperscript{47} The Z’ factor was determined based on the guidelines (Equation 2-1) provided by National Institutes of Health (NIH),\textsuperscript{52} resulting in Z’ factor = 0.8, which suggests our assay can be directly applied to the HTS format.

![Graph showing assay robustness test](image_url)

Figure 2-11. Assay robustness test by FP measurements of free and bound Fl-C\textsubscript{35} controls

\[ Z' = 1 - \frac{3(\delta p + \delta n)}{|\mu p - \mu n|} \]

\textbf{Equation 2-1. Equation for calculating Z’ factor} Values of Z’ between 0.7 – 0.9 correspond to a good assay while a Z’ value of 0.5 corresponds to the minimum acceptable value for the HTS. Means and standard deviations of both positive (p) and negative (n) controls (\(\mu_p\), \(\delta_p\), and \(\mu_n\), \(\delta_n\))
Small molecule fusion inhibitors tested in 5HB-based competitive FP assays

As discussed earlier, since the hydrophobic cavity was identified as a potentially attractive drug target, several small molecules that prevent hRSV infection in cell studies have been reported (Table 2-2 and Fig. 1-12).

Table 2-2. Human respiratory syncytial virus fusion inhibitors

<table>
<thead>
<tr>
<th>Drug candidates</th>
<th>Type of compounds</th>
<th>Development status</th>
<th>Administration</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMC353121</td>
<td>Viral fusion inhibitor</td>
<td>Preclinical, ongoing</td>
<td>Inhalation or oral delivery</td>
<td>53,54</td>
</tr>
<tr>
<td>BMS433771</td>
<td>Viral fusion inhibitor</td>
<td>Discontinued</td>
<td>Oral delivery</td>
<td>10,55,56</td>
</tr>
<tr>
<td>RFI641</td>
<td>Viral fusion inhibitor</td>
<td>Discontinued</td>
<td>Inhalation</td>
<td>57</td>
</tr>
<tr>
<td>VP14637</td>
<td>Viral fusion and replication inhibitor</td>
<td>Discontinued</td>
<td>Inhalation</td>
<td>9,58,59</td>
</tr>
<tr>
<td>BTA9881</td>
<td>Viral fusion inhibitor</td>
<td>Insufficient safety in Phase I</td>
<td>Oral delivery</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 2-12. Structures of hRSV fusion inhibitors tested in this study
Even though the mechanisms of the antiviral activity of these compounds are poorly understood, it is predominantly believed that the small molecules bind to the hydrophobic cavity on the inner core of HRA helices, thereby preventing the 6HB formation and consequently blocking viral fusion. Recently, it was suggested that a small molecule inhibitor of hRSV F entry may act not by blocking the 6HB formation, but by distorting the final 6HB conformation. This study indicates that the inhibitor (TMC353121) engages both HRA and HRB regions and may thereby enhance the 6HB assembly into a non-functional structure.

Since the Fl-C₃₅ probe in our FP assay covers the hydrophobic pocket, we can determine whether the small molecules bind the hydrophobic pocket (and displace the

![Graph](image_url)

**Figure 2-13. Competitive FP assays of small molecule fusion inhibitors** Unlabeled C₃₅ was at 10 μM used to show 100% inhibition of 5HB·Fl-C₃₅ bound. As a negative control (0% inhibition), %5HB·Fl-C₃₅ bound was measured in the absence of inhibitors (no cmpd). Each compound at 0, 10, and 100 μM was tested in the presence of 5 nM Fl-C₃₅ and 20 nM 5HB. (Cmpd; compound, cmpd1; TMC353121, cmpd2; JNJ240868, cmpd3; Trimeris, cmpd4; BMS433771, and cmpd 5; Biota)
probe) or bind elsewhere (and do not disturb the probe). Therefore, we examined several hRSV F fusion inhibitors (Fig. 2-12) using the 5HB-based FP assay (Fig. 2-13). Surprisingly, none of small molecule inhibitors showed an inhibitory effect on the binding of Fl-C_{35} to the 5HB, indicating these compounds do not prevent the 6HB formation by binding to the hydrophobic pocket. We still do not fully understand how these compounds including TMC353121 can block viral fusion without blocking the 6HB formation. However, we believe that in addition to its role as a drug discovery-screening tool our 5HB-based FP assay could serve as a mechanism evaluation tool for these and other compounds. Notably, the use of an alternate probe, such as a 35-mer that does not cover the hydrophobic pocket, could enable further in-depth mechanistic studies. In any case, we anticipate that the 5HB system will provide a new rapid screening platform to specifically identify small molecules that prevent hRSV F 6HB formation.

**Conclusions and future prospects**

In this study, we demonstrated that a protein-based assay can be used as a direct screening platform for identifying potential antivirals against hRSV using fluorescence polarization (FP), which is a well-proven tool for direct and rapid measurement of molecular interactions. Our competitive FP assay using the 5HB for mimicking the formation of the hRSV 6HB can measure biological activities of short hRSV F-derived peptides over a wide range of binding affinity, suggesting that this system is sufficiently sensitive to screen weak binders to the 5HB as potential antiviral candidates that could target not only the hydrophobic pockets but also the groove
formed by two neighboring HRA helices. Moreover, this assay could be suitable for an initial high through-put screening effort to prioritize inhibitor candidates prior to tissue culture-based assays. Since current screening strategies require week-long cell-based assays using live virus, our rapid protein-based assay is significantly lower in cost and can enable more comprehensive drug discovery efforts at a given level of expenditures. This 5HB construct from the hRSV F protein may additionally prove useful for other viruses within the paramyxovirus family for future drug discovery efforts. Recent tests of small molecule fusion inhibitors using our 5HB-based FP assay suggest that this 5HB assay may also allow us to specifically screen small molecules that block or distort the 6HB formation of hRSV F.
Chapter Two: Design and evaluation of a structure-guided screening platform for peptide-based hRSV entry inhibitors

Materials and Methods

Protein synthesis

5-Helix Bundle cloning: The 5HB DNA construct is composed of three N$_{57}$ and two C$_{49}$ helices, representing residues 126 to 182 (HRA) and 476 to 524 (HRB) of the hRSV F protein, respectively. Each fragment was amplified by PCR using the hRSV strain A2 genome as a template and connected using short linkers: N$_{57}$ was joined using a linker (PPPELGGP) to C$_{49}$ to generate a heterodimer, N$_{57}$-C$_{49}$; two heterodimers were connected with a short linker (KGSSK); the final N$_{57}$ was linked after the second C$_{49}$ via the linker (KGSSK) (Fig. 2-4A). The engineered gene encoding 5HB was cloned between the Nde I and BamH III restriction sites of the hexahistidine expression vector pET-15b (Novagen, San Diego, CA, USA). The resulting plasmid carrying the complete 5HB construct was transformed into *E.coli* strain BL21 (DE3) for protein expression.

5-Helix Bundle expression and purification: Protein was recombinantly expressed in *E.coli* strain BL21 (DE3) grown to an OD of 0.8 at 600 nm at 37 °C in Luria-Bertani (LB) medium. Protein expression then was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (ITPG), and cells were grown for an additional 20 hrs at 20 °C, to enhance the solubility of protein.$^6$ The cells were harvested by centrifugation at 4500 x g for 15 minutes, and the resulting cell pellet was resuspended in 20 mM phosphate buffered saline (PBS) and stored at -80 °C. Cells were lysed in lysis buffer (CellLytic™ B cell lysis reagent [cat. no. C8740, Sigma Aldrich, Milwaukee, WI, USA], 20 mM PBS at pH 7.4, 1 mM phenylmethylsulphonyl fluoride [PMSF],
protease inhibitor cocktail [Sigma Aldrich, Milwaukee, WI, USA], 1% Triton X-100, 500 mM NaCl and 0.2 mg/ml lysozyme) and incubated for 1 hr at room temperature. Cell lysate was then clarified by centrifugation at 18,000 x g for 30 min. The soluble fraction was immediately incubated with a Nickel-immobilized chelating sepharose fast flow resin (cat. no. 17-0575-02, GE Healthcare, Piscataway, NJ, USA) at room temperature for 30 min with a gentle agitation. The protein-bound resin was washed out with more than 10 column volumes (CV) of a wash buffer (20 mM PBS at pH 7.4, 100 mM imidazole, 1% Triton X-100 and 500 mM NaCl). The 5HB was eluted with an elution buffer (20 mM PBS at pH 7.4, 300 mM imidazole and 500 mM NaCl). The purity of protein was assessed by SDS-PAGE and the protein was used without further purification. Protein concentration was determined by using the BCA protein Assay (cat. no. 23225, Pierce, Rockford, IL, USA). The final yield of soluble 5HB was approximately 1 mg/L of cell culture with batch-to-batch variation.

*Trx-C₄₉ cloning, expression and purification:* The gene sequence referred to as rec-Trx-C₄₉ was obtained by PCR and contains residues 476-524 of the HRB domain (Fig. 2-6A). The constructed gene was cloned into expression vector pET-32a at the HindIII-XhoI restriction site. The resulting plasmid tagged by Thioredoxin was transformed into *E.coli* BL21 (DE3) for protein expression. Thioredoxin is known to enhance the solubility and allow the high level production of small peptides. The cells were grown in LB media to an optical density (at 600 nm) of 0.8 before induction with IPTG (1.0 mM) for 3 hrs at 37 °C. Bacterial cells were harvested and then resuspended in PBS and subsequently frozen at –80 °C until use. Thawed
resuspensions were lysed with addition of lysis buffer and centrifuged to separate the soluble fraction from insoluble fraction. The soluble protein was incubated with a Talon resin (Clontech, Mountain View, CA, USA) at room temperature for 1 hr. Protein was eluted in 20 mM PBS at pH 7.4 containing 500 mM NaCl, and 100 mM imidazole. The purity of protein was judged by SDS-PAGE and protein was used without further purification.

**ELISA experiment**\(^{39,40}\)

*Non-competitive ELISA:* The wells of a microtiterplate were coated with the 5HB overnight at 4 °C. After washing wells out with TBS-T and blocking with 5% milk in TBS-T for 1 - 2 hrs at 4 °C, serially diluted Trx-C\(_{49}\) was added into each well followed by 2 hrs of the incubation at room temperature with agitation. Unbound Trx-C\(_{49}\) was then washed away and a Trx-C\(_{49}\) specific anti-Stag antibody conjugated to alkaline phosphatase (AP) was added and the plate was incubated for 1 hr at room temperature. After the addition of the AP substrate, p-nitrophenyl phosphate (pNPP), absorbance at 405 nm was measured.

*Competitive ELISA:* The wells of a microtiterplate were coated with the 5HB followed by the incubation with sample solution containing 4 nM of Trx-C\(_{49}\) and increasing concentrations of Fl-C\(_{35}\). Unbound Trx-C\(_{49}\) was then rinsed off and a Trx-C\(_{49}\) specific anti-Stag antibody conjugated to AP was added. Subsequent steps are the same as described above.
Circular Dichroism spectroscopy

CD spectra were obtained with a Jasco J-815 spectrophotometer (JASCO, Easton, MD, USA). Sample was prepared in 20 mM PBS, pH 7.4 in concentration of 35 μM. Data were recorded from 195 to 260 nm with a scanning speed of 20 nm/min and a bandwidth at 1.0 nm in a 0.1 cm path-length quartz cell. Each CD spectrum was an average of 3 measurements and corrected for buffer blank obtained under identical conditions. The resulting data was converted to per-residue molar ellipticity units, [θ] (deg cm² dmol⁻¹ residue⁻¹), and the secondary structure content was analyzed with the Dichroweb software package. The thermal stability of 5HB was monitored by measuring its molar ellipticity between 0 °C and 85 °C at 222 nm. 2.5 μM of 5HB was used for this study. The rate of temperature change was 2.5 °C/min with a scanning speed of 50 nm/min and a bandwidth of 1.0 nm.

Peptide synthesis

Peptide synthesis reagents were purchased from Applied Biosystems (Foster city, CA, USA) or Sigma-Aldrich (Milwaukee, WI, USA). Resins and Fmoc-protected amino acids were purchased from NovaBioChem (San Diego, CA, USA) or Anaspec (San Jose, CA, USA). Solvents for HPLC were purchased from Fisher Scientific (Pittsburgh, PA, USA). All chemicals were used without additional purification. Fluorescently-labeled peptide (Fl-C₁₅) and truncated peptides (C₃₀ and C₃₅) of 95% purity were commercially obtained (EZBiolab, Carmel, IN, USA and Bio Basic, Markham, ON, Canada) and used without further purification. The remaining truncated peptides (C₂₀, C₁₇ and N₁₅) were synthesized in the laboratory using standard
Fmoc chemistry on solid support (preloaded Wang resin, Novabiochem, San Diego, CA, USA) with an ABI 433A automated peptide synthesizer (Applied Biosystems, Foster city, CA, USA). After synthesis, the peptides were cleaved from the resin and deprotected in trifluoroacetic acid (TFA)/water/triisopropylsilane (TIPS)/thionisole (90:5:2.5:2.5 v/v) for 1.5 hr at room temperature. Peptides were purified by preparation RP-HPLC on a C18 column using a linear gradient of 5-99% solvent B in solvent A over 60 min (solvent A is 0.1% (v/v) TFA in water and solvent B is 0.1% (v/v) TFA in acetonitrile). Final purities of synthetic peptides were confirmed to be >95% by analytical RP-HPLC and the molecular weight of the purified product was confirmed by electrospray mass spectrometry (ESI) at the Stanford University Mass Spectrometry (SUMS) facility.

**FP measurements**

FP measurements were performed using a Synergy4 (Biotek, Winooski, VT, USA) plate reader with a tungsten lamp as a light source with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Fluorescently labeled 35 aa peptide, Fl-C_{35}, was chosen as a tracer due to its inhibitory potency (EC_{50} of 0.051 μM).\(^6\) Lyophilized Fl-C_{35} was dissolved in 20 mM PBS (at pH 7.4) and subsequent dilutions were carried out in FP buffer (20 mM PBS at pH 7.4, 500 mM NaCl, 0.01% (v/v) Tween-20, and 0.05 mg/ml bovine gamma globulin [BGG]). Specific control groups included free Fl-C_{35} (Fl-C_{35} in the absence of 5HB, negative control), bound Fl-C_{35} (Fl-C_{35} in the presence of 5HB, positive control), and FP buffer for every measurement, allowing accurate estimation of specific polarization.
**Saturation Binding FP assays:** The saturation binding experiments of Fl-C\textsubscript{35} to 5HB were performed under the following condition: each well in a black 96-well plate (Corning Inc. Lowell, MA, USA) contained a final concentration of 5 nM of Fl-C\textsubscript{35} (tracer) and increasing concentrations ranging from 0 to 500 nM of 5HB in a final volume of 185 µL in FP buffer. The polarization in millipolarization units (mP) was measured after 1 hr incubation at room temperature. Data obtained were analyzed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) to calculate a binding dissociation constant (K\textsubscript{d}) by fitting the experimental data using a one-site specific binding model. Experiments were performed in duplicate.

**Competitive FP binding assays:** Each well in a black 96-well plate (Corning Inc. Lowell, MA, USA) contained 20 nM of 5HB and increasing concentrations (0.001 to 200 µM) of each truncated peptide in FP buffer in a final volume of 185 µL. After 1hr incubation at room temperature, Fl-C\textsubscript{35} was added to 5 nM followed by 30 min incubation at room temperature. The FP response was measured in duplicate with controls including free Fl-C\textsubscript{35}, bound Fl-C\textsubscript{35}, and FP buffer. All experimental data were plotted using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). The percentage of inhibition (% Inhibition) was calculated using the following equation:

\[
\% \text{ Inhibition} = 100 \times \left[\frac{(mP-mP_f)}{mP_b-mP_f}\right]
\]

where mP\textsubscript{f} is the millipolarization of the free Fl-C\textsubscript{35} control, mP\textsubscript{b} is the millipolarization of the bound Fl-C\textsubscript{35} control and mP is the millipolarization of the bound inhibitor to the 5HB.
References


Chapter Three

NMEGylation: A novel modification to enhance the bioavailability of therapeutic peptides*

Limitations in using synthetic peptides as therapeutics

With the monumental advances in biotechnology over the past few decades, peptides and proteins have become key players in the drug market as therapeutic candidates with undeniably high specificity and low toxicity compared to conventional synthetic small molecule drugs, resulting in more than 60 biotherapeutics with sales reaching US $40 billion.\(^1\,^2\) However, several unfavorable biophysical properties still remain as challenges for clinical uses and manufacturing of peptide/protein-based medicines. One of the major obstacles for the peptide-based therapeutics is that they are extremely susceptible to proteolytic degradation, resulting in rapid renal clearance with short in vivo circulation half-lives (2 – 5 min). Efforts have been made to increase the half-lives of biotherapeutics without sacrificing their efficacy, but these efforts have yielded only a few notable successes.\(^3\,^8\)

PEGylation and its pressing challenges

PEGylation, the conjugation of PEG (poly-ethylene glycol) chains to druggable materials, has been intensively studied, clinically proven and is an acceptable method for modifying peptide/protein-based medicines to increase their

* This chapter is adapted from the following publication:
Park, M. *et al.* (2011) “NMEGylation: A novel modification to enhance the bioavailability of therapeutic peptides” Biopolymers (Peptide Science). *Accepted*
protease resistance. The decrease in proteolysis reduces renal clearance rates and enhances therapeutic efficacy. PEGylation is known to improve physical and thermal stability as well as solubility of biopharmaceuticals, which makes PEGylation suitable for drug delivery and formulation.⁹-¹² Since Adagen® (PEGylated adenosine deaminase, Enzon) was introduced as the first FDA-approved PEGylated therapeutic agent in 1990, several PEGylated protein-based drugs have reached the market, including PEGasys® (PEGylated α-interferons for Hepatitis C treatment, Hoffmann/Roche) and Cimzia® (PEGylated Anti-TNF Fab for rheumatoid arthritis and Crohn’s disease, UBC) (Table 3-1).⁹,¹³

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Target diseases</th>
<th>Target protein</th>
<th>Year to market</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adagen®</td>
<td>Enzon</td>
<td>Severe combined immunodeficiency</td>
<td>Adenosine deamidase</td>
<td>1990</td>
</tr>
<tr>
<td>Oncaspar®</td>
<td>Enzon</td>
<td>Acute lymphoblastic leukemia</td>
<td>Asparaginase</td>
<td>1994</td>
</tr>
<tr>
<td>Doxil®</td>
<td>Novartis</td>
<td>Kaposi’s sarcoma and ovarian cancer</td>
<td>PEGylated liposomal doxorubicin</td>
<td>1995</td>
</tr>
<tr>
<td>PEGasys®</td>
<td>Hoffmann/Roche</td>
<td>Hepatitis C</td>
<td>IFNα-α2a</td>
<td>2002</td>
</tr>
<tr>
<td>Neulasta®</td>
<td>Amgen/Nektar</td>
<td>Neutropenia</td>
<td>G-CSFb</td>
<td>2002</td>
</tr>
<tr>
<td>Macugen®</td>
<td>Pfizer</td>
<td>Ocular vascular disease</td>
<td>Anti-VEGFc aptamer</td>
<td>2004</td>
</tr>
<tr>
<td>Cimizia®</td>
<td>UCB</td>
<td>Rheumatoid arthritis and Crohn’s disease</td>
<td>Anti TNFd Fab</td>
<td>2008</td>
</tr>
</tbody>
</table>

⁹IFN = Interferon  
¹⁰G-CSF = Granulocyte colony-stimulating factor  
¹¹VEGF = Vascular endothelial growth factor  
¹²TNF = Tumor necrosis factor
PEGylation, however, still presents challenges such as the heterogeneity (polydispersity) of the PEG polymers and as a result, the mixture of PEGylated products has become an issue in clinically using PEGylated drugs.\textsuperscript{14} To avoid variable extent in PEGylation, PEGylation requires a complicated and multi-step manufacturing process, which leads to higher costs.\textsuperscript{15} In addition to many different approaches to improve specificity and homogeneity in PEGylation,\textsuperscript{16,17} efforts to develop alternatives to PEG are also being pursued, resulting in currently limited but promising outcomes.\textsuperscript{18-20}

\textit{A novel peptide/protein modification: NMEGylation}

\textit{N}-methoxyethylglycine (NMEG) is a hydrophilic peptoid monomer and has a similar chemical moiety (ethylene oxide unit) to that of PEG (Fig. 3-1). Peptoids are peptidomimetics based on a peptide backbone that can resist proteolytic degradation due to the peptoid side chains being attached to the backbone nitrogen instead of the \( \alpha \)-carbon (Fig. 3-2).\textsuperscript{21} Over the past few years peptoids have been widely used in many biological research areas including gene delivery,\textsuperscript{22} drug delivery,\textsuperscript{23} and ligand design with improved binding affinity.\textsuperscript{24-26} Any primary amine can be easily incorporated into the peptoid backbone using a straightforward submonomer approach, which has

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure3_1.png}
\caption{Structure of (A) \textit{N}-methoxyethylglycine (NMEG) and (B) poly-ethylene glycol (PEG)}
\end{figure}
enabled the use of a diverse set of side chains in the peptoid research (Fig. 3-2).\(^{27}\)

Previously the Barron group has reported that oligoNMEG (n = 10, 20 and 30) can serve as (1) antifouling agents, which prevent non-specific protein binding to surfaces\(^{28}\) and (2) friction-generating moieties for solution-based DNA separations.\(^{29}\) Even though NMEGylation, a covalent attachment of oligoNMEG, should provide protease resistance to peptide/protein drugs,\(^{30,31}\) this possibility has not yet been investigated. Thus, we explored NMEG as a promising PEG-like material in this study. We previously identified a C\(_{20}\) peptide as a potential antiviral agent using a protein-based screening assay as a part of an effort to identify potential viral entry inhibitors targeting human respiratory syncytial virus (hRSV) infection (see Chapter \textbf{2}).\(^{32}\) The C\(_{20}\) peptide showed a low micromolar binding affinity to the 5HB; however, the solubility and stability of this short peptide are not ideal. Therefore, using the C\(_{20}\) peptide as a parent peptide, a series of NMEGylated C\(_{20}\) analogs were prepared with different numbers of NMEG monomers and their biophysical properties and biological activities were examined. We believe these results may open a new opportunity to use peptoids as a modification method for biotherapeutics with broad applications in biology and medicine.
Figure 3-2. Synthetic approaches to prepare peptoids, peptides and peptomers (R = peptoid side chain, R’ = peptide side chain, Prt = protecting group, DIC = N,N'-diisopropyl carbodiimide, DMF = dimethylformamide, NMP = methylpyrrolidone, SPPS = Solid phase peptide synthesis)
Synthesis, purification and characterization of NMEGylated C\textsubscript{20} peptides

The C\textsubscript{20} peptide was previously identified from peptide truncation studies showing a low micromolar binding affinity to a 5-Helix Bundle (5HB), a genetically engineered protein construct derived from hRSV fusion protein F.\textsuperscript{32} however, because of its relatively short length and unstructured conformation,\textsuperscript{33-35} the C\textsubscript{20} peptide has a high susceptibility to proteases and poor solubility in aqueous solution. Although PEGylation may improve the desirable biophysical features of the C\textsubscript{20} peptide, PEGylation was not pursued because the large increase in molecular weight of the PEGylated C\textsubscript{20} products and potential steric shielding might impede the binding of C\textsubscript{20} to the 5HB. Thus we decided to explore NMEG as a potential alternative to PEG, because NMEG is hydrophilic like PEG but monodisperse compared with many other commercially available PEG derivatives. Importantly, because peptoids (e.g., NMEG) cannot be recognized by proteases, we anticipated that the resulting peptoid-peptide hybrids (peptomers) formed using NMEGylation would be less susceptible to proteolytic degradation. As shown in Table 3-2, a series of NMEGylated C\textsubscript{20} peptides were prepared by attaching NMEG oligomers (n = 1 – 10) at either the N- or C-terminus of C\textsubscript{20}. The resulting NMEGylated peptides were extremely soluble in aqueous buffer (up to > 10 mg/mL) compared to C\textsubscript{20} (< 2 mg/mL). The relative hydrophilicity of NMEGylated C\textsubscript{20} analogs based on percent acetonitrile at elution was evaluated by RP-HPLC (Table 3-2).\textsuperscript{36,37} The decrease in percent acetonitrile of at elution of NMEGylated analogs demonstrates that NMEGylation dramatically enhances the hydrophilicity of the C\textsubscript{20} peptide, notably, even with very short NMEGylation (n = 1).
### Table 3.2: NMEGylation of C28 peptide sequences tested in this study with molecular weight (MW), purity, solubility, hydrophilicity and their IC$_{50}$ values

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Sequences (amino to carboxy)</th>
<th>Molar mass (Da)</th>
<th>Purity (%)</th>
<th>% AcN at elution$^a$</th>
<th>IC$_{50}$$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{28}$</td>
<td>ISQVNEKINWILAFIRKSDE</td>
<td>2319.6/2319.0</td>
<td>&gt; 95</td>
<td>78</td>
<td>35 ± 1</td>
</tr>
<tr>
<td>NMEG$<em>3$-$C</em>{28}$</td>
<td>NMEG$_3$-ISQVNEKINWILAFIRKSDE</td>
<td>2664.9/2664.4</td>
<td>&gt; 95</td>
<td>60</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>NMEG$<em>{10}$-$C</em>{28}$</td>
<td>NMEG$_{10}$-ISQVNEKINWILAFIRKSDE</td>
<td>3470.3/3471.8</td>
<td>&gt; 95</td>
<td>57</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>$C_{28}$-NMEG$_{10}$</td>
<td>ISQVNEKINWILAFIRKSDE-NMEG$_{10}$</td>
<td>3468.9/3469.8</td>
<td>&gt; 95</td>
<td>57</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>$C_{28}$-NMEG$_3$</td>
<td>ISQVNEKINWILAFIRKSDE-NMEG$_3$</td>
<td>2894.2/2894.1</td>
<td>&gt; 95</td>
<td>54</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>$C_{28}$-NMEG$_5$</td>
<td>ISQVNEKINWILAFIRKSDE-NMEG$_5$</td>
<td>2663.9/2663.7</td>
<td>&gt; 95</td>
<td>52</td>
<td>&gt; 200</td>
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<tr>
<td>NMEG$<em>1$-$C</em>{28}$</td>
<td>NMEG$_1$-ISQVNEKINWILAFIRKSDE</td>
<td>2434.7/2334.0</td>
<td>&gt; 95</td>
<td>52</td>
<td>&gt; 200</td>
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<tr>
<td>$C_{28}$-NMEG$_1$</td>
<td>ISQVNEKINWILAFIRKSDE-NMEG$_1$</td>
<td>2433.7/2432.9</td>
<td>&gt; 95</td>
<td>52</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>NMEG$<em>{10}$-$C</em>{28}$-NMEG$_1$</td>
<td>NMEG$_{10}$-ISQVNEKINWILAFIRKSDE-NMEG$_1$</td>
<td>2548.8/2547.9</td>
<td>&gt; 95</td>
<td>55</td>
<td>&gt; 150</td>
</tr>
<tr>
<td>NMEG$<em>{1}$-Gly-$C</em>{28}$</td>
<td>NMEG$_{1}$-G-ISQVNEKINWILAFIRKSDE</td>
<td>2491.7/2491.1</td>
<td>&gt; 90</td>
<td>55</td>
<td>81 ± 2</td>
</tr>
</tbody>
</table>

$^a$ % AcN = percent acetonitrile at RP-HPLC elution of each compound.

$^b$ The mean of IC$_{50}$ with standard error in μM.
Biological activity of NMEGylated C_{20} peptides

Despite greatly increased hydrophilicity and solubility, NMEGylation of C_{20} with more NMEG monomers (n = 3, 5, and 10) was accompanied by significant loss of binding affinity to the target protein, 5HB, as evaluated using our previously reported protein-based fluorescence polarization (FP) assay (Table 3-2).^{32} Since the binding of C_{20} to the 5HB involves a large interface, steric hindrance caused by the attached NMEG oligomers might be occurring, suggesting the need for optimizing the number of NMEG oligomers. Therefore, NMEGylated peptides with only one NMEG at either or both termini of the C_{20} peptide were prepared, and their biophysical properties and biological activities against the 5HB construct were evaluated. Although addition of even a single NMEG to C_{20} greatly improves solubility and hydrophilicity, binding affinity is still significantly diminished (Fig. 3-3, Table 3-2).

![Graph](image-url)

**Figure 3-3. Resulting data from competitive FP assays** Binding of NMEGylated C_{20} peptomers to the 5HB was determined using FP. % Inhibition represents the displacement capability of NMEGylated peptomers over a tracer (Fl-C_{35}) to the target protein (5HB) and is presented as a function of the concentration of NMEGylated C_{20} peptides.
To recover the biological activity of NMEGylated C\textsubscript{20}, we decided to use an NMEG bound through a flexible linker to allow the C\textsubscript{20} enough space to properly form \(\alpha\)-helical conformation upon binding to the 5HB. On the other hand, a linker that is too flexible would lose too much entropy upon binding and consequently deleteriously affect binding affinity. Glycine was chosen as the linker because we anticipated that glycine would be flexible but would not disrupt the peptide backbone structure. To determine the number of glycines that maximizes C\textsubscript{20} binding to the 5HB, \(N\)-terminally NMEGylated C\textsubscript{20} (\(n = 1\)) with different numbers of glycine residues (\(n = 0, 1, 2, \) and 3) were synthesized and the binding affinity of each peptomer was examined (Fig. 3-4).

![Graph](image)

**Figure 3-4. Optimization of the length of the glycine linker** (A) Binding NMEGylated C\textsubscript{20} peptomers with different numbers of glycines to the 5HB were determined using competitive FP assay. (B) Comparison of 5HB•FITC-C\textsubscript{35} bound in the presence of peptomers (100 \(\mu\text{M}\)) with a different number of glycines residues as linkers. The binding affinity of the parent C\textsubscript{20} is shown in blue for ease of comparison.

NMEG-glycine-C\textsubscript{20} (NMEGGC\textsubscript{20}) showed significantly tighter binding affinity to the 5HB than the rest of the NMEGylated C\textsubscript{20} analogs with greatly improved solubility and hydrophilicity (Table 3-2, Fig. 3-4A). Because glycine is known to be a
helix breaker,\textsuperscript{38,39} it is possible that longer glycine linkers might impede the binding affinities of peptomers to the 5HB by increasing the flexibility of the peptide backbone structure (Fig. 3-4B). The benefits of NMEGuylation of potential biotherapeutics should increase when the NMEG attachment points directly away from the likely binding interface. Since the C\textsubscript{20} peptide likely forms an \( \alpha \)-helix upon binding to the 5HB,\textsuperscript{40} a direct attachment of peptoid residues may disrupt the \( \alpha \)-helix formation due to the lack of structural rigidity.\textsuperscript{41} The secondary structural analysis of the NMEGylated peptomers will be discussed later in this chapter.

**Serum stability of NMEGylated C\textsubscript{20} peptides**

As discussed earlier, PEGylation is clinically proven to extend the half-life of biotherapeutics, thereby improving their efficacy. We therefore examined whether NMEGuylation can provide a similar beneficial effect, focusing on singly-NMEGylated peptomers with greatly improved aqueous solubility. NMEGylated C\textsubscript{20} peptides were incubated with human serum, which contains many proteases (e.g., trypsin and elastase), at room temperature and then analyzed the mixture by RP-HPLC to determine the amount of remaining intact peptomers. Our resulting data (Fig. 3-5) indicate that NMEGuylation indeed enhanced the serum stability of NMEGylated peptides compared to the parent peptide, C\textsubscript{20}. Interestingly, the protease stability of NMEGGC\textsubscript{20} was the most greatly improved, suggesting that despite a slight loss of binding affinities, NMEGGC\textsubscript{20} could present comparable therapeutic efficacy to the C\textsubscript{20} peptide due to a prolonged half-life.
Figure 3-5. Peptide stability in presence of serum  NMEGylated peptides were incubated with human plasma at 37 °C and sampled at the indicated time points, followed by RP-HPLC analysis. The amount of remaining peptomer at each time point was quantified against an enzyme-stable internal organic standard (benzyl alcohol).

Secondary structure analysis of NMEGylated C_{20} peptides in different solvent systems

As mentioned earlier in this chapter, the C_{20} peptomers with longer NMEG chains showed diminished binding to the 5HB, although they possessed greatly enhanced solubility and hydrophilicity compared to the parent peptide, C_{20}. To better understand how NMEGylation influences the secondary structures of peptomers, CD spectra of NMEGylated peptomers were monitored in different solvent systems such as aqueous solution (e.g., Tris) or mixtures of aqueous and organic solvents (e.g., 50% acetonitrile in Tris). Per residue molar ellipticity (Θ, deg·cm^{2}·dmol^{-1}) of NMEGylated C_{20} analogs (100 μM) was measured at 222 nm, and the results of these studies
suggest that the longer the NMEG chain, the more irregular the secondary structure (Fig. 3-6). Attachment of one NMEG does not perturb the secondary structure of the C<sub>20</sub> peptide, whereas longer oligoNMEG (n > 3) have a significantly greater impact on the C<sub>20</sub> structure. These observations provided useful information to correlate the probable structures of these peptomers with their binding affinities to the 5HB.

![Figure 3-6. Comparison of helical propensity of NMEGylated C<sub>20</sub> peptomers with C<sub>20</sub> peptide at 222 nm](image)

100 µM of each compound was prepared in 10 mM TBS containing 50 mM NaCl at pH 7.4 and CD spectra at 222 nm were obtained at room temperature.

We also investigated the solvent polarity dependency of NMEGylated C<sub>20</sub> in different solvent systems, focusing on C<sub>20</sub>NMEG<sub>3</sub> as intermediate in length of NMEGylation and secondary structure. Due to poor aqueous solubility, stock solution of the C<sub>20</sub> peptide were prepared in organic solvent (e.g., acetonitrile) and then diluted in an aqueous solution (e.g. Tris) to obtain the desired concentrations. Since acetonitrile is less polar than water, we varied the concentration of acetonitrile in Tris buffer to alter the polarity of the solvent mixture. The resulting CD spectra of C<sub>20</sub>NMEG<sub>3</sub> in Fig. 3-7 indicate that as the concentration of acetonitrile decreased, the
NMEGylated peptide tends to show an increase in the intensity of the very strong band at 200 nm band and a decreased intensity in the band at 222 nm, consistent with possible loss of a regular secondary structure. Additionally, the spectra were shifted to shorter wavelengths (blue shift) when the solvent polarity increased, suggesting the destabilization of structured conformations.\textsuperscript{42}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3-7.png}
\caption{Solvent polarity dependency in CD spectra of 100 \textmu{M} of C\textsubscript{20}NMEG\textsubscript{3} The polarity of the solvent mixture is increased from 0 to 75\% acetonitrile, which are indicated in dotted black, solid gray, and black lines. Blue arrow indicates a blue shift of 200 nm-negative band and red arrow represents a decrease in the intensity of 222 nm-negative band.}
\end{figure}

To examine the possibility of aggregation of the NMEGylated peptides that might lead to unexpected structure formation, the concentration dependence of the secondary structure of C\textsubscript{20}NMEG\textsubscript{3} was tested. However, neither concentration-dependent structural changes nor signs of aggregation were observed (Fig. 3-8), suggesting that the NMEGylated peptides are present in monomeric forms and that
the secondary structure of NMEGylated C\textsubscript{20} peptomers might be governed primarily by the solvent polarity rather than by undesirable aggregation.

Figure 3-8. CD spectra of different concentrations of C\textsubscript{20}Nmeg\textsubscript{3} ranging from 10 to 200 µM in various solvent systems (A) 0%, (B) 50%, and 75% acetonitrile in Tris buffer
Chapter Three: NMEGylation as a potential method for enhancing the bioavailability of peptide-based therapeutics

Conclusions and future prospects

In this study, we demonstrate that NMEGylation enhances multiple therapeutically desired properties of peptides including the solubility, hydrophilicity and serum stability. Our data also suggest that modifying the molecular weight of the target peptide, C20, by less than 5% with NMEGylation is sufficient to achieve favorable biophysical properties. Furthermore, optimizing the length of the NMEG and glycine linkers enabled the NMEGylated peptomer to greatly recover the binding affinity to its biological target. While PEGylation is still a useful technique for large proteins, it requires post-synthetic conjugation and multiple purification steps to obtain the desired PEGylated products.\textsuperscript{13} In contrast, NMEGylation can be used to modify short therapeutic peptides as well as proteins, quickly yielding highly monodisperse products at lower costs. Since NMEGylation is intrinsically compatible with solid phase peptide/peptoid synthesis and offers both low-cost, short synthesis times, and site-specific incorporation as part of the synthesis protocols, NMEGylated peptide/peptoid libraries could be designed at an early stage of molecular optimization by varying both the number and position of NMEG monomers and linker units. Therefore, NMEGylation may prove a new, broadly useful method of peptoids for future modification of therapeutically attractive peptides and proteins.
Methods and Materials

General materials

Reagents for peptide and peptomer synthesis were purchased from Applied Biosystems (Foster city, CA, USA) or Sigma-Aldrich (Milwaukee, WI, USA). Resins and Fmoc-protected amino acids were purchased from NovaBioChem (San Diego, CA, USA) or Anaspec (San Jose, CA, USA). Solvents for analytical and preparative RP-HPLC were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used without additional purification.

NMEGylated peptomers and C20 peptide synthesis

The C20 and its NMEGylated analogs were synthesized in the laboratory using standard Fmoc chemistry and a submonomer peptoid synthesis27 (Fig. 3-2) with an automated ABI 433A peptide synthesizer (Applied Biosystems, Foster city, CA, USA). After synthesis, the peptides were cleaved off the resin and deprotected in trifluoroacetic acid (TFA)/water/triisopropylsilane/thionisole (90:5:2.5:2.5 v/v) for 1.5 hr at room temperature with agitation. Peptide and peptomers were purified by preparative RP-HPLC on a C18 column using a linear gradient of 5-99% solvent B in solvent A over 60 min (solvent A is 0.1% (v/v) TFA in water and solvent B is 0.1% (v/v) TFA in acetonitrile). Final purities of the synthetic peptides were confirmed to be > 95% by analytical RP-HPLC, and the molecular weight of the purified product was confirmed by electrospay mass spectrometry (ESI) at the Stanford University Mass Spectrometry facility.
Selected structure of NMEGylated peptomers (A; C_{20}, B; NmgG_{C_{20}}, C; NMEG_{3}C_{20}, and D; C_{20}NMEG) (C_{20} peptide in black, glycine spacer in blue and NMEG in red)
Chapter Three: NMEGylation as a potential method for enhancing the bioavailability of peptide-based therapeutics

Selected HPLC traces of NMEGylated peptomers (A; C20, B; NmgGC20, C; NMEG3C20, and D; C20NMEG3)
**Percent acetonitrile measurement**

The relative hydrophilicity (percent acetonitrile at elution) of NMEGylated peptides was compared by analytical RP-HPLC, previously reported as a reliable method for determining the contribution of the hydrophilicity of amino acids to the retention time of peptides.\(^{36,37}\) The purified C\(_{20}\) and its NMEGylated analogs were analyzed using RP-HPLC on a Phenomenex (Torrance, CA, USA) C\(_{18}\) column (250 mm x 2.00 mm) with 5 \(\mu\)m resin size using a linear gradient of 5-95% solvent B over 30 or 60 min (solvent A is water with 0.1% (v/v) TFA and solvent B is acetonitrile with 0.1% (v/v) TFA).

**Serum stability assay\(^{43}\)**

Peptides (~ 0.4 mg) were dissolved in 20 mM PBS at pH 7.6 (400 \(\mu\)L containing 0.1% v/v benzyl alcohol as internal standard). Equal volumes of 50% human serum plasma (Sigma-Aldrich, Milwaukee, WI, USA) and peptides solutions were combined and incubated at 37°C. Samples were taken in triplicate (3 \(\times\) 50 \(\mu\)L) at 0, 30, and 240 min and placed on ice. Each sample (including the t = 0 time point) was prepared for HPLC analysis in the following way. First, 20 \(\mu\)L of 0.5 M lysine monohydrochloride was added, followed by 65 \(\mu\)L of acetonitrile. The samples were then cooled on ice for 10 min and subsequently centrifuged for 10 min at 3000 rpm. A 50 \(\mu\)L aliquot of the resulting samples was taken from the supernatant, diluted with 100 \(\mu\)L of water and analyzed by analytical RP-HPLC. The ratio of the disappearing peptomter peak area relative to the benzyl alcohol peak area was calculated for each time point and
normalized against the t = 0 time point (representing 100% peptide remaining) as a percentage of the peptide remaining.

**Competitive fluorescence polarization (FP) binding assay**

Competitive FP binding assays of NMEGylated C20 peptomers were carried out as previously described.\(^3\) Briefly, using a 5HB protein construct and a fluorescently labeled 35 amino acid-long peptide (Fl-C\(_{35}\): YDPLVFPSDEFDASISQVEKINQSL AFIRKSDDEL) as a target and a tracer respectively, the binding affinities of NMEGylated peptomers were individually evaluated. Both the C\(_{20}\) and C\(_{35}\) peptide sequences are derived from HRB domain of hRSV F protein and mimic the 6-helix bundle formation of hRSV F protein by occupying the binding site on the 5HB. The binding of Fl-C\(_{35}\) peptide to the 5HB in absence of peptomers provides 100% 5HB-Fl-C\(_{35}\) bound as a positive control. Each well in a black 96-well plate (Corning Inc. Lowell, MA, USA) contained 20 nM of the 5HB and increasing concentrations of each NMEGylated peptomer in FP buffer (20 mM PBS at pH 7.4, 500 mM NaCl, 0.01% (v/v) Tween-20, and 0.05 mg/ml bovine gamma globulin) in a final volume of 185 µL with 1 hr incubation at room temperature. Fl-C\(_{35}\) was then added to a final concentration of 5 nM followed by 30 min incubation at room temperature. The FP responses were monitored using a Synergy4 plate reader (Biotek, Winooski, VT, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All experimental data were obtained in duplicate and the percentage of inhibition (% Inhibition) was calculated using the following equation: % Inhibition = 100×([mP-
mPf)/(mPb-mPf)], where unbound Fl-C35 (mPf), bound Fl-C35 (mPb) and the bound inhibitor to the 5HB (mP) are used accordingly.

**Secondary structural analysis by CD spectroscopy**

CD spectra were obtained with a Jasco J-815 spectrophotometer (JASCO, Easton, MD, USA). Sample was prepared in 10 mM Tris containing 50 mM NaCl (pH 7.4) in concentrations ranging from 10 – 200 µM. In some cases, 50 – 75% of acetonitrile was used to test solvent effect on the secondary structures of NMEGylated peptomers. Data were recorded from 195 to 260 nm with a step size of 0.2 nm, at a rate of 100 nm/min, a bandwidth of 1.0 nm with a scanning speed of 20 nm/min in a 0.1 cm path-length quartz cell. The response time was 0.5 s. Each CD spectrum was an average of 3 measurements and the baseline was corrected by subtracting the spectrum of a buffer blank obtained under identical conditions. The resulting data was converted to per-residue molar ellipticity units, [θ] (deg cm² dmol⁻¹ residue⁻¹).
References


Chapter Four

Knowledge-based approaches for designing peptoid-peptide hybrids and their structurally constrained analogues

Background and motivation for this study

As discussed in Chapter 1, synthetic peptides have recovered their reputation as therapeutics due to their undeniably advantageous features (e.g., high specificity) for medical applications; however, manufacturing synthetic peptides is still hindered by high production costs due to the laborious chemical synthesis. Additional challenges for broader clinical use of therapeutic synthetic peptides are their poor biophysical properties including rapid renal clearance, low cell-permeability, variable solubility, high immunogenicity, and limited stability.\(^1\)\(^-\)\(^3\) We previously demonstrated a fluorescence polarization assay using an engineered hRSV F protein, 5-Helix Bundle (5HB), to screen potential hRSV fusion inhibitors and identified the C\(_{20}\) peptide, which has a low micromolar binding affinity to the 5HB (see Chapter 2 for details).\(^4\)

To enhance the biophysical properties of C\(_{20}\), we explored proteolytically resistant peptoids (N-substituted glycines) for designing peptide-peptoid hybrid (peptomer) hRSV inhibitors. As discussed in Chapter 1, peptoids are bioinspired polymers with side chains attached to the peptide backbone nitrogen that possess many attractive features such as altered conformations and chemical versatility.\(^5\) In Chapter 3, we tested peptoid modification to peptides by decorating the “outside” of the C\(_{20}\) peptide sequence (i.e., at N- or C-terminus).
In this chapter, we take this approach even further by incorporating peptoids directly “within” the peptide sequences. Since there are no straightforward ways to convert biologically active peptides into peptoids or peptomers with retention of biological activity, often many variants have to be synthesized, tested, and progressively optimized.\textsuperscript{6-9} This laborious and time-consuming approach has unsurprisingly led to relatively few successes in transforming therapeutically interesting peptides into peptoids.\textsuperscript{10-15} Thus, in addition to improving the biological properties of the C\textsubscript{20} peptide, we are at least equally interested in developing a broadly-applicable approach for converting biologically active peptides into peptoids and peptomers.

\textit{A combined approach of alanine, proline, and sarcosine scans}

When substituting individual residues in peptides with regular amino acids, an “alanine scan” is used to elucidate the relative importance of each side chain;\textsuperscript{16,17} a series of analogues is prepared where each amino acid is replaced in turn by alanine, which has a minimal side chain, and the impact on the activity of the peptide is assessed. In replacing peptide residues with peptidomimetics, however, the peptide backbone itself is typically altered, and thus more sophisticated approaches need to be used.

Specifically, the substitution of peptoid residues into peptides must address three criteria: first, the importance of the original amino acid and its side chain, as can be elucidated with an alanine scan; second, the importance of the amide hydrogen, which is missing in peptoids, and could be investigated using sarcosine (Fig. 4-1), the
peptoid equivalent of alanine; third, particularly if substituting peptoid residues into a helix, the tolerance of the peptide α–helix for the peptoid polyproline type I (PPI)-like helix structure needs to be assessed. Since peptoid helices form a PPI-like helix, proline itself could quite reasonably serve as a probe of the suitability of the parent helix to incorporate this structure. Whereas alanine scans are both routine and ubiquitous, there are almost no literature reports of proline and sarcosine scans, and no reports of combining these approaches.\textsuperscript{18-20}

![Structural comparison of alanine, proline, and sarcosine](image)

**Figure 4-1. Structural comparison of alanine, proline, and sarcosine**

Therefore, we used the alanine, proline and sarcosine scans together on the C\textsubscript{20} peptide, establishing a novel, comprehensive approach to identify peptoid-tolerable residues in the C\textsubscript{20} peptide. Substitution of each amino acid along the C\textsubscript{20} peptide sequence successively by alanine, proline and sarcosine was carried out, generating 60 peptides in total. After evaluating the binding affinity of these peptides to the 5HB using our competitive FP assay as discussed below, we found that several substituted peptides could maintain moderate 5HB-binding affinities. Subsequently, we incorporated peptoid residues with identical side chains to the peptide amino acid being replaced into the positions determined by the combined scans, creating peptomeric anti-hRSV fusion inhibitors.
Although one could have theoretically utilized the peptoid residues directly with the same side chains instead of sarcosine in a “peptoid scan”, we intentionally used the sarcosine scan for two reasons. First, although peptoid synthesis is entirely compatible with peptide synthesis, it is not as universally used; in contrast, sarcosine is available as Fmoc-sarcosine and could thus be immediately used in solid phase peptide synthesis protocols without modification, thereby enabling wider adoption of this scanning technique. Second, although not pursued in this study, the data from this combined approach could also serve as a starting point for a library screening approach that would investigate peptoid side chains different from that of the original amino acid being substituted and that would take advantage of the near limitless chemical diversity of peptoids.

We believe that the combination of the alanine and sarcosine scans in particular could become a universal approach for preparing peptoid and peptomer therapeutics. Furthermore, our novel comprehensive approach could be applied to other peptidomimetic systems with altered peptide backbones, such as by combining alanine and β–alanine to generate β–alanine containing therapeutic peptides, or by combining multiple such assays, e.g., alanine, sarcosine, and β–alanine scans to determine the best peptidomimetic substitutions for any position in a therapeutic peptide.
Chapter Four: Knowledge-based approaches to design peptoid-peptide hybrids and their structurally constrained analogues

Alanine substitution study

To evaluate the importance of the side chains in the interaction of the C20 peptide with the 5HB, 20 alanine-substituted peptides were synthetically prepared (Table 4-1) and the binding affinity of each peptide to the 5HB was examined at the concentrations of 100 and 200 µM using the 5HB-based competitive FP assay. The results were subsequently converted to % inhibition of Fl-C35 binding to the 5HB, which is summarized in Table 4-1 and Fig. 4-2.

Table 4-1. Sequences of alanine-substituted peptides tested in this study and their inhibitory effect on binding of Fl-C35 to the 5HB at 200 µM

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences (amino to carboxy)</th>
<th>MW (Da)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20</td>
<td>ISQVNEKINQSLAFIKSDE</td>
<td>2319.6</td>
<td>100</td>
</tr>
<tr>
<td>A1</td>
<td>ASQVNEKINQSLAFIKSDE</td>
<td>2277.5</td>
<td>16</td>
</tr>
<tr>
<td>A2</td>
<td>IAQVNEKINQSLAFIKSDE</td>
<td>2304.6</td>
<td>24</td>
</tr>
<tr>
<td>A3</td>
<td>ISAVNEKINQSLAFIKSDE</td>
<td>2263.5</td>
<td>27</td>
</tr>
<tr>
<td>A4</td>
<td>ISQANEKINQSLAFIKSDE</td>
<td>2291.8</td>
<td>40</td>
</tr>
<tr>
<td>A5</td>
<td>ISQVAEKINQSLAFIKSDE</td>
<td>2277.6</td>
<td>26</td>
</tr>
<tr>
<td>A6</td>
<td>ISQVNAEKINQSLAFIKSDE</td>
<td>2261.6</td>
<td>89</td>
</tr>
<tr>
<td>A7</td>
<td>ISQVNEAINTQSLAFIKSDE</td>
<td>2262.5</td>
<td>100</td>
</tr>
<tr>
<td>A8</td>
<td>ISQVNEKANQSLAFIKSDE</td>
<td>2277.5</td>
<td>14</td>
</tr>
<tr>
<td>A9</td>
<td>ISQVNEKIAQSLAFIKSDE</td>
<td>2276.6</td>
<td>31</td>
</tr>
<tr>
<td>A10</td>
<td>ISQVNEKINASLAFIKSDE</td>
<td>2262.6</td>
<td>11</td>
</tr>
<tr>
<td>A11</td>
<td>ISQVNEKINQNALAFIKSDE</td>
<td>2303.6</td>
<td>33</td>
</tr>
<tr>
<td>A12</td>
<td>ISQVNEKINQSAFIRKSDE</td>
<td>2277.5</td>
<td>44</td>
</tr>
<tr>
<td>A13</td>
<td>ISQVNEKINQSLAFIRKSDE</td>
<td>2319.6</td>
<td>100</td>
</tr>
<tr>
<td>A14</td>
<td>ISQVNEKINQSLAAIRKSDE</td>
<td>2243.5</td>
<td>25</td>
</tr>
<tr>
<td>A15</td>
<td>ISQVNEKINQSLAFARKSDE</td>
<td>2277.5</td>
<td>21</td>
</tr>
<tr>
<td>A16</td>
<td>ISQVNEKINQSLAFIAKSD</td>
<td>2234.5</td>
<td>54</td>
</tr>
<tr>
<td>A17</td>
<td>ISQVNEKINQSLAFIRASDE</td>
<td>2262.5</td>
<td>100</td>
</tr>
<tr>
<td>A18</td>
<td>ISQVNEKINQSLAFIRKADE</td>
<td>2303.6</td>
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</tr>
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<td>A19</td>
<td>ISQVNEKINQSLAFIRKSAE</td>
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<td>32</td>
</tr>
<tr>
<td>A20</td>
<td>ISQVNEKINQSLAFIRKSDA</td>
<td>2261.6</td>
<td>29</td>
</tr>
</tbody>
</table>
Alanine substitutions at Glu6, Lys7, and Lys17 that point away from the expected binding interface are the most well-tolerated, suggesting these peptide residues could be candidates for peptoid substitution. Interestingly, we observed that the further the alanine substitutions occur from the binding interface, the more well-tolerated they are (Table 4-1). These observations are consistent with the extreme stability of the helical bundle to denaturing (as discussed in Chapter 2) and suggest that the hydrophobic residues involved in the direct interaction between the C\textsubscript{20} peptide and the 5HB are critical for binding of the C\textsubscript{20} peptide to the 5HB and should not be altered.

![Figure 4-2. % Inhibition of alanine-substituted peptides at 100 and 200 \mu M.](image)

Each peptide was competed with 5 nM Fl-C\textsubscript{55} in the presence of 20 nM 5HB and its ability to displace Fl-C\textsubscript{55} was monitored by FP. The FP response was converted to % inhibition.
**Proline substitution study**

As the only naturally occurring N-substituted amino acid, proline has been recognized as natural candidate for peptoid substitution in various studies. For example, prolines in WW and SH3 domain binding peptide ligands have been replaced with various peptoid monomers leading to improved binding affinity.\textsuperscript{10,11} In addition, proline substitution with peptoid monomers in proline-containing antimicrobial peptides increased their antimicrobial activity\textsuperscript{21,22} as well as the binding affinity of plant peptide hormones.\textsuperscript{14} Inspired by these exciting findings in using proline as a prototypical peptoid, we substituted each amino acid in the C\textsubscript{20} peptide with proline one by one to identify peptoid-tolerant peptide residues. The resulting data shown in Fig. 4-3 and Table 4-2 indicate that proline substitutions greatly detract from the binding affinities of the C\textsubscript{20} peptide analogues, yielding minimal % inhibition values even at 200 μM.

**Table 4-2. Sequences of proline-substituted peptides tested in this study and their inhibitory effect on binding of Fl-C\textsubscript{35} to the 5HB at 200 μM**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences (amino to carboxy)</th>
<th>MW (Da)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>PSQVNEKINQSLAFIRKSDE</td>
<td>2303.5</td>
<td>26</td>
</tr>
<tr>
<td>P2</td>
<td>IPQVNEKINQSLAFIRKSDE</td>
<td>2329.6</td>
<td>23</td>
</tr>
<tr>
<td>P3</td>
<td>ISPVNEKINQSLAFIRKSDE</td>
<td>2288.5</td>
<td>14</td>
</tr>
<tr>
<td>P4</td>
<td>ISQPEKINQSLAFIRKSDE</td>
<td>2317.5</td>
<td>12</td>
</tr>
<tr>
<td>P5</td>
<td>ISQVPEKINQSLAFIRKSDE</td>
<td>2302.6</td>
<td>34</td>
</tr>
<tr>
<td>P6</td>
<td>ISQVPEKINQSLAFIRKSDE</td>
<td>2287.6</td>
<td>26</td>
</tr>
<tr>
<td>P7</td>
<td>ISQVNEPINQSLAFIRKSDE</td>
<td>2288.5</td>
<td>-</td>
</tr>
<tr>
<td>P8</td>
<td>ISQVNEPKNQSLAFIRKSDE</td>
<td>2303.5</td>
<td>11</td>
</tr>
<tr>
<td>P9</td>
<td>ISQVNEKIPQSLAFIRKSDE</td>
<td>2302.6</td>
<td>33</td>
</tr>
<tr>
<td>P10</td>
<td>ISQVNEKPNSLAFIRKSDE</td>
<td>2288.5</td>
<td>9</td>
</tr>
<tr>
<td>P11</td>
<td>ISQVNEKNPQSLAFIRKSDE</td>
<td>2329.6</td>
<td>9</td>
</tr>
<tr>
<td>P12</td>
<td>ISQVNEKINQPSAFIRKSDE</td>
<td>2303.5</td>
<td>22</td>
</tr>
</tbody>
</table>
P13  ISQNEKINQSLPFIRKSDE  2345.6  10
P14  ISQVNEKINQSLAPIRKSDE  2269.5  13
P15  ISQVNEKINQSLAFRKSDE  2303.5  4
P16  ISQVNEKINQSLAFPKSDE  2260.5  4
P17  ISQVNEKINQSLAFIRPSDE  2288.5  6
P18  ISQVNEKINQSLAFIRPDE  2329.6  0
P19  ISQVNEKINQSLAFIRKSPD  2301.6  84
P20  ISQVNEKINQSLAFIRKSDP  2287.6  31

Figure 4-3. % Inhibition of proline-substituted peptides at 100 and 200 μM Each peptide was competed with 5 nM Fl-C35 in the presence of 20 nM 5HB and its ability to displace Fl-C35 was monitored by FP. The FP response was converted to % inhibition.

The decreased binding affinities of proline-substituted peptides can be explained by proline’s intrinsic structure. Proline is known to induce a kink in α-helices, often leading to unusual conformations and significant flexibility in the α-helix as well as the rapid cis/trans isomerization. Despite these reasons, the PPI-like helical structure adopted by bulky α-chiral peptoid residues may have proved entropically favorable, which is why the proline scan was pursued. It should be noted
that the greater rigidity of the α-chiral peptoid residues should still be useful to improve the binding affinity of non-helical peptides, and that a proline scan would be more relevant in such systems. The findings from proline substitution studies on the C₂₀ peptide confirmed that maintaining α-helical conformation is crucial for the C₂₀ peptide to bind to the 5HB and thus peptomers with α-chiral peptoid residues were not synthesized. Notably, this is the first time that the hypothesis of proline as the representative of structure-promoting peptoids has been tested in the context of designing peptoid-based peptidomimetics.

**Sarcosine substitution study**

Sarcosine, also known as N-methylglycine, has already been used to improve the pharmacokinetic properties of biologically intriguing peptides including metabolic stability and permeability, and to test the tolerance to conformational changes in short peptides. Moreover, as the simplest peptoid monomer, sarcosine has been used to identify pharmacophores in peptoid ligands from combinatorial libraries of peptoids, in a direct parallel of the alanine scan in peptides. We thus expected that the sarcosine substitution study of the C₂₀ peptide would reveal which peptide residues can be replaced with peptoid monomers without severely impacting the binding affinity to the 5HB. Twenty sarcosine-substituted peptides were commercially obtained and their binding affinities to the 5HB were investigated using 5HB-based competitive FP assays (Table 4-3 and Fig. 4-4).
Table 4-3. Sequences of sarcosine-substituted peptides tested in this study and their inhibitory effect on binding of Fl-C35 to the 5HB at 200 μM

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequences (amino to carboxy)</th>
<th>MW (Da)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sar1</td>
<td>SarSQVNEKINQSLAFIRKSDE</td>
<td>2277.5</td>
<td>15</td>
</tr>
<tr>
<td>Sar2</td>
<td>ISarSQVNEKINQSLAFIRKSDE</td>
<td>2303.6</td>
<td>22</td>
</tr>
<tr>
<td>Sar3</td>
<td>ISSarQVNEKINQSLAFIRKSDE</td>
<td>2262.6</td>
<td>11</td>
</tr>
<tr>
<td>Sar4</td>
<td>ISQarNEKINQSLAFIRKSDE</td>
<td>2291.6</td>
<td>1</td>
</tr>
<tr>
<td>Sar5</td>
<td>ISQVSSarQVNEKINQSLAFIRKSDE</td>
<td>2276.6</td>
<td>23</td>
</tr>
<tr>
<td>Sar6</td>
<td>ISQVSSarQVNEKINQSLAFIRKSDE</td>
<td>2261.6</td>
<td>100</td>
</tr>
<tr>
<td>Sar7</td>
<td>ISQVINESarQVNEKINQSLAFIRKSDE</td>
<td>2262.6</td>
<td>100</td>
</tr>
<tr>
<td>Sar8</td>
<td>ISQVNEKIsarQVNEKINQSLAFIRKSDE</td>
<td>2277.5</td>
<td>26</td>
</tr>
<tr>
<td>Sar9</td>
<td>ISQVNEKIsarQVNEKINQSLAFIRKSDE</td>
<td>2276.6</td>
<td>13</td>
</tr>
<tr>
<td>Sar10</td>
<td>ISQVNEKInSarQVNEKINQSLAFIRKSDE</td>
<td>2262.6</td>
<td>21</td>
</tr>
<tr>
<td>Sar11</td>
<td>ISQVNEKINQsarQVNEKINQSLAFIRKSDE</td>
<td>2303.6</td>
<td>13</td>
</tr>
<tr>
<td>Sar12</td>
<td>ISQVNEKINQssarQVNEKINQSLAFIRKSDE</td>
<td>2277.5</td>
<td>7</td>
</tr>
<tr>
<td>Sar13</td>
<td>ISQVNEKINQSSarQVNEKINQSLAFIRKSDE</td>
<td>2319.6</td>
<td>4</td>
</tr>
<tr>
<td>Sar14</td>
<td>ISQVNEKINQSLAsarQVNEKINQSLAFIRKSDE</td>
<td>2243.5</td>
<td>4</td>
</tr>
<tr>
<td>Sar15</td>
<td>ISQVNEKINQSLAFsarQVNEKINQSLAFIRKSDE</td>
<td>2277.5</td>
<td>30</td>
</tr>
<tr>
<td>Sar16</td>
<td>ISQVNEKINQSLAFISarQVNEKINQSLAFIRKSDE</td>
<td>2234.5</td>
<td>0</td>
</tr>
<tr>
<td>Sar17</td>
<td>ISQVNEKINQSLAFISarQVNEKINQSLAFIRKSDE</td>
<td>2262.5</td>
<td>84</td>
</tr>
<tr>
<td>Sar18</td>
<td>ISQVNEKINQSLAFIsarQVNEKINQSLAFIRKSDE</td>
<td>2303.6</td>
<td>21</td>
</tr>
<tr>
<td>Sar19</td>
<td>ISQVNEKINQSLAFIRKSSarE</td>
<td>2275.6</td>
<td>59</td>
</tr>
<tr>
<td>Sar20</td>
<td>ISQVNEKINQSLAFIRKssarE</td>
<td>2261.38</td>
<td>100</td>
</tr>
</tbody>
</table>

From the result, sarcosine substitutions of Glu6, Lys7, Asp19, and Glu20 showed minimal disruption in the original binding activity of the C20 peptide, whereas most of other sarcosine-substituted peptides dramatically lost their binding affinities to the 5HB. This study reveals essentially the importance of the amide hydrogens in the backbone of the C20 peptide for maintaining the binding affinity to the 5HB. The loss of the hydrogen bonds that are necessary for the α-helix in residues 6 and 7 does not seem to greatly diminish the binding affinity to the 5HB, and may result from
unusually high local enthalpic contributions from the neighboring residues at the hydrophobic interface. While proline has previously served as a beacon inviting substitution by structure-promoting peptoid residues, this data reveals sarcosine as an effective screen for substituting peptide residues with any possible peptoid monomers.

**Figure 4-4. % Inhibition of sarcosine-substituted peptides at 100 and 200 μM** Each peptide was competed with 5 nM Fl-C_{35} in the presence of 20 nM 5HB and its ability to displace Fl-C_{35} was monitored by FP. The FP response was converted to % inhibition.

**Synthesis and characterization of peptomeric C_{20} analogue**

As a proof of concept and to confirm the predictions of the alanine, proline, and sarcosine scan results experimentally, we incorporated peptoid monomers with identical side chains to the peptide residues being replaced into the C_{20} peptide. Since sarcosine substitutions at Glu6, Lys7, and Glu20 had relatively less impact on the binding affinity of the C_{20} to the 5HB, we created an initial peptomer by replacing with Lys7 and Glu20 with N-(4-aminobutyl)glycine (NLys) and N-(2-
carboxyethyl)glycine (NGlu) to structurally mimic Lys and Glu, respectively (Fig. 4-5). Glu6, Lys17, and Asp19 were not replaced with peptoids due to the variation in % inhibition values. After the synthesis, the peptomer (NLysNGluC20) was purified by preparative RP-HPLC with > 90% purity.

Figure 4-5. (A) Structures of peptoid residues corresponding to Lys and Glu residues, respectively and (B) structure of NLysNGluC20 peptomer (peptoid residues in shaded in dotted boxes)

The binding affinity of this peptomeric C20 analogue to the 5HB was monitored using our 5HB-based competitive FP assay (Fig. 4-6). The stock solution of this peptomer was prepared in water in the presence of 30% acetonitrile to reduce the viscosity and diluted into the FP buffer prior to use. Unfortunately, the binding affinity to the 5HB significantly decreased down to a % inhibition of only ~ 30 at 200 μM, presumably due to the additive effects of two substitutions. We are now synthesizing
singly-substituted peptomers with substitutions both at residues predicted as tolerating peptoid substitutions and at other locations, since such a comparison is a more appropriate evaluation of the scanning results.

![Graph](image.png)

**Figure 4-6. Competitive FP assay of NLysNGluC20** The FP response of Fl-C35 was monitored in the presence of the 5HB as the concentration of NLysNGluC20 increased.

Notably, this is the first example of rationally creating peptomers based on a data set obtained from a systematic scanning approach. Theoretically, one can imagine that by replacing amino acids with their counterpart peptoid monomers, proteolytic degradation could be minimized, thereby counteracting a decreased binding affinity with a longer circulation half-life.
Importance of $\alpha$-helices in biological settings

To find better usage for our combined alanine, proline and sarcosine scans, it is important to note that peptides derived from the HRB domain such as the C$_{20}$ peptide are poorly structured in monomeric form, but upon binding to the innercore HRA helices, they adopt $\alpha$-helix conformation.$^{31,32}$ In general, short peptides are not able to maintain well-defined 3D-structures in water, implying that there can be an immense number of conformational isomers present whose loss upon binding is entropically unfavorable. Thus, the lack of a defined structure in the C$_{20}$ peptide greatly weakens its binding affinity (IC$_{50} = 35$ $\mu$M) to the 5HB compared to longer peptides such as C$_{35}$ ($K_d = 38$ nM) and also leads to high susceptibility to proteolytic degradation.

$\alpha$-Helices are one of the most abundant structural features of proteins and many proteins recognize binding partners through contacts with the surface of $\alpha$-helices. However, typically the $\alpha$-helix does not form on its own, and instead the peptide tends to have a floppy structure, leading to a high entropy penalty associated with adopting an ordered structure upon binding to the target protein.$^{33,34}$ To overcome this unfavorable entropy penalty, numerous approaches to enforce $\alpha$-helix formation have been tested including helical capping,$^{35,36}$ intermolecular cyclization via disulfide bonds,$^{37-39}$ lactam bridges between Lys and Asp or Lys and Glu,$^{37-41}$ Intramolecular cyclization via click chemistry,$^{42-44}$ and hydrocarbon stapling.$^{45-49}$ These efforts have yielded notable success in increasing biological activities of peptides and overcoming unfavorable biophysical properties, which has helped revive the prospects of peptides becoming drug candidates.
We thus further utilized the knowledge gained from the combined scan approach to design C\textsubscript{20} peptide analogues that can adopt a stable \(\alpha\)-helical conformation and retain its binding affinity to the SHB. In this study, two separate strategies were explored, as discussed below.

\textit{Structurally constrained C\textsubscript{20} peptide derivatives via hydrocarbon stapling}

One method we investigated to stabilize the secondary conformation of the C\textsubscript{20} peptide is by hydrocarbon stapling. The incorporation of hydrocarbon-staple links into peptides at \((i, i + 4)\) or \((i, i + 7)\) positions has been widely investigated in various fields, yielding great success\textsuperscript{45-47}. For introducing hydrocarbon stapling into the C\textsubscript{20} peptide, two “designer amino acids” which have both an \(\alpha\)-methyl group and an \(\alpha\)-alkenyl group (e.g., (S)-2-(4’-pentenyl) alanine) were incorporated at the positions determined based on the results from the alanine, proline, and sarcosine scanning studies (Fig. 4-7). Because the efficiency of the stapling reaction depends on the

![Figure 4-7. Predicted stabilized helical conformations by hydrocarbon stapling (A) SUMP1 and (B) SUMP2 Non natural amino acids ((S)-2-(4’-pentenyl) alanine) were incorporated at \((i, i + 4)\) within the C\textsubscript{20} peptide, indicating that reactive residues would be located on the non-binding face of the \(\alpha\)-helix.](image-url)
positions in the peptide sequence, it is important to determine the right positions for the designer amino acids. The resulting two stapled peptides with \((i, i + 4)\) stapling on the solvent-exposed helix space spanning one turn of the helix were commercially obtained with > 90% purity.

- **SUMP1** (NH\(_2\)-ISQVNEKINASLAFIXKSDX-NH\(_2\), X = hydrocarbon-stapled positions) was designed to staple one turn of the helix at the C-terminus of the C\(_{20}\) peptide. This (S)-2-(4’-pentenyl) alanine was incorporated at Arg16 and Glu20 identified from the alanine and sarcosine substitution studies, respectively. Unlike SUMP2, the C-terminus of SUMP1 was amidated to overcome difficulty in the stapling reaction.

- **SUMP2** (NH\(_2\)-ISQVNEKINASLXFIRXSDE-CO\(_2\)H, X = hydrocarbon stapled positions) was initially designed to have a helix turn induced by the hydrocarbon stapling close to the middle of the peptide. Ala13 and Lys17 from the alanine and alanine/sarcosine substitution studies, respectively, were chosen for (S)-2-(4’-pentenyl) alanine incorporation.

![Figure 4-8. CD spectra of (A) SUMP1 and (B) SUMP2](image-url)

The helicity of each stapled peptides were calculated based on CD spectra presented. TFE was used as a helical-promoting solvent.
To evaluate if hydrocarbon stapling increases the helicity of the C$_{20}$ as desired, the secondary structure of each stapled peptide was monitored by CD spectroscopy (Fig. 4-8 and Table 4-4). All stock solutions were prepared in 50% acetonitrile in water for solubility reasons, and were then diluted with an equal volume of either water (0% TFE) or TFE (50% TFE). The CD spectra indicate that SUMP2 is significantly more helical in the absence of TFE than SUMP1. As previously reported, α-helicity tends to be more easily promoted when the hydrocarbon stapling is introduced in the middle of the peptide.$^{50}$ Therefore, our observation appears to be reasonable. We also examined the helical propensity of stapled peptides using trifluoroethanol (TFE), a well-known helix forming solvent.$^{33}$ At 50% (v/v) TFE, both stapled peptides showed extremely helical conformations with over 80% α-helicity.

Table 4-4. Ratio of molar ellipticities (deg·cm$^{-2}$·dmole$^{-1}$) at 222 and 208, and percent helicity calculated for stapled peptides and clicked peptomers

<table>
<thead>
<tr>
<th>Peptides /Peptomers</th>
<th>Solvent</th>
<th>[Θ]$<em>{222}$/[Θ]$</em>{208}$</th>
<th>% Helicity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{20}$</td>
<td>0% TFE</td>
<td>-$^a$</td>
<td>8$^c$</td>
</tr>
<tr>
<td></td>
<td>50% TFE</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>SUMP1</td>
<td>0% TFE</td>
<td>0.52</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>50% TFE</td>
<td>0.69</td>
<td>84</td>
</tr>
<tr>
<td>SUMP2</td>
<td>0% TFE</td>
<td>0.84</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>50% TFE</td>
<td>0.89</td>
<td>88</td>
</tr>
</tbody>
</table>

$^a$ Since the absorbance was too weak, [Θ]$_{222}$/[Θ]$_{208}$ was not calculated.

$^b$ % Helicity was calculated using K2D2$^{51,52}$ based on data points ranging from 190 and 240 nm.

$^c$ Data points from 200 to 240 nm was used.
To investigate if the helical propensity correlates with the binding affinity of the stapled peptides, the 5HB-based FP assays of SUMP1 and SUMP2 were performed. Unfortunately, introducing the hydrocarbon stapling into the peptide significantly lowers its aqueous solubility, particularly for SUMP1. After many different combinations of solvents (e.g., methanol and acetonitrile) were tested, the stock solution of SUMP1 still appeared to be very viscous, thereby exhibiting extremely high polarization values, which makes the FP assay impractical. Efforts to find a proper solvent system for SUMP1 are on-going. Concentrations ranging from 0.1 to 200 \( \mu \text{M} \) of SUMP2 were tested using the 5HB-based competitive FP assays (Fig. 4-9), demonstrating that SUMP2 (IC\(_{50} = 27 \mu \text{M}\)) could bind to the 5HB slightly better than the parent C\(_{20}\) peptide (IC\(_{50} = 36 \mu \text{M}\)).

![Figure 4-9. Competitive FP assay of SUMP2](image)

The FP response of Fl-C\(_{35}\) was monitored in the presence of the 5HB as the concentration of SUMP2 increased.
Overall, SUMP2, which has high helical content either in the presence or absence of TFE, has a lower IC\textsubscript{50} value for binding to the 5HB compared to C\textsubscript{20}, although the increase in the binding affinity was not as dramatic as expected.

**Structurally constrained peptomers using click chemistry**

The click chemistry method (a coupling of an azide and an alkyne to forma triazole) has been widely applied to many research disciplines,\textsuperscript{53} and successfully improved pharmacokinetic features of peptides (e.g., enhanced bioactivity and increased protease resistance) by constraining the structures.\textsuperscript{42-44} In addition, peptoids have also been subjects for click chemistry, resulting in linear/cyclic peptoids with multiple clicked functionalities,\textsuperscript{54-57} and induced helical conformations.\textsuperscript{58} However, click chemistry has not been applied to peptomers yet.

![Figure 4-10. Predicted stabilized helical conformation of the C\textsubscript{20} peptide using click reaction](image)

Nonnatural amino acids with clickable functional groups were incorporated at (i, i + 4) within the C\textsubscript{20} peptide, indicating that reactive residues would be located on the safe face of the \(\alpha\)-helix.

Therefore, the second strategy we employed is the use of the click chemistry method on peptomeric C\textsubscript{20} analogues. This approach is expected to achieve enhanced protease resistance by incorporating two peptoid monomers with clickable functional groups, i.e., azido and alkyne groups and simultaneously to stabilize the helical
conformation of the peptomers (Fig. 4-10). Arg16 and Glu20 were chosen based on the data resulting from the alanine and sarcosine scans, and were replaced with peptoid residues with azidopropyl and propargyl side chains, respectively (Fig. 4-11). Since these selected residues are at \((i, i + 4)\) position, it is expected that one-turn of the helix at the C-terminus of the \(C_{20}\)-based peptomer \((C_{20}, C)\) can be formed by click chemistry cyclization.

![Azidopropyl side chain and Propargyl side chain](image)

**Figure 4-11. Structures of peptoid residues with clickable functional groups**

To enhance the efficiency of the click reaction, microwave irradiation was used and all reagents were freshly prepared prior to the experiments. To avoid multimerized products and possible steric hindrance from the rest of peptide fragment that might impede the completion of click reaction, the click chemistry was performed on-resin on partially synthesized peptomer before completion of the peptomer synthesis.

The completion of the click reaction can be monitored by various methods including the growth of a new band at \(1684\ \text{cm}^{-1}\) in IR (infrared) spectroscopy with, corresponding to the triazole ring,\(^{59}\) HPLC analysis with retention time (Rt) difference,\(^{43,55}\) and a modified Kaiser method.\(^{60}\) Since the click reaction was
performed on resin, IR spectroscopy would not have been effective, and thus we decided to use a modified Kaiser method, which utilizes triphenyphosphine (TPP) to reduce unreacted azide to amine and ninhydrin to detect free amine group (see Materials and methods). Following the completion of the click reaction between the two peptoid monomers, the synthesis of rest of the peptide sequence was completed and the clicked peptomer was purified by preparative RP-HPLC with > 90% purity, yielding C_{20-C-C} (Fig. 4-12B). Unclicked peptomer was also prepared as a control (C_{20-C-U}) (Fig. 4-12A).

Figure 4-12. Structures of (A) C_{20-C-U} and (B) C_{20-C-C} with desired molecular weights. Azide and alkyne groups are presented in red and blue, respectively. The molecular weight of each peptomer is the same, suggesting additional method is required to confirm the completion of the click reaction and the synthesis.
To examine if the clicked peptomer could demonstrate increased helical content, the secondary structure of each peptomer was analyzed by CD spectroscopy. The CD spectra indicated that both C\textsubscript{20} C\textsubscript{2} C\textsubscript{6} and C\textsubscript{20} C\textsubscript{2} U were poorly helical in water with 25\% (v/v) AcN, exhibiting [\Theta]\textsubscript{222} values of approximately -5,000 deg\textsuperscript{-1}cm\textsuperscript{2}dmol\textsuperscript{-1} and insignificant % helicity (Fig. 4-13 and Table 4-5). The helical propensity of each peptomer in 50\% (v/v) TFE was also studied, suggesting that the clicked C\textsubscript{20} C\textsubscript{2} C\textsubscript{6} showed higher helical content than its unclicked analogue, C\textsubscript{20} C\textsubscript{2} U and C\textsubscript{20}, which confirms that our attempt to stabilize peptomer secondary structure using the click chemistry was successful.

Figure 4-13. CD spectra of (A) C\textsubscript{20} C\textsubscript{2} U and (B) C\textsubscript{20} C\textsubscript{2} C\textsubscript{6} The helicity of each peptomer were calculated based on CD spectra presented. TFE was used as a helical-promoting solvent to determine how easily the helical conformation of the stapled peptides can be promoted.
Table 4-5. Ratio of molar ellipticities (deg·cm²·dmole⁻¹) at 222 and 208, and percent helicity calculated for stapled peptides and clicked peptomers

<table>
<thead>
<tr>
<th>Peptides /Peptomers</th>
<th>Solvent</th>
<th>[Θ]222/[Θ]208</th>
<th>% Helicity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₂₀</td>
<td>0% TFE</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>50% TFE</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>C₂₀₋C₋U</td>
<td>0% TFE</td>
<td>0.60</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>50% TFE</td>
<td>0.76</td>
<td>67</td>
</tr>
<tr>
<td>C₂₀₋C₋C</td>
<td>0% TFE</td>
<td>0.56</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>50% TFE</td>
<td>0.77</td>
<td>78</td>
</tr>
</tbody>
</table>

<sup>a</sup> % Helicity was calculated using K2D2<sup>51,52</sup> based on data points ranging from 190 and 240 nm.

To evaluate the correlation between the helical propensity and the binding affinity of the clicked and unclicked peptomers, the 5HB-based competitive FP assays were carried out using C₂₀₋C₋C and C₂₀₋C₋U. Although the clicked C₂₀₋C₋C, which has the highest helical content in 50% (v/v) TFE, seemed to bind to the 5HB less tightly compared to the C₂₀, C₂₀₋C₋C exhibited slightly higher binding affinity to the
5HB than its unclicked analogue, C$_{20}$C$_{U}$ (Fig. 4-14).

Although the helix-stabilization strategy using click chemistry on peptomers seemed effective given the CD spectroscopy results, it is possible that the highly structured peptomers might be in an inactive conformation, and thereby have poor binding affinity to the 5HB. It is also well-known that in many cases introducing helix constraints in the middle of a helix instead of near the terminus is more effective, and we are in the process of synthesizing these derivatives.
Conclusion and future prospects

Here we propose a generally applicable method using alanine, proline, and sarcosine for determining peptoid-replaceable peptide residues and attempt to prove the uniqueness and usefulness of the combined scan using \( \text{C}_{20} \) as a model peptide. This combined approach has generated useful data sets related to the conformational tolerance and importance of individual side chains, which cannot be easily provided by structural studies such as an X-ray crystallography.

![Figure 4-15. Helical wheel representation of the \( \text{C}_{20} \) peptide](image)

The \( \text{C}_{20} \) peptide is presented as a helical wheel projection and peptoid-replaceable peptide residues are in red circle. The positions for hydrocarbon stapling and click reaction were connected with dotted line. The top view of the 5HB is included, showing three inner HRA helices in green and two outer HRB helices in red.
The results we obtained through this approach are summarized in Fig. 4-15, implying that peptide residues pointing to the binding interface are crucial to retain the binding affinity, while amino acids facing away from the binding site are better candidates for peptoid replacement and further structural modifications. A prototype peptomer, NLysNgluC20, were first tested, proving that it is plausible to predict peptoid-replaceable peptide residues in the target peptide sequence without transforming each peptide residue to the corresponding peptoid monomer experimentally. Furthermore, we investigated two different strategies to promote the α-helical conformation of the C20 peptide via either hydrocarbon stapling between nonnatural amino acids with olefinic side chain or click chemistry cyclization between clickable peptoid monomers. The structurally constrained C20 analogue, SUMP2, was more helical and exhibited increased binding affinity to the 5HB as compared to C20. Although another conformationally restricted C20 analogue, the clicked C20 C C shows helicity that is clearly improved by the click reaction, its binding affinity to the 5HB was not improved (Fig. 4-16).

![Figure 4-16. Comparison of % Inhibition and helical propensity](image-url)

Figure 4-16. **Comparison of % Inhibition and helical propensity** of structurally constrained peptomer (C20 C C) and peptide (SUMP2) are presented in comparison with those of C20. %Inhibition was calculated at 200 µM of each compound and for helical propensity, per residue molar ellipticity at 222nm was measured in 50%(v/v) TFE.
Our findings suggest that it is helpful but apparently not sufficient in all cases to have high helical propensity (i.e., how easily the helix can be promoted). Additionally, we recognize that the carbon chain formed by the click reaction in our study is one carbon shorter than the hydrocarbon stapling approach, which might interfere with the ability of the peptomer to adopt a properly formed α-helical conformation.

Our system, which predominantly consists of an α-helix, is suboptimal in proving the true value of the combined scanning approach to develop peptidomimetics. Protein interactions through α-helices require a precise spacing between peptide residues from each binding partner, with less than ideal tolerance of incorporating peptoids into the peptide sequences (e.g., the conformational difference of the peptoid helix and α-helix). Beyond the immediate application to the hRSV system, we hope that our novel strategy presented here will be more useful and powerful on other systems (e.g., β-turn mimics and miniature protein scaffolds) and provide a critical design paradigm for future therapeutic peptidomimetics.
Chapter Four: Knowledge-based approaches to design peptoid-peptide hybrids and their structurally constrained analogues

Materials and Methods

General materials
Reagents for peptide and peptomer synthesis were purchased from Applied Biosystems (Foster city, CA, USA) or Sigma-Aldrich (Milwaukee, WI, USA). Resins and Fmoc-protected amino acids were purchased from NovaBioChem (San Diego, CA, USA) or Anaspec (San Jose, CA, USA). Solvents and reagents for peptoid monomer synthesis and for analytical and preparation RP-HPLC were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used without additional purification.

Alanine/proline/sarcosine substituted peptides synthesis
Peptides with alanine, proline, and sarcosine substitution were commercially obtained with 95% purity (Aapptec, Louisville, KY, USA) and the sequences are listed in Table 4-1, 2, and 3. Final purities and the molecular weight of synthetic peptides were confirmed by Aapptec and analytical HPLC traces and data sheet of electrospray mass spectrometry (ESI) were also provided.

Stock solution preparation
In the case of using dimethyl sulfoxide (DMSO) for the peptide stock solution in a high concentration (e.g., 5 mM), immediate use is recommended. At room temperature, peptide stock solutions in DMSO appeared to be clear with no sign of precipitation. However, after being stored at 4 °C, the solutions may become frozen due to low melting point of DMSO (18.5 °C) and stay turbid and viscous, even after being
incubated at room temperature over 1 hr. This will interfere with polarization measurement. To overcome this solubility issue and enable to store the solutions at 4 (~ days) or -20 °C (~ weeks), 50% of acetonitrile is recommended.

**Sample preparation for FP measurements**

Few peptides were extremely insoluble compared to other peptides. Because the testing concentrations of alanine/proline/sarcosine-substituted peptides for the 5HB-based competitive FP assays were relatively high (100 and 200 μM), precipitations were often observed. To reduce loss of peptides due to their poor solubility, vortexing and/or sonication were carried out. In addition, to minimize undesirable perturbation in polarization measurement caused by remaining precipitates, the solution of each sample was spun down and then transferred to the 96-well plate with care, followed by 10 sec delay before measurements.

**Stapled peptides preparation**

Stapled peptides were synthetically prepared with 90% purity (Anaspec, San Jose, CA, USA). The peptide residues for hydrocarbon stapling were chosen based on the results from our alanine/proline/sarcosine scans and the synthesis of hydrocarbon-stapled peptides was carried out as previously reported. Final purities and the molecular weight of synthetic peptides were confirmed by provided analytical HPLC traces and data sheet of ESI.
**Monomer preparation: 3-Azidopropylamine**

Synthesis of 3-azidopropylamine (2) is shown in Scheme 1. Commercially available 3-chloropropylamine (1) is refluxed with 3 eq. of sodium azide in water at 80 °C overnight to complete the conversion of (1) to (2). The reaction was then cooled and treated with NaOH (10 M) until pH became basic. The aqueous solution was extracted using chilled ethyl ether three times and then the combined organic layer was dried over Na₂SO₄, and filtered. Due to the low boiling point of the desired product, the organic solution was concentrated under weak N₂ flow for over 1 hr. Resulting 3-azidopropylamine and H¹ NMR confirmed that (2) was obtained with reasonable purity. After concentration was determined, (2) was diluted in N-Methylpyrrolidone (NMP) as a final concentration of 1.0 M for peptoid synthesis. H¹ NMR was used to determine the concentration of the solution; solvent was further evaporated if necessary. H¹ NMR (500 MHz, CD₃OD) δ 3.40 (t, J = 7.0, 2H, N₃CH₂), 2.79 (t, J = 7.0, 2H, H₂NCH₂), 1.77 (m, J = 7.0, 2H, CH₂CH₂CH₂). H¹ NMR of 3-azidopropylamine was performed by Dr. Sungyoung Seo (Stanford University).

![Scheme 1. Synthesis of 3-azidopropylamine](image-url)
Synthesis of peptomeric C\textsubscript{20} analogue

Peptomeric C\textsubscript{20} analogue, NLys/Glu\textsubscript{20}, was synthesized in the laboratory using standard Fmoc chemistry for peptide residues and a submonomer peptoid synthesis\textsuperscript{62} with peptoid monomers using an automated ABI 433A peptide synthesizer (Applied Biosystems, Foster city, CA, USA). After synthesis, the peptides were cleaved off from the resin and deprotected in trifluoroacetic acid (TFA)/water/TIPS/thionisole (90:5:2.5:2.5 v/v) for 1.5 hr at room temperature. Peptomers were purified by preparation RP-HPLC on a C18 column using a linear gradient of 5-99% solvent B in solvent A over 60 min (solvent A is 0.1% (v/v) TFA in water and solvent B is 0.1% (v/v) TFA in acetonitrile). Final purities of synthetic peptides were confirmed to be > 95% by analytical RP-HPLC, and the molecular weight of the purified product was confirmed by ESI at the Stanford University Mass Spectrometry (SUMS) facility.

Synthesis of peptomeric C\textsubscript{20} analogues for click reaction

Peptomeric C\textsubscript{20} analogues (C\textsubscript{20}_C_C and C\textsubscript{20}_C_U) were synthesized in the laboratory as described above. For an unclicked peptomer as a control, the synthesis is completed without any interruption. On the other hand, for a clicked peptomer, microwave-assisted click chemistry was carried out after first 6 residues including peptoid monomers were synthesized. The synthesis for the rest of the C\textsubscript{20} peptide was then completed. The cleavage and purification of these peptomers were done as described above.
Microwave-assisted click chemistry

Click chemistry (1,3-dipolar cycloaddition) between the azide and alkyne functional groups was performed on solid support (rink amide resin). Stock solutions were prepared: (i) 1 M of CuSO$_4$·5H$_2$O (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in H$_2$O; (ii) 1M of L-(+)-sodium ascorbate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was dissolved in t-BuOH/H$_2$O (1:1). For a microwave-assisted reaction, 0.13 mmole of partially synthesized peptide-bound resin in 75 mL of t-BuOH/H$_2$O (1:1) was placed in the reaction tube and subsequently L-(+)-sodium ascorbate (520 µmol) and CuSO$_4$·5H$_2$O (130 µmol) were added. The reaction tube was sealed and heated in the microwave reactor for 1 hr (70 °C, absorption level: high). The reaction mixture was pale yellow after microwave heating. The clicked and unclicked peptomers were purified by preparation RP-HPLC on a C18 column using a linear gradient of 5-99% solvent B in solvent A over 60 min (solvent A is 0.1% (v/v) TFA in water and solvent B is 0.1% (v/v) TFA in acetonitrile).

Scheme 2. Microwave-assisted Cu (II) catalyzed click chemistry on resin Azido and alkyne functional groups in peptoid side chains are shown in red and blue, respectively. To enhance the reaction efficiency of the click chemistry, on-resin click chemistry is adopted in this study.
As shown below, the resulting clicked peptomer (Rt = 16 min) was eluted faster then the unclicked one (Rt = 18 min) analyzed by analytical RP-HPLC on a C18 column using a linear gradient of 5-99% solvent B in solvent A over 30 min (solvent A is 0.1% (v/v) TFA in water and solvent B is 0.1% (v/v) TFA in acetonitrile).

**Comparison of the analytical HPLC traces of purified C20_C_C and C20_C_U**

![HPLC traces comparison](image)

**Modified Kaiser method**

To verify the completion of the click chemistry, a simple colorimetric method based on Kaiser test was carried out. 200 µL of 5% triphenylphosphine (TPP) was added on to the peptomer-bound resin and then the solution were vortexed, followed by the addition of 5% ninhydrin in ethanol. The mixture was heated at 130 °C till the strong colors showed up (dark purple). Unclicked peptide-bound resin and resin only were
also tested as controls. For best results, it is highly recommended that TPP solutions should be prepared prior to use and/or stored under inert atmosphere to minimize air oxidation.

![Diagram showing the modified Kaiser method to determine the completion of the click reaction](image)

**Scheme 3. Modified Kaiser method to determine the completion of the click reaction**

**Competitive fluorescence polarization (FP) binding assay**

Competitive FP binding assays of the C_{20} derivatives were carried out as previously described. Briefly, using a 5HB protein construct and a fluorescently labeled 35 amino acid-long peptide (Fl-C_{35}) as a target and a tracer respectively, the binding affinity of each C_{20} analogue prepared in this study was individually evaluated. Each well in a black 96-well plate (Corning Inc. Lowell, MA, USA) contained 20 nM of the
5HB and increasing concentrations of each peptomer in FP buffer (20 mM PBS at pH 7.4, 500 mM NaCl, 0.01% (v/v) Tween-20, and 0.05 mg/ml bovine gamma globulin) in a final volume of 185 µL with 1hr incubation at room temperature. Fl-C35 was then added to a final concentration of 5 nM followed by 30 min incubation at room temperature. The FP responses were monitored using a Synergy4 plate reader (Biotek, Winooski, VT, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The percentage of inhibition (% Inhibition) was calculated using the following equation: % inhibition = 100×[(mP-mPf)/(mPb-mPf)], where unbound Fl-C35 (mPf), bound Fl-C35 (mPb) and the bound inhibitor to the 5HB (mP) are used accordingly.

**Secondary structural analysis by CD spectroscopy**

CD spectra were obtained with a Jasco J-815 spectrophotometer (JASCO, Easton, MD, USA). Sample was prepared in water with 25% (v/v) acetonitrile and 50% TFE for studying the helical promotion potentially induced by hydrocarbon stapling and click reaction. The concentrations ranging from 25 – 50 µM were used. Data were recorded from 190 to 260 nm with a step size of 0.2 nm, at a rate of 100 nm/min, a bandwidth of 1.0 nm with a scanning speed of 20 nm/min in a 0.1 cm path-length quartz cell. The response time was 0.5 s. Each CD spectrum was an average of 3 measurements and the baseline was corrected by subtracting the spectrum of a blank obtained under identical conditions. The resulting data was converted to per-residue molar ellipticity units (deg·cm²·dmol⁻¹·residue⁻¹) and the secondary structure content was analyzed with a web-based software, K2D2.⁵¹,⁵²
References


Chapter Five

Identifying short peptide-based hRSV entry inhibitors from a phage-displayed peptide library and their peptidomimetic analogues

In previous chapters we discussed our knowledge-based approaches to the identification and optimization of peptide and peptomer therapeutic candidates. These candidates were all guided by the known sequence and structure of the hRSV F protein 6-Helix Bundle. To ensure that we had left “no stone unturned,” we wanted to complement our knowledge-based approaches with unbiased library screens, and therefore turned to phage displayed peptide libraries.

Overview of bacteriophages

Bacteriophages, or simply phages, are single-stranded DNA viruses that infect a variety of Gram-negative bacteria using pili as receptors.1 Various engineered phage particles have been intensively studied for medical and therapeutic applications as well as for the development of new materials and nanostructures.2-4 As a research tool, the filamentous phage, M13, is one of the most

Figure 5-1. Schematic diagram of a filamentous phage (M13) Coat proteins are “displayed” on pIII or pVIII.
widely used phages, because it contains genetically easily modifiable genes for coat proteins, which are useful and versatile for display purposes. The M13 genome consists of 11 genes, expressing about 2,700 copies of the pVIII coat protein and 3 to 5 copies of the pIII coat protein (Fig. 5-1). Although infection with M13 phages is not lethal to bacterial cells because it is a non-lytic virus (i.e., M13 phages neither lyse nor destroy the bacterial cells after replication), the rate of cell growth is detrimentally affected when M13 phages infect bacteria.

**Phage display technology**

Phage display was first introduced in 1985 as an effective method to map epitope-binding sites of antibodies by screening phage-displayed peptide libraries against an immobilized immunoglobulin. The basic principle of phage display is to literally “display” desirable peptides or proteins on the surface of the phage. After exposure to a target protein, specific clones of phages displaying peptides that bind to the target protein can be easily selected and amplified. To construct the phage-displayed peptide libraries, DNA sequences of interest with variable regions are inserted in the genome of the phage such that the encoded protein is expressed as a fusion product to one of the phage coat proteins (e.g., pIII). Therefore, several billion peptide variants can be constructed simultaneously instead of modifying individual genetic material, expressing, purifying and analyzing each variant one-by-one. The advantage of phage-displayed peptide libraries over chemically synthesized peptide libraries is that the physical connection between the surface-displayed peptides and the
internal genetic coding allows successive rounds of optimization to be carried out without regaining laborious sequencing and identification at each round.

The length of the peptides displayed on phages was historically limited to 6 – 15 residues, due to the possible interference with the coat proteins’ endogenous function and poor display efficiency. However, this limitation has been overcome by using a helper phage or encoding an additional copy of coat protein genes into a phagemid vector.\(^7\) The sequences of the peptides of interest can be randomly or rationally designed to increase the odds of obtaining optimal sequences (e.g., by fixing positions of certain amino acids or by introducing structural constraints such as cyclization of the peptides via disulfide bond).\(^8\) Typical phage-displayed library sizes range from \(10^7\) to \(10^9\) of individual transformants, though libraries up to \(10^{11}\) have been produced.\(^9\)

Once the library is obtained, the phage particles are exposed to the target proteins in an immobilized or solubilized form to identify specific ligands or enrich previously selected ligands with enhanced binding affinity. After non-specific binders are removed, the phages bound to the target are recovered and subsequently amplified for further rounds of the selection. This \textit{in vitro} selection process called “panning” is typically repeated for 3 – 5 rounds until enrichment of the specific phages is achieved (Fig. 5-2).\(^9\) After the panning, DNA is extracted from individual phages and sequenced to identify the protein phenotype of each clone.
When the peptide binders are enriched and their sequences are revealed, the consensus sequence among the identified peptides are often found, which are considered as more important since they represent a “convergent evolution” solution that likely serves as the optimized endpoint of multiple initial designs. For this purpose, the consensus sequences can serve as a useful scaffold for building more focused libraries for affinity maturation, if necessary. Once the peptides with desired properties including high selectivity and biological activity are chosen, synthetic peptides are prepared and then further investigated to obtain insight on the relationships between their structures and biological functions, which can be used to design peptide, non-peptide, or peptidomimetic ligands.
Applications of phage display technology to antiviral development

Since the first phage display library was produced, phage display technology has become a powerful approach to discover therapeutically useful peptides and improve their biophysical properties as suitable pharmaceuticals. Many novel peptides identified by phage display are now in clinical trials as therapeutics to treat a variety of diseases are used as diagnostic tools in various biological and medical research areas, including the antibody identification, enzyme inhibitors, gene delivery, cancer targeting, protein-binding peptides, DNA-binding peptides, and tissue-specific peptides. Specifically, considerable efforts have been made to identify antiviral peptides targeting various viruses. For instance, antiviral peptides targeting West Nile virus (WNV) envelope protein (E) have been isolated from phage-displayed peptide libraries. The E protein is known to mediate virus attachment to the host cells and subsequent viral fusion with the host cell membrane, is therefore an attractive therapeutic target. Screening of phage-displayed peptides and proteins derived from murine brain cDNAs against the WNV E protein yielded several short peptides ranging from 8 to 26 amino acid in length with the potential of crossing the mouse blood-brain barrier. HIV-1 gp120 bound peptides were also identified from three randomly designed peptide libraries. The functional properties of the identified dodecameric peptides were investigated, suggesting that they would stabilize HIV-1 gp120 conformations and might be useful as leads for designing HIV-1 entry inhibitors.

Another interesting application of phage display is mirror-image phage display, which has yielded successful D-peptide ligands for diagnostic and therapeutic
applications.\textsuperscript{25} There is a growing interest in the therapeutic usage of $D$-peptides, particularly because they are highly protease-resistant and thereby present prolonged \textit{in vivo} half-lives compared to $L$-peptides. Briefly, the strategy employed for the mirror-image phage display begins with a synthetically generated therapeutic $D$-enantiomeric form of the target protein followed by screening the phage-displayed $L$-peptide library against them. After identification of efficacious $L$-peptides against the $D$-target, equivalent $D$-peptides are synthesized for use against the actual biological $L$-target (Fig. 5-3). Specifically, the $D$-peptide HIV-1 entry inhibitors targeting HIV-1 gp41 have been identified using mirror-image phage display library with high potency ($IC_{50} = 250$ pM) and binding affinity to the natural protein targets.\textsuperscript{26-29}

\textbf{Figure 5-3. Concept of mirror-image phage display} The $D$-enantiomeric form of target peptides are used for phage display. Once $L$-enantiomeric peptides bound to the $D$-enantiomeric targets are isolated, the $D$-enantiomeric form of selected $L$-peptides can be synthesized, providing enzymatically stable peptides.
**hRSV F protein as a target for phage-displayed peptide library**

Although phage display has allowed for the rapid and cost-effective screening of a huge number of peptide libraries, attempts to identify antiviral peptides to control hRSV infection using phage display libraries have not been pursued yet. Previously, we reported that we can easily screen potential hRSV entry inhibitors using a protein-based fluorescence polarization (FP) assay, which is scalable to a high-throughput screening (HTS) format (see the details in Chapter 2). hRSV F protein-mediated viral fusion is the most essential step of the hRSV infectious cycle. As discussed in Chapter 1, this viral fusion occurs extracellularly, and is therefore an attractive drug target because of its relatively easy access to antivirals.

In this study, we applied the Ph.D.™-12 phage display peptide library (New England Biolabs, Ipswich, MA, USA) to an engineered hRSV F protein (5-Helix Bundle, 5HB) to identify peptide-based inhibitor candidates to block hRSV F-mediated viral fusion. After isolating phage-bound peptides that specifically bind to the target (5HB), peptides were individually synthesized and their binding affinities to the 5HB were examined using the 5HB-based FP assay. Even with the short length (12 amino acid-long), which might cause a poorly structured conformation and high susceptibility to proteolytic degradation, the selected peptides showed modest binding affinities to the 5HB. To reduce the protease susceptibility of these peptides, peptoid-peptide hybrids were synthesized and tested. Furthermore, to understand the interaction of the identified peptides with the 5HB, the effort of co-crystallization of these peptides with the 5HB is being pursued and is expected to provide insight into
how these short peptides can bind to the 5HB and thus provide a design basis for peptidomimetic antivirals.

**Phage-displayed peptide library panning results and ELISA tests**

With the aim of isolating peptides that specifically bind a hydrophobic groove where a missing 6th helix of hRSV F protein 6-helix bundle (6HB) binds, the 5HB was used as a target during the panning process. Random 12-mer peptides from Ph.D.™-12 phage display peptide library with a capacity of ~ 2.7 x 10^9 sequences were tested against the 5HB directly. After 3 rounds of the panning, the DNA extracted from 30 plaques of individual phage clones was sequenced, resulting in 16 unique phage clones and 2 wild type phages (pIII protein only). The sequences and their frequency of isolation during the panning are shown in Table 5-1.

**Table 5-1. Sequences of phage-bound peptides isolated by screening against the 5HB**

16 different peptide sequences were identified.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence (amino to carboxy)</th>
<th>Frequency of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-2</td>
<td>WHWSWQPQHRSP</td>
<td>3</td>
</tr>
<tr>
<td>P1-5</td>
<td>WHWSVPWAPLHE</td>
<td>1</td>
</tr>
<tr>
<td>P1-7</td>
<td>VAAPAKATMSST</td>
<td>3</td>
</tr>
<tr>
<td>P1-8</td>
<td>THKYANYQWQPR</td>
<td>4</td>
</tr>
<tr>
<td>P3-1</td>
<td>WHWFPTAPSYRA</td>
<td>1</td>
</tr>
<tr>
<td>P3-4</td>
<td>FPQMHNGPSTRT</td>
<td>2</td>
</tr>
<tr>
<td>P3-8</td>
<td>WHWQPYVPWTFR</td>
<td>1</td>
</tr>
<tr>
<td>P4-1</td>
<td>KCCYPDIPQNSR</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence (amino to carboxy)</th>
<th>Frequency of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4-2</td>
<td>VLAAPSISHRTL</td>
<td>1</td>
</tr>
<tr>
<td>P4-3</td>
<td>HLHALSSLPTPL</td>
<td>1</td>
</tr>
<tr>
<td>P4-6</td>
<td>KLHQRVMPTPLW</td>
<td>1</td>
</tr>
<tr>
<td>P4-7</td>
<td>DARIMPRPLGYPY</td>
<td>2</td>
</tr>
<tr>
<td>P4-9</td>
<td>KVWLPHNPTLNI</td>
<td>1</td>
</tr>
<tr>
<td>P5-1</td>
<td>GLKIWSLPPHHG</td>
<td>2</td>
</tr>
<tr>
<td>P5-3</td>
<td>QPIKVMPMGWAT</td>
<td>1</td>
</tr>
<tr>
<td>P5-7</td>
<td>RCHPNVPEISA</td>
<td>1</td>
</tr>
</tbody>
</table>

*aEach clone is named by the plate number that it was selected followed by the clone number.*
Figure 5-4. Binding of selected phage-bound peptides to immobilized 5HB Binding abilities of phage-bound peptides identified from the panning process were measured by using the ELISA assay. BSA was used as a control. The data present mean values of duplicate measurements.

To further assess the selectivity and specificity toward the 5HB, the peptide-bound phages were amplified and then tested using the ELISA methods. Serially diluted peptide-bound phages ranging from $10^4$ to $10^{11}$ pfu/mL were incubated in the 5HB- and BSA-coated wells, respectively. BSA was used as a negative control to prevent isolating non-specific binding peptides. The ELISA tests with the 5HB resulted in four phage-bound peptides with significantly higher binding affinity to the 5HB compared to BSA (Fig. 5-4), corresponding to the sequences WHWSWQPQRHSP (P1-2), WHWSVPWAPLHE (P1-5), VAAPAKATMSST (P1-7), and THKYANYQWQPR (P1-8). During the panning and ELISA experiments, the protein-coated wells were filled with a blocking solution to the top of each well to avoid the possibility of isolating plastic-binding phages. However, we still observed
a high frequency of aromatic residues such as Trp in the identified peptide sequences. Although the enrichment of aromatic residues could be an indication of plastic-binding sequences, the abundance of these residues should not be considered as an absolute diagnostic of plastic binding since there are many reports on the specific binding of aromatic residue-rich peptides to target proteins such as HIV-1 gp120, and the hydrophobic binding pocket of the 5HB may be a target as well (see Chapter 2).

**Competitive FP assays of selected synthetic peptides**

Even with numerous benefits gained from phage display, there are a number of technical limitations in the panning process: first, the elution conditions of selectively bound phages should be optimized because overly stringent conditions would reduce yields of candidate peptides in early stages. Second, the avidity effect may lead to the selection of tight-binding peptides in the context of the phage virion that may not bind with high-affinity in the context of synthesized monomeric peptides. The avidity due to the polyvalent display magnifies an intrinsic affinity to the target, so that even weak-binding peptides with poor affinity can bind to the target, which leads to unnecessarily widening of the pool of potential candidates. To eliminate these intrinsic limiting factors in using the phage display libraries: we first progressively increased the elution stringency as the panning progressed. Second, to minimize multivalent avidity effects, we prepared synthetic peptides with 95% purity (Table 5-2) and further tested the peptides. The 5HB-based FP assay here served as a secondary assay to select the monomeric peptide ligands with high specificity and also enabled
efficient quantification of binding affinity of peptides identified from the phage-displayed peptide library.

Table 5-2. Peptides individually synthesized and used in this study Proline residues are in bold and similar sequences found in at least two peptides are underlined.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sequence (amino to carboxy)</th>
<th>Molecular weight (Da)</th>
<th>pI$^a$</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-8</td>
<td>THKYANYQWQPR</td>
<td>1591.5</td>
<td>9.70</td>
<td>138</td>
</tr>
<tr>
<td>P1-2</td>
<td>WHWSWPQRHSP</td>
<td>1631.7</td>
<td>9.76</td>
<td>NM$^b$</td>
</tr>
<tr>
<td>P1-5</td>
<td>WHWSVWPWPLHE</td>
<td>1544.7</td>
<td>6.02</td>
<td>27</td>
</tr>
<tr>
<td>P1-7</td>
<td>VAAPAKATMSST</td>
<td>1134.3</td>
<td>8.72</td>
<td>NM</td>
</tr>
<tr>
<td>P5-3</td>
<td>QPIKVMPHGAT</td>
<td>1358.6</td>
<td>8.75</td>
<td>NM</td>
</tr>
</tbody>
</table>

$^a$pI (Isoelectric point) was predicted with the ProtParam algorithm (http://expasy.org/tools/protparam.html).

$^b$NM = Non measurable

Each synthetic peptide’s ability to prevent the missing 6$^{th}$ helix (fluorescently labeled C$_{35}$ peptide probe, Fl-C$_{35}$) binding to the 5HB was determined at the concentrations of 100 and 200 μM, Fig. 5-5). Only two peptides (P1-5 and P1-8) showed good % inhibition for Fl-C$_{35}$ binding to the 5HB, showing an IC$_{50}$ value of 27 and 138 μM, respectively (Table 5-2 and Fig. 5-6). On the other hand, the other peptides substantially lost their binding affinities (Table 5-2). As briefly discussed above, it is not surprising that some of the synthetic peptides showed weaker binding activities than those of peptide-bound phages, because of high local concentrations of the peptides due to the avidity effect of polyvalently expressed peptides on the phage.$^{34}$
Figure 5-5. % Inhibition of Fl-C35 binding to the 5HB by resynthesized peptides using a competitive FP assay A polarization value of 5HB-Fl-C35 in the absence of inhibitors serve as 0% inhibition and unbound Fl-C35 was used as a 100% inhibition value. The polarization values obtained in the presence of inhibitors at 100 and 200 μM of concentrations were normalized based on the polarization values of 0 and 100% inhibition.

Figure 5-6. Competitive FP assays of P1-5 and P1-8 The binding affinities of two 12-mer peptides, P1-5 and P1-8 were fully evaluated using the 5HB-based competitive FP assays.
**Secondary structure analysis of selected synthetic peptides**

Since these peptides could displace Fl-C35 for the binding site of the 5HB with a low micromolar binding affinity, we speculated that the peptides should bind to a hydrophobic groove formed by two neighboring HRA helices within the 5HB. However, no significant sequence homology was found between these selected 12-mer peptides and hRSV F protein or even among selected peptides. We thus pursued the structural studies on selected synthetic peptides. Even with no obvious sequence consensus between the different peptides, it is noteworthy that proline residues are frequently found in identified peptide sequences (Table 5-2). Particularly, P1-5 contains two prolines with a polyproline II (PPII) structure type sequence motif (PXXP: P = proline and X = other amino acids) suggesting that P1-5 might have a PPII helical structure. This PPII is typically a short left-handed helix with characteristic CD spectral features of a negative band at around 200 – 210 nm and a weaker positive band at 225 – 235 nm (Fig. 5-7A). It is also known to play an important role in hydrophobic interactions and is commonly found in many protein-protein interactions including SH3 and WW domains. Because the PPII helix contains no intramolecular H-bonds, its structure can be more open and flexible than α-helices, allowing the conformation to easily adopt an appropriate structure when it needs to bind to any target proteins.

To test if P1-5 or other selected peptides have the PPII type helical structure, the secondary structure of P1-5 was examined by CD spectroscopy. The CD spectrum of P1-8 peptide with one proline was also analyzed due to its promising binding affinity to the 5HB. P1-5 did not present a strong indication as the PPII helix
signature, which can be easily confused with random coil (unstructured or unordered) conformations, and the spectrum of P1-8 was a too weak to be assigned as any distinctively structured conformation.

![Figure 5-7. CD spectra of (A) reference spectra of secondary structures and (B) P1-5 and P1-8](image-url)

In the reference spectra, α-helical (α), β-sheet (β), random-coil (r), and polyproline type II (PPII) conformations in green solid line are indicated (adapted from Rath et al. (2005) Biopolymers 80, 179). The characteristic bands of PPII at a negative and a positive peak are marked in dotted and solid line, respectively in both graphs.

**Efforts to cocrystallize selected peptides with the 5HB**

To better understand how selected peptides form a surface contact with the target (5HB), co-crystallization or NMR spectroscopy can be useful. The 3-D structural information can be further combined with functional studies on the selected peptides, potentially providing valuable insights for more focused libraries for affinity maturation. We therefore decided to investigate the interaction of P1-5 and P1-8 with the 5HB using X-ray crystallography. Our initial aim for structural studies was to obtain co-crystals of these peptides with the 5HB. After more than 300 crystallization
conditions were screened, the crystallization effort was still not as fruitful as desired.

In examining related hRSV F protein constructs that successfully yielded the diffracting crystals leading to structural information,26,40,41 we noted that other protein constructs are composed of individually chemically synthesized HRA and HRB peptides. Since the 5HB is a single-chain polypeptide generated by alternatively connecting three HRA and two HRB helices using short peptide linkers, these peptide linkers might be placed differently depending on how the helices align in the bundle. Theoretically, both right-turning and left-turning bundles can occur equally with our construct, which may provide two different configurations of the 5HB (Fig. 5-8). The presence of more than one 5HB conformation might explain the difficulties in co-crystallizing the 5HB with selected peptides.

**Figure 5-8. Two different handedness of the 5HB bundling** The right- and left-turning 5HB can be formed equally with unwanted heterogeneity, which may interfere with the 5HB crystallization. (Linker 1 in green: PPPELGGP, Linker 2 in red: KGSSK)
Alternative ways to cocrystallize selected peptides with the GCN construct

To obtain a structurally homogeneous protein construct, the crystallization strategy was thus changed to create a simple construct mimicking the innercore HRA helices of the hRSV F 6HB. Previously, it has been shown that the HRA domains easily aggregate in the absence of the HRB domains, due to the hydrophobic groove presented on the HRA helices.\textsuperscript{42} The GCN domain was adapted to stabilize the trimerization and solubilize the protein constructs, thereby preventing the possible uncontrolled aggregation of the HRA helices (Fig. 5-9).\textsuperscript{26,43} The GCN domain is 32 amino acid-long (ARMKQIEDKIEILSKYHIENEIARIKKLIGEA) and a parallel trimer-forming domain with high thermal stability ($T_m > 100$ °C). Two constructs were designed to consist of the HRA helix fused to the GCN domain both $N$- and $C$-terminally (GHG) or $C$-terminally only (HG). These constructs were expected to yield soluble and stable trimerized HRA helices, providing a sufficient binding site to peptides of interest (Fig. 5-9).

![Figure 5-9](image-url)

**Figure 5-9.** (A) Schematic diagram of GCN constructs (HG and GHG) for crystallization study and (B) illustration of trimerized GCN constructs (arrowhead in red represents TEV protease cleavage site for His$_8$-tag removal)
Purified GCN constructs were tested using the 5HB-based FP assay with Fl-C$_{35}$ to determine if the binding site is exposed as designed. As shown in Fig. 5-10, Fl-C$_{35}$ seemed to bind to both GCN constructs reasonably well, but a difference in increased polarization values was observed. This can be explained by the following reasons: first, to have Fl-C$_{35}$ tightly bind to the GCN constructs, the binding site needs to be well formed when Fl-C$_{35}$ is introduced. The GHG construct thus is expected to be more stable due to two GCN domains being attached to both the $N$- and $C$-terminus of the HRA domain. Therefore, the higher polarization value from Fl-C$_{35}$ binding to the GHG construct is reasonable. Second, the molecular weight of the GHG construct (16.2 KDa when trimerized) is higher than that of the HG construct (12.6 KDa when trimerized). In FP, a larger target protein exhibits a larger increase in polarization value upon a probe binding to the target due to slower tumbling rate. Therefore it is understandable that the GHG construct showed higher polarization value than the HG construct in the presence of Fl-C$_{35}$.

**Figure 5-10. FP response of GCN constructs in the presence of Fl-C$_{35}$ peptide** 5 nM of Fl-C$_{35}$ was used and 30 µM of C$_{35}$ peptide was included for a positive control.
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The competitive FP assays of Fl-C35 binding to the GCN constructs in the presence of a unlabeled C35 peptide at 30 µM were also tested (Fig. 5-10). Even with an excess amount of the unlabeled C35 peptide (IC_{50} = 38 nM, see Chapter 2 for details) used, we observed only ~ 80% decrease in the polarization value compared to the GCN constructs in the absence of Fl-C35, for both GHG and HG. Since the GCN constructs theoretically expose three binding sites, it might be difficult to displace all Fl-C35 bound to the GCN constructs at once. For example, if only two-thirds of the binding sites that are occupied with Fl-C35 are displaced with the unlabeled C35, one can still observe a significantly high polarization value compared to completely unbound Fl-C35, which represents 100% inhibition.

Currently, we are examining various co-crystallization conditions of selected peptides with both of the GCN constructs, which should provide an insight for understanding the working mechanism of these short peptides.

**Preliminary data for antiviral activities of selected peptides**

The antiviral activity of P1-5 and P1-8 was investigated by collaborating with Dr. Ron Geller in Prof. Judith Frydman’s lab at Stanford University. Briefly, hRSV A2 strain was pre-mixed with 50 µM of each peptide and then added to Hep2 cells to infect the cells. P5-7 and C35 peptides were included as negative and positive controls, respectively. P5-7 (RCCHPNVPEISA) was initially identified from the panning process; however, it has no measurable binding affinity to the 5HB as judged by the FP assay. After 48 hours of infection, the media containing viral particles was diluted and transferred to cells that were previously cultured in a 96-well plate. After 5 – 6
days of incubation, each plate was observed by a microscope for any signs of syncytial formation in the wells. Preliminary data suggest that P1-5 and P1-8 showed some inhibition in syncytial formation as an indication of antiviral activity (Fig. 5-11). Although large batch-to-batch variations made it difficult to draw definitive conclusions, it was obvious that these peptides lost some of their biological activity in the cellular antiviral activity assay.

![Peptide inhibition graph](image)

**Figure 5-11. Peptides (P1-5 and P1-8) inhibition of hRSV infection in Hep2 cells** 50 μM of P1-5 and P1-8 were tested. 50 μM of P5-7 and C35 were included as negative and positive controls, respectively.

**Peptomeric hRSV inhibitors and their binding activity toward the5HB**

The low antiviral activities of P1-5 and P1-8 in the cellular assays might be caused by their relatively short length and consequently rapid proteolytic degradation during the long course of the assays. This led us to explore the possibility of designing peptomers based on selected 12-mer peptides by introducing peptoid residues to the peptides sequences. As discussed earlier (Chapter 4), proline is the only naturally occurring N-substituted cyclic amino acid, lacking a backbone NH group. For this
reason, proline has been recognized as a representative of peptoids in several studies.\textsuperscript{44-48} To examine the effect of peptoid residue substitution at the proline residue on the binding activity, we first synthesized analogues of P1-8 in which one proline residue is located close to its C-terminus instead of testing P1-5, which contains two proline residues.

As a starting point, NMEG and NPhe were selected (Fig. 5-12). NMEG is known to be very hydrophilic, which was discussed in depth in \textbf{Chapter 3} for its potential applications in protein modification, whereas NPhe is expected to maintain a pattern of the P1-8 sequence, which contains alternating aromatic and charged residues. These two peptomers, P1-8-NPhe and P1-8-NMEG, were manually synthesized and then purified to > 90\% purity (Fig. 5-13). Using the 5HB-based competitive FP assay, the binding activities of these two peptomers at the concentration of 75 \( \mu \text{M} \) were determined (Fig. 5-14). Even though the concentration used in this study was lower than the IC\(_{50}\) value of P1-8 (138 \( \mu \text{M} \)), we expected both P1-8 mimics to bind to the 5HB with similar binding affinity based on our observation (Fig. 5-6). However, both P1-8-NPhe and P1-8-NMEG significantly lost binding affinity to the 5HB, indicating that even single peptoid substitution can affect the binding affinity of the peptide greatly. It might also suggest that the determination of peptoid replacement needs more sophisticated methods. For example, since P1-5 and P1-8 are unlikely to be helical, they may be
more amenable to the systematic screening approach described in **Chapter 4**. Thus it may still be useful to investigate the potential of P1-8 (and possibly P1-5) as a design scaffold for creating peptidomimetic antivirals.

(A)

![Chemical structure](image)

(B)

![Chemical structure](image)

**Figure 5-13. Structures of P1-8 mimics** Proline in P1-8 peptide was replaced with peptoid residues of NPhe and NMEG, yielding (A) P1-8-Nphe and (B) P1-8-NMEG, respectively.

**Figure 5-14. Resulting data of P1-8 mimics using a competitive FP assay** The binding of Fl-C_{35} to the 5HB was monitored in the presence and absence of 75 µM of each peptomer.
Conclusions and future prospects

In this study we identified two promising 12-mer peptides, P1-5 and P1-8, from a phage-displayed peptide library using our previously reported 5HB-based FP assays. Even with their relatively short length, these peptides showed moderate binding affinity to the 5HB. We also demonstrated that these dodecameric peptides may inhibit viral replication by preventing syncytium formation in a cell-based assay, however, their antiviral activity as hRSV fusion inhibitors needs to be further confirmed.

Efforts to crystallize a complex of each 12-mer peptide with the GCN constructs are currently being made. This will lead us to better understanding of the binding mode of these peptides with the 5HB and enable maximizing their performance as potential anti-hRSV agents.

Additionally, the binding of the peptomeric P1-8 mimics to the 5HB was tested to prove the hypothesis that the proline residues can be replaced with peptoid residue. Our rather disappointing results from a limited set of derivatives suggests that transforming peptides to peptoids or peptomers will require more comprehensive evaluation on each peptide residue depending on the system and furthermore, in-depth side-chain optimization should be performed.
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Materials and Methods

Materials for phage selection (Panning)

Ph.D.™-12 phage display peptide library was purchased from New England Biolabs (Ipswich, MA, USA). The reagents for phage selection are as following: blocking buffer (0.1 M NaHCO₃, 5 mg/mL BSA, 0.02% NaN₃ at pH 8.6), elution buffer (0.2 M Glycine-HCl, 1 mg/mL BSA at pH 2.2), neutralization buffer (1 M Tris-HCl at pH 9.1), agarose top (10 g Bacto-Tryptone, 5 g yeast extract, 5 g NaCl, 1g MgCl₂, 7 g agarose in 1 L water, the mixture is autoclaved, and then aliquoted into 50 mL tubes. Solidified agarose top should be stored at room temperature, and melted in a microwave prior to use.), LB/IPTG/Xgal plates (1 L LB medium, 15 g/L agar, 5 mg IPTG in DMF, 4 mg Xgal [5-bromo-4-chloro-3-indoly-β-D-galatoside], the mixture is autoclaved and then poured into the plate. The plate should be stored at 4 °C in the dark), LB-Tet plates (1 L LB medium, 15 g/L agar, 20 mg tetracycline, the plate should be stored at 4 °C.), PEG/NaCl solution (20% (w/v) polyethylene glycol-8000, 2.5 M NaCl), iodide buffer (10 mM Tris-HCl, 1.0 mM EDTA, 4 M NaI at pH 8.0), TBS (50 mM Tris-HCl, 150 mM NaCl at pH 8.6), TBS-T (TBS with Tween-20).

Phage selection

Ph.D.™-12 phage display peptide library consists of ~ 2.7 x 10⁹ electroporated sequences, amplified once to yield ~55 copies of each sequence in 10 μL of the supplied phage. Peptides are fused to the N-terminus of the protein of gene III, with a GGS spacer and expressed on the phage surface in five identical copies. For phage selection, wells in the 96-well plate were coated with 150 μL of 40 μg/mL 5HB at 4
℃ overnight. Microplates were then blocked with 200 µL of blocking solution for 1 hr at 4 °C. The plate was then washed with TBS-T (0.1%) and incubated with the phage suspension for 1 hr at room temperature. Unbound phages were removed by washing with TBS-T containing 0.1% (1st panning) and 0.5% (2nd and 3rd panning) of Tween-20. Bound phage were eluted with 100 µL of 0.2 M glycine (pH 2.2) and neutralized with 15 µL of 1 M Tris (pH 9.1). After amplification, DNA of eluted phage was extracted and purified. DAN sequencing for each phage clone was done by Sequestech (Mountain View, CA USA) using a -96 gIII primer (5’-CCCTCATAGTTAGCGT AACG-3’, NEB).

**Peptide synthesis**

Peptide synthesis reagents were purchased from Applied Biosystems (Foster city, CA, USA) or Sigma-Aldrich (Milwaukee, WI, USA). Fluorescently labeled peptide (FL-C35) with 95% purity was commercially obtained (EZBiolab, Carmel, IN, USA) and used without further purification. Individual 12-mer peptides identified from the panning were synthetically prepared with 95% purity (Bio Basic, Markham, Ontario, Canada) and used without purification.

**GCN constructs design, expression and purification**

Synthetic genes for HRA-GCN (HG), and GCN-HRA-GCN (GHG) were commercially prepared by Genescript (Piscataway, NJ, USA). Genes were subsequently cloned into pET-15b using NdeI-BamHI restriction enzyme sites and the resulting plasmids were transformed into Rosetta gami 2 (DE3). The resulting protein
sequences are following: for the HG construct, **AVSKLHLEGEVNKIKSALLSTN KAVVSLNGVSVLTSKVLK**NKIDKQLLPIV**NKIKQIEDKIEILS**K**I**YHIEEIl** ARIKLIGEAGSG**E**NYFQG**GSSG**H**H**HHHHH, and for the GHG construct, ARIKQIEDK**I**EILS**K**IYHIEEIA**R**IKKLAV**S**K**LHLEG**E**V**N**KIKSALLSTN**K**A VVSLSNGVSVLTSKVLKN**K**IDKQLLPIV**NK**IKQIEDKIEILS**K**I**YHIEEIA**R**IKKLIGEAGSG**E**NYFQG**GSSG**H**H**HHHHH**H**H**H**H** (HRA domain in bold, GCN domain in underlined, Tabacco Etch Virus (TEV) cleavage site in underlined bold and His tag in underlined italic). Proteins were bacterially expressed with 1 mM IPTG for 3 hrs at 37 °C. The cells were harvested by centrifugation at 4500 g for 15 minutes, and the cell pellet was resuspended in 20 mM phosphate buffered saline (PBS) and stored at -80 °C until use. Cells were lysed with lysis buffer (CelLytic™ B cell lysis reagent [Sigma Aldrich, Milwaukee, WI, USA], 20 mM PBS at pH 7.4, 1 mM PMSF, protease inhibitor cocktail [Sigma Aldrich, Milwaukee, WI, USA], 1% Triton X-100, 500 mM NaCl and 0.2 mg/ml lysozyme) and incubated for 1 hr at room temperature. Cell lysate was then clarified by centrifugation at 18,000 g for 30 min. The soluble fraction was immediately incubated with a Nickel-immobilized chelating sepharose fast flow resin (GE Healthcare, Piscataway, NJ, USA) at room temperature for 30 min with a gentle agitation. The protein-bound resin was washed out with more than 10 column volumes (CV) of a wash buffer (20 mM PBS at pH 7.4, 10 mM imidazole, 1% Triton X-100 and 500 mM NaCl). The GCN constructs were eluted with an elution buffer (20 mM PBS at pH 7.4, 150 mM imidazole and 500 mM NaCl). The purity of the protein was assessed by SDS-PAGE gel and the protein was used without further purification.
Phage binding assays using ELISA experiments

Binding studies were performed using ELISA to evaluate the specificity of binding of selected 12-mer peptides to the 5HB. A microtiter plate was coated with 100 uL of 100 ug/mL of the 5HB in 0.1 M NaHCO₃ (pH 8.6) and incubated at 4 °C overnight. After rinsing wells with TBS-T (0.1%) and blocking with 5% milk in TBS-T (0.1%) for 1 hr at 4 °C, serially diluted phages ranging from 10^{12} to 10^{8} phages/mL were applied, followed by incubation for 2 hrs at room temperature with gentle agitation. Unbound phage were removed by washing the plates 6 times with TBS-T (0.5%). 200 μL of horseradish peroxidase (HRP) conjugated anti-M13 antibody (Sigma Aldrich) diluted in blocking buffer (1:5,000) was added and incubated at room temperature for 1 hr to detect phage interaction with the 5HB. After the addition of HRP substrate (3,3’, 5,5’-tetramethylbenzidine, TMB) was added to each well, absorbance at 405 – 415 nm was measured. BSA was used as a negative control.

Competitive FP binding assay

Competitive FP binding assays of selected 12-mer peptides were carried out as previously reported.³⁰ Briefly, using a 5HB protein construct and a fluorescently labeled 35 amino acid-long peptide (Fl-C₃₅) as a target and a tracer respectively, the binding affinities of dodecameric peptides were individually evaluated. Each well in a black 96-well plate (Corning Inc. Lowell, MA, USA) contained 20 nM of the 5HB and increasing concentrations of each 12-mer peptide in FP buffer (20 mM PBS at pH 7.4, 500 mM NaCl, 0.01% (v/v) Tween-20, and 0.05 mg/ml bovine gamma globulin) in a final volume of 185 μL with 1 hr incubation at room temperature. Fl-C₃₅ was then
added to a final concentration of 5 nM followed by 30 min incubation at room temperature. The FP responses were monitored using a Synergy4 (Biotek, Winooski, VT, USA) plate reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The percentage of inhibition (% Inhibition) was calculated using the following equation: % Inhibition = 100×[(mP-mPf)/(mPb-mPf)], where unbound Fl-C_{35} (mPf), bound Fl-C_{35} (mPb) and the bound inhibitor to the 5HB (mP) are used accordingly.

**Secondary structural analysis by CD spectroscopy**

CD spectra were obtained with a Jasco J-815 spectrophotometer (JASCO, Easton, MD, USA). Samples were prepared in 20 mM PBS, pH 7.4 in concentration of 50 μM. Data were recorded from 195 to 260 nm with a scanning speed of 20 nm/min and a bandwidth at 1.0 nm in a 0.1 cm path-length quartz cell. Each CD spectrum was an average of 3 measurements and corrected for a buffer blank obtained under identical conditions. The resulting data was converted to per-residue molar ellipticity units, [Θ] (deg·cm²·dmol⁻¹·residue⁻¹).

**Peptomer synthesis**

Each peptomer (P1-8-NPhe and P1-8-NMEG) were manually synthesized in the laboratory using standard Fmoc chemistry for peptide residues and a submonomer peptoid synthesis⁴⁹ for peptoid monomers manually on rink amide resin. After synthesis, the peptides were cleaved off the resin and deprotected in TFA/water/TIPS/thionisole (90:5:2.5:2.5 v/v) for 1.5 hr at room temperature with agitation.
Peptomers were purified by preparative RP-HPLC on a C18 column using a linear gradient of 5-99% solvent B in solvent A over 60 min (solvent A is 0.1% (v/v) TFA in water and solvent B is 0.1% (v/v) TFA in acetonitrile). Final purities of synthetic peptomers were confirmed to be > 90% by analytical RP-HPLC, and the molecular weight of the purified product was confirmed by electrospray mass spectrometry (ESI) at the Stanford University Mass Spectrometry (SUMS) facility.
References


Chapter Six

Conclusion

Conclusions and future prospects

In this thesis, we have validated a protein-based HTS-compatible fluorescence polarization (FP) assay as a successful screening platform for hRSV fusion inhibitors, and evaluated the potential of short peptides and their peptoid-based peptidomimetics using this assay. To obtain therapeutically favorable properties including improved solubility and enhanced half-lives, we also have demonstrated a broadly-applicable methodology for creating peptoid-based biostable therapeutic peptidomimetics.

In Chapter 2, we discussed that the 5HB-based FP assay might also provide a key to study small molecule antivirals whose mechanism of action is still poorly understood. Our recent findings in testing several small molecule fusion inhibitors using the 5HB-based FP assay suggest that small molecules might not block the 6HB formation of hRSV F as others have claimed,\(^1\text{-}^5\) but likely trap the 6HB in a non-functional form.\(^6\) Because of the relatively small size of the antivirals, it is likely that the bulk of the HRB helices can still interact with the HRA helices to form a partially functional 6HB in the presence of the bound small molecule, while the remaining part of the HRB helices covered over the small molecules bound to the hydrophobic pocket. Simply by varying the length of the probe peptide or the region where the probe is binding (e.g., covering the hydrophobic pocket or not), it might be possible to narrow down the possible binding mode of the small molecules. However, to prove the
hypothesized mechanism of the small molecules using the 5HB, the most important question to answer is whether or not these small molecules bind to the hydrophobic pocket (i.e., the known binding site for Fl-C$_{35}$) in the 5HB. One simple test can be to monitor the small molecule binding to the 5HB in the presence and absence of C$_{35}$ or shorter derivatives by thin layer chromatography (TLC), which has been widely used for the separation and identification of biochemical compounds such as carbohydrates, steroid hormones and amino acids.\textsuperscript{7} After incubation, the unbound small molecules can be removed from the mixture by a desalting column and only the 5HB-bound small molecule-bound 5HB will be recovered. On a TLC plate, small molecules will be separated from the sample spot at the baseline and will migrate with the mobile phase (e.g., dichloromethane), allowing then to be detected.

Subsequently, the structural studies of the small molecule inhibitors with the whole hRSV F protein should also be pursued to understand the interaction of small molecules with the hRSV F protein in depth. Electron microscopy (EM) studies might be a simple experiment to begin with, since EM studies of the F proteins from other paramyxoviruses have shown that the pre- and post-fusion structures of the F protein can be easily distinguished and the sample preparation would be relatively straightforward compared to other methods (e.g., NMR studies).\textsuperscript{8} In the presence and absence of the small molecules, the hRSV F protein is expected to behave differently upon activation to the post-fusion form. To gain further insight on the interaction between the small molecules and the hRSV F protein at the atomic level, X-ray structural studies of a complex of the small molecules with the full length hRSV F should be pursued as a long term strategy.
In Chapter 3, we suggest that NMEGylation can be applied to modify a simple, short therapeutic peptide as well as proteins yielding highly monodisperse products with saved time and costs. NMEGylated peptide/peptoid libraries could be designed at an early stage of molecular optimization varying both the number and position of Nmgs and any spacers due to the intrinsic compatibility of NMEGylation with solid phase peptide/peptoid synthesis.

Our proposal in this study can be further confirmed if we can prove how having a linker helps to retain the binding affinity of the C20 peptide. To fully understand how placing a glycine linker in between NMEG and the C20 peptide sequence affects the binding affinity, structural studies using X-ray crystallography might provide useful information. We generated a possible 3-D model of NMEG-Gly-C20 binding to the 5HB using Pymol. The model reveals a possible H-bond between the hydrogen atom at N-terminus (donor) and the nitrogen atom at Lys191 (acceptor) from one of the HRA helices, showing a distance of 2.7 Å (Fig. 6-1). This postulated H-bond enabled by the glycine linker might be very weak since it occurs between primary and secondary amine, however, it might act as a stabilizing factor to place Isoleucine (the first residue of the C20) in the right position to easily initiate binding of the C20 to the 5HB, which may explain why NMEG-Gly-C20 could recover most of the binding affinity to the 5HB compared to the other linker-less NMEGylated peptides.
Figure 6-1. Stabilized the binding of NMEG-Gly-C$_{20}$ to the 5HB by H-bond  Three HRA helices are shown in green ribbons and the C$_{20}$ peptide is presented as white ribbon. The first residue of the C$_{20}$ peptide, Isoleucine, is represented by red, and Lysine at position 191 in the HRA helix is shown in magenta. The possible hydrogen bond between NMEG residue and Lys191 are shown in dotted line.

Additionally, we reasoned that since a large open contact surface is involved in the binding of the C$_{20}$ to the 5HB, direct conjugation of oligoNMEG led to impeded binding of NMEGylated peptides to the 5HB due to possible steric hindrance. As illustrated in Fig. 6-2, we believe NMEGylation will more successfully improve the biophysical properties of ligands that involve a narrow binding cleft (e.g., substrate and enzyme) without severely impacting their biological activity. Such a finding would further demonstrate the potential of NMEGylation as a biotherapeutic modification tool.
Figure 6-2. Retaining biological activity of NMEGylated ligands and steric hindrance (A) When the binding site is narrow and limited to several active residues, oligoNMEG in red can be directly attached to a target ligand (e.g., the C_{20} peptide and substrates) without deteriorating the biological activity of ligands. (B) The high steric hindrance in dotted circle may cause the lower binding affinity of NMEGylated ligands that interact a large binding interface on a receptor (e.g., the 5HB and enzymes)

The combined peptide scan approach using alanine, proline, and sarcosine for determining peptoid-replaceable peptide residues in therapeutic peptides that we proposed in Chapter 4 can be applied to design peptidomimetic hRSV fusion inhibitors using the dodecameric peptides identified in Chapter 5 as a design basis. Two promising 12-mer peptides, P1-5 and P1-8, showed a moderate binding affinity (27 and 138 μM respectively) to the 5HB, which is comparable with the C_{20} peptide. To improve the likely high susceptibility to proteases caused by their short length, simple P1-8 analogues (Pro \rightarrow peptoids) were tested, however, in part because of the small number of peptomers, the results were rather disappointing. Therefore, alanine/proline/sarcosine substitution studies will provide critical information on each residue to design better peptoid-based peptidomimetic peptides based on the 12-mer peptides.

As discussed in Chapter 5, the avidity effect caused by multivalency of phage-bound peptides has been propose to explain the lower biological activity of
individually synthesized peptide with the target proteins. However, if we can mimic
the avidity effect by intentionally multimerizing peptides, it may be possible to gain
positive and desirable impact on the biological activity.

First, dimerization via a disulfide bond\(^\text{10}\) and native chemical ligation
methods\(^\text{11,12}\) have been widely used to provide increased structural stability and thus
enhance biological activity. However, since none of 12-mer peptides contain cysteine,
it should be critical to find optimal sites for cysteine mutation or insertion. The
stabilizing effect of the disulfide bond in peptides or proteins can be varied depending
on where the disulfide bond introduced.\(^\text{13}\) Once cysteine-contained peptides are
prepared, the disulfide bonds are formed simply by air oxidation of a solution of
reduced peptides.

Second, trimeric peptides can be prepared using a scaffold that provides three
functional groups to tether the target peptide. This strategy has been applied to
peptides derived from HIV-1 gp41 HRA and HRB helices, generating 3-helix bundle
mimetics of HIV-1 gp41. (Fig. 6-3).\(^\text{14}\) Trimeric 12-mer peptides can be generated by
using a trimeric scaffold such as TBB (Tris-(bromomethyl) benzene) or TREN (Tris-
(2-aminoethyl)amine) (Fig. 6-4). TREN has been used as an effective structural
scaffold for the assembly of triple helical collagen mimetic structures.\(^\text{15}\) As compared
to TBB, TREN is expected to provide a flexible tripodal structure, which allows for a
better accommodation of the three-peptide chains.
Third, selected 12-mer peptides can even further multimerized using a peptide dendrimer approach (more than 3 branches), which has been recognized for potential therapeutic applications including drug delivery,\textsuperscript{16} multiple antigenic peptides,\textsuperscript{17} antiviral peptides\textsuperscript{18,19} and antimicrobial peptides.\textsuperscript{20} Since dendrimers have highly branched macromolecules synthesized from a highly structured core (Fig. 6.5), they can present multiple ligands or binding sites, which mimics the avidity effects.\textsuperscript{17,21}
It is anticipated that multimerized peptides using the strategies described above will present high protease resistance and increased local concentration, thereby having longer half-lives and tighter binding to the target protein.

![Image of peptide dendrimer motif examples](A) poly-(prolylene imine) core is currently commercially available and (B) lysine-based core

In summary, we describe a novel and effective high-throughput screening compatible platform for screening potential viral fusion inhibitors (Chapter 2). We used both knowledge-based (Chapter 2) and phage-displayed peptide library (Chapter 5) approaches to generate possible "hits" for such inhibitors. We then report two completely novel and broadly-applicable approaches for optimizing therapeutic candidate peptides into peptomers. We report NMEGylation as a method to “decorate” peptides for improved solubility and protease resistance (Chapter 3) and a comprehensive Ala/Pro/Sar scan method for identifying optimal residues in a peptide for a peptoid substitution. The work has greatly focused on creating enabling tools, such as the 5HB screening platform and the peptomer optimization strategies, and thus expect that this work will have a wide and lasting influence.
References


