SPONTANEOUS MEMBRANE TRANSLOCATING PEPTIDES:
CHARACTERIZATION OF COMMON MOTIFS AND DESIGN OF NOVEL SCREENS
FOR DELIVERY OF BIOACTIVE CARGOES

AN ABSTRACT

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BY

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ABSTRACT

Cell penetrating peptides (CPPs) are a unique family of peptides capable of crossing the selectively-permeable lipid bilayer membrane of cells. The mechanism of entry remains an important characterization for CPPs if they are to be used for therapeutic and biotechnological tools. Two often non-exclusive processes are involved with CPPs crossing the lipid bilayer; energy-dependent or energy-independent mechanisms of entry. CPPs that rely heavily on energy-dependent mechanisms of entry are unfavorable for the advancement of CPPs as therapeutic and biotechnological applications because of the problems faced with endosomal sequestration and lysosomal degradation of a CPP-cargo conjugate. Thus, developing CPPs reliant on energy-independent mechanisms of entry increases the usefulness of CPPs to deliver cargoes directly across the lipid bilayer and into the cytosol of cells. Recently, our lab developed a high-throughput orthogonal screen capable of identifying spontaneous membrane translocating peptides (SMTPs) from a 10,000-peptide member library. The sequences identified in the screen, which were observed to cross lipid bilayers of synthetic vesicles using energy-independent mechanisms, shared a common LRLLR sequence motif. The readily occurring LRLLR motif prompted further investigation if the common sequence motif was necessary and sufficient for spontaneous membrane translocation. In addition, the LRLLR motif was used to rationally design arginine spacing variants of the motif to test the effects that arginine positioning had on spontaneous translocation. Elucidation of the mechanisms involved during spontaneous translocation for the motif and arginine spacing variants were also conducted. The results gained from the guided rational design of motif variants and the mechanisms responsible for spontaneous translocation will inform designs of future peptide libraries, which will undergo iterative screenings to expand the repertoire of SMTPs.
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CHAPTER 1

Introduction

Cell penetrating peptides (CPPs) are a part of a larger class of membrane active peptides that also includes membrane permeabilizing peptides. By their definition, CCPs gain access to the interior of the cell by crossing the selectively permeable barrier created by the lipid bilayers of the cell membranes. CPPs were first described when the transactivator of transcription protein of HIV (TAT) was observed to cross the cell membrane, enter the nucleus, and induce viral replication. 

Truncation of the TAT protein revealed the minimal peptide sequence necessary to maintain cell penetration, and thus the first CPP was discovered 20 years from the time this was written. Since then, hundreds of CPPs have been described in the literature. The physical chemical properties of CPPs vary in hydrophobicity, amphipathicity, and length ( > 30 amino acids), however, most are cationic and contain arginine (Figure 1.1). There have been attempts to classify CPPs mainly by how the peptide was discovered (protein derived, modeled from existing CPP, or rationally designed) with more recent classification attempts focusing on physical chemical properties: identifying classes such as cationic, amphipathic, and hydrophobic CPPs.

The unique ability of CPPs to cross the cell membrane provides a useful delivery system for therapeutic molecules that on their own would not cross the cell membrane due to their size, polarity, and/or hydrophobicity. For delivery, cargoes can either be covalently attached or associated with the peptide by non-covalent electrostatic interactions. Typical cargoes include covalently attached fluorescent dyes and to a lesser extent, useful bioactive cargoes such as DNA,
Figure 1.1* Physical Chemical Properties of Experimentally Verified CPPs

Displayed here are the physical chemical properties displayed for a total of 747 experimentally verified CPPs. The average length (A), charge (B), interfacial activity due to hydrophobicity, electrostatics, and/or amphipathicity (C), fraction of sequence containing cationic residues (Arg or Lys), amphipathicity due to α-helix (E) or β-sheet secondary structure (F).

*Figure taken from:
RNA, proteins/peptides, or other membrane-impermeant molecules. Because the physical chemical properties of CPPs dictate whether cell penetration can occur, it is reasonable to expect that cargo can impact the ability of a CPP to cross the lipid bilayer. Nevertheless, CPPs have demonstrated that cellular delivery of membrane-impermeant cargoes is possible.

CPPs use two broad, non-exclusive mechanisms of cell penetration; energy independent or energy-dependent\(^7\) (Figure 1.2). The mechanism of cell penetration a peptide utilizes can be influenced by the experimental conditions such as peptide concentration, experimental design, and cargo properties\(^8\). Mechanism of cell entry must be known for a CPP delivering a cargo since energy-dependent mechanisms convene to an endosome that can then fuse with a lysosome and promote degradation of the peptide and cargo\(^9\). Therefore, CPPs with more reliance on energy-independent mechanisms of entry avoid being sequestered inside of an endo/lysosome, thus making delivery of a cargo directly to the cell cytosol potentially more advantageous.

CPPs with a desired energy-independent mechanism of entry remain underrepresented. Efforts to rationally design CPPs with a defined mechanism of action are difficult due to the large physical chemical property space one must consider. One solution came from an orthogonal screen of a peptide library that found peptides able to cross synthetic lipid bilayers of vesicles spontaneously without disrupting the membrane (i.e. pore formation). The screen yielded *Spontaneous Membrane Translocating Peptides* (SMTPs), a class of peptides that from their discovery relied on an energy-independent mechanism of entry\(^10\). Further characterization in cell models showed that SMTPs delivered cell impermeant fluorescent cargoes directly into cells under non-physiological temperatures and thus spontaneously.

The interactions between peptides and the cell membrane influence the mechanistic behavior of a CPP. How SMTPs and other CPPs cross the cell membrane is best understood by
5

Energy-Dependent Entry

A.

1. Phagocytosis
2. Macropinocytosis
3. Clathrin-mediated endocytosis
4. Caveola-mediated endocytosis
5. Clathrin and caveolin independent endocytosis

B.

Direct translocation
6. "Carpet-like"
7. Inverted micelle
8. Membrane thinning
9. Pore formation

Energy-Independent Entry
Figure 1.2* Proposed Mechanisms of Cell Entry for CPPs

Mechanisms of entry are described as energy-dependent processes (A) and/or energy-dependent processes (B). Those processes that rely on energy-dependent mechanisms of cell entry all convene to further sequestration into an endosome. Energy-independent mechanisms of entry directly cross the cell membrane; however, some processes are more toxic than others (numbers 9 > 6 > 7 > 8 > 10) with “carpet like” (9) and pore formation (6) processes more prevalent with pore forming and antimicrobial peptides.

*Figure taken from:

studying the physical chemical properties of the lipid bilayer. The next section will cover the physical interactions between peptides and the lipid bilayer that influence the ability of a peptide to translocate across the lipid bilayer.

**Peptide Interactions with the Lipid Bilayer**

*What Makes a Membrane Active Peptide a Cell-Penetrating Peptide, a Pore-forming Peptide or an Antimicrobial Peptide?*

Defining a membrane active peptide as either a CPP, pore-forming peptide or antimicrobial peptide (AMP) is difficult due to overlapping properties. The differences of lipid composition of eukaryotic versus bacterial membranes may determine how membrane active peptides adopt lytic activity against bacteria but not against eukaryotic cells. For instance, the overall neutral charge of eukaryotic membrane lipids and the presence of cholesterol to “seal” the membrane may serve as intrinsic properties that allow AMPs to only lyse the overall negatively charged bacterial membrane better than eukaryotic membranes\textsuperscript{11}. However, at a certain concentration, CPPs that crossed the cell membrane without lysis can become cytotoxic as peptide aggregates on the surface to promote disruption of the membrane\textsuperscript{12}. Interestingly, one key distinguishing feature that may define membrane active peptides as either CPPs or pore-forming and AMPs is the lack of self-assembly on or in the membrane during the translocation event (i.e. translocation as a monomer)\textsuperscript{13}. The next section will give an overview of peptide-membrane interactions and how these interactions can result in a peptide translocating across the eukaryotic membrane.

*Peptide Interactions with the Eukaryotic Lipid Membrane*

The majority of the eukaryotic cellular membrane is comprised of various glycerophospholipids. Phospholipids spontaneously form a bilayer membrane with their
hydrophilic head groups creating 15Å interfacial regions of the outer and inner-leaflets of the membrane while their hydrophobic acyl chains orient inward to form a 30Å hydrophobic core\textsuperscript{14}. This arrangement of lipids creates a selectively impermeant barrier able to exclude mostly polar and hydrophilic molecules. The overall ratios of phospholipid species varies by cell-type; however, zwitterionic phosphatidylcholine (POPC) is the major component of eukaryotic cell membrane\textsuperscript{15}. The various species of phospholipids differ mostly by their hydrophilic head groups, which influence the spatial area the lipid exists in as well as the surface charge\textsuperscript{15}.

Translocation of a peptide across the membrane is a stepwise process with partitioning into the interfacial domain of the outer membrane leaflet considered to be the first step a peptide must experience prior to crossing the bilayer (Figure 1.3). Most CPPs are cationic\textsuperscript{3} making it is easy to conceptualize peptide electrostatically interacting with the outer-leaflet of the membranes containing negatively charged constituents such as glycoconjugates. Further partitioning of peptides into the interfacial domain of the outer-leaflet are less reliant on electrostatic interactions due to the eukaryotic cell maintaining an asymmetric distribution of negatively charged lipids located to the inner-leaflet of the lipid bilayer membrane\textsuperscript{15}. The hydrophobic interactions between the peptide and the interfacial domain of the membrane begin to take a more prevalent role due to various solvation states present in the interfacial region. The Wimley-White scale of hydrophobicity measures the whole residue contributions of each amino acid to partition into the highly dynamic interfacial region of the membrane\textsuperscript{14}. This hydrophobicity scale has been validated to explain a possible translocation mechanism for a well-known CPP, TP10\textsuperscript{16}. A peptide sequence can therefore be assessed for its likelihood of having favorable hydrophobic interactions with the interfacial domain of the membrane (the initial step for translocation). Once partitioned into the interfacial domain of the
**Figure 1.3* Partitioning Energetics for Peptide Insertion into a Lipid Bilayer Membrane**

Peptide interact first with the interfacial domains of the lipid bilayer. Interfacial regions are a complex portion of the lipid bilayer where peptides can adopt secondary structure to lessen their energetic costs to further translocate into the hydrophobic core of the membrane.

*Figure taken from:

membrane, a peptide can undergo conformational changes brought on by the unique environment at the membrane interface, which can further lessen the energetic cost of partitioning into the hydrophobic core (Figure 1.3)\textsuperscript{14}. It is here as the peptide partitions into the interfacial region of the membrane where another possible point of distinction between a membrane active peptide behaving as a CPP, pore-forming peptide, or AMP can be made. Peptides that tend to have rigid and defined secondary structures and orientations when interacting with membrane surfaces have been shown to be more lytic than those peptides that are “conformationally polymorphic”\textsuperscript{17,18}. The “Interfacial Activity Model” for membrane active peptides further describes how stable, long-term interactions within the interfacial domain of the membrane can lead to peptide-induced lipid rearrangements resulting in membrane disruption, a characteristic synonymous with pore forming peptides and AMPs\textsuperscript{19}. As mentioned previously, most CPPs are cationic with arginine comprising of the majority of positively charged residues\textsuperscript{20}. Arginine contains a side-chain guanidinium group capable of bidentate bonding between the phosphates of the phospholipid head-groups within the interfacial domain of the membrane (Figure 1.4)\textsuperscript{21,22}.

\textit{The Importance of Arginine for CPP Translocation}

Although charged at neural pH (pK\textsubscript{a} > 12) and highly hydrophilic, arginine can partition into the hydrophobic environment of the lipid bilayer presumably by charge neutralization provided by phosphates of the phospholipid head-groups\textsuperscript{23–25}. CPPs containing arginine likely make bidentate hydrogen bonds to the interfacial phospholipids for direct and energy-independent translocation. A few proposed models describe direct translocation of arginine containing CPPs across the membrane, all of which perturb the membrane with various degrees of potential cytotoxicity\textsuperscript{26}. The least potentially cytotoxic mechanisms of direct translocation involve the
**Figure 1.4** Multidentate Interactions Between Arginine and Phospholipids

The multidentate interactions between arginine side chains with membrane constituents. The multiple hydrogen bond donor/acceptor pairs between the guanidinium group of arginine allow interactions with phosphate from phospholipid head groups as well as glucosylaminoglycans (GAGs).

*Figure taken from:

arginines of CPPs forming inverted micelles within the bilayer membrane, or having lipid molecules chaperone the CPP across the hydrophobic core without the aid of micelle formation (also known as lipid flip-flop)²⁶,²⁷.

CPPs with arginine comprising the majority of their sequence are overwhelmingly taken up by cells via energy-dependent mechanisms, most likely macropinocytosis⁷. The behavior of mostly arginine containing CPPs can take on what appears to be an energy-independent mechanism of cell penetration, however, these results are obtained by increasing peptide concentration to cause extracellular entry of calcium²⁸ and cause a high degree of membrane defects³⁹. Previous work has shown that increasing the hydrophobicity of arginine-rich CPPs with a terminal hexanoic acid moiety resulted in direct translocation across a lipid bilayer³⁰. A delicate balance between hydrophobic and electrostatic interactions with the membrane must be achieved by a CPP to avoid a single mechanism of cell penetration that is reliant on a specific peptide concentration or experimental condition.

**Peptide Interactions with Other Cell Membrane Structures**

Membrane constituents other than lipids such as proteins, cholesterol and glycosaminoglycans (GAGs) effect the ability of a CPP to translocate across a cellular membrane. GAGs allow electrostatic clustering of polycationic CPPs on the membrane surface and depletion of GAGs from the cell surface greatly reduces the ability of several CPPs to cross the cell membrane³¹,³². Depletion of cholesterol from the cell membrane with cycloextrin removes liquid-order (lipid-raft) domains on the membrane surface resulting in a more fluid membrane. Less rigidity of the membrane has been reported to decrease translocation³³, however, this observation may be an artifact due to cycloextrin interfering with the available peptide in the experiment³¹. Digestion of membrane surface proteins with protease followed by treatment of CPPs showed significant loss
of translocation, suggesting that the presence of membrane proteins are important for CPP translocation\textsuperscript{34}.

\textit{Peptide Interactions with Local Alterations to the Cell Membrane}

The physical state of the membrane can affect peptide translocation. Oxidative stress causes the lipid bilayer to change its biophysical properties resulting in an increase of translocation for polycationic (thus the vast majority of) CPPs\textsuperscript{35}. The lipids of the cell membrane can undergo peroxidation to produce negatively charged head groups and shorter acyl chain lipids that lead to both increased electrostatic interactions with CPPs as well as altered packing/organization of the bilayer; both facilitate translocation across the membrane\textsuperscript{36}. Local sites of peptide aggregation on the membrane can induce physical curvature changes to the membrane\textsuperscript{37}. CPPs able to induce an outward budding or positive curvature of the membrane are observed to directly penetrate cells with increased efficiency\textsuperscript{38,39}.

\textbf{Spontaneous Membrane Translocating Peptides (SMTPs)}

Methods for designing CPPs specifically for energy independent (i.e. spontaneous) cellular entry are under investigated. The proposed utility for CPPs as cargo delivery agents relies on the mechanism by which the peptide gains access to the cell interior. In particular, avoidance of the degradative pathways associated with energy-dependent mechanisms of entry via lysosomal degradation should be a desired trait for the delivery of many types of cargoes with CPPs. Rational design of a CPP that spontaneous translocates across the lipid bilayer is a difficult task due to the lack of sequence homology among CPPs responsible for a given mechanism of entry as well as the possibility of creating a lytic cytotoxic peptide.

Previously, spontaneous or energy-independent translocating peptides were selected from a peptide library synthesized using a split and recombine synthesis strategy. The peptide library
contained over 10,000 unique 12-residue peptides and was screened for members that translocated across synthetic lipid bilayer vesicles without permeabilization. The design of the peptide library included several examples of CPPs shown to have both energy-dependent and energy-independent mechanisms of cellular entry. The screen simultaneously measured peptide translocation and membrane perturbation in a synthetic lipid bilayer system devoid of any cellular energetics. Those peptides that showed high translocation and low membrane perturbation were deemed Spontaneous Membrane Translocating Peptides (SMTPs). Capabilities of SMTPs to deliver cargo into cells was initially assayed by conjugating a fluorescent cell impermeant dye, TAMRA, with multiple SMTPs found from the screen. Dye conjugated to SMTPs showed diffuse cytosolic distribution under both physiological and non-physiological temperatures, suggestion spontaneous delivery of the cargo. A SMTP discovered from the original screen was also shown to deliver a peptide cargo consisting of an α-helical portion of the green fluorescent protein (GFP) into cells that expressed a truncated GFP which once again fluoresce by incorporating the missing α-helical peptide supplied by the SMTP.

Further biophysical characterization of a SMTP found from the screen, TP2, revealed that this class of peptides can penetrate the lipid bilayer without significant membrane perturbations. Steady-state partitioning of TP2 into POPC membranes was weak with a loosely defined β-sheet secondary structure upon partitioning into lipid membranes. Neutron diffraction of deuterated TP2 showed that the peptide was distributed closer to the lipid bilayer center while not causing spacing alterations of the membrane. This type of behavior is contrast to other membrane active peptides classified as pore forming and antimicrobial peptides that have a high degree of lipid rearrangements and interfacial activity. TP2 and other SMTPs found from the same screen thus
possess the necessary physical chemical interactions with the lipid bilayer for spontaneous translocation to occur.

Interestingly, most SMTPs found from the screen possessed a common **LRLLR** motif. The design of the peptide library allowed for a variety of other amino acids resulting in 144 different motifs at the positions where the **LRLLR** motif was present. This motif is present in nature as part of the voltage sensing S4 helix of potassium voltage gated ion channels which is proposed to translocate into the bilayer upon voltage gating\(^43\). The segment of the S4 helix alone was shown to spontaneously translocate into synthetic vesicles with an impermeant polar cargo\(^43\). Therefore, the presence of a common motif in most of the SMTPs found from the screen provides a possible minimal peptide sequence capable of spontaneous translocation\(^42\). Efforts to rationally design improved SMTPs could focus on using the **LRLLR** motif as promising starting point which was derived from a nonbiased screen for peptides that crossed a synthetic bilayer spontaneously. Alternative strategies less reliant on rational design to find additional SMTPs are possible by creation of another peptide library modeled after the results of the original SMTP screen. This iterative process of finding more gain-of-function variants from results of previous library hits is referred to as “synthetic molecular evolution”\(^44,45\).

**Therapeutic Opportunities for CPPs; The End Goal**

Drug design strategies for intracellular targets must consider the barrier the cell membrane presents for any possible therapeutic compounds. Current high-throughput drug design strategies manage the barrier posed by the cell membrane by adopting a set of criteria known as the “Lipinski Rule of 5”\(^46\) which predicts therapeutic compounds that passively cross the lipid bilayer. The “Rule of 5” describes molecules that have less than 5 hydrogen bond donors, less than 10 hydrogen bond
acceptors, an octanol/water partitioning LogP value less than 5 and less than 500Da in size as being ideal compounds for drug design.

Unfortunately, consideration of only those therapeutic compounds that can cross the membrane passively hampers drug design strategies for other classes of molecules that do not cross the membrane passively. Useful bioactive molecules such as large polar compounds, peptides, DNA/RNAs, antibodies, proteins, etc. are often abandoned for possible drug design for intracellular targets. Reliance on smaller lipophilic molecules for drug design sets a finite limit of possible drug candidates. Since smaller compounds also result in fewer possible interactions with their targets when compared to larger molecules, small lipophilic molecules also have unintended off-target interactions which may lead to side-effects. The expanded annotation of the human proteome reveals ~20,000 proteins are encoded from our genome. Of these 20,000 proteins encoded by our genes, an even smaller number possess ideal binding pockets that small lipophilic molecules heavily rely on for drug interactions, commonly referred to as the “druggable genome”. Thus, CPPs can serve drug design strategies by expanding candidate drug compounds to include larger polar molecules with more target interactions and overall improved therapeutic outcomes. This end goal will most likely require CPPs such as SMTPs that can translocate the cell membrane barrier safely and directly that are not sequestered/degraded inside of an endosome.

The discovery of additional SMTPs by using the results from the original screen as a guided rational design was attempted. This guided rational design strategy offers a technique able to produce peptide sequences that from their conception have a known mechanism of entry that involves energy-independent translocation. Recognizing the presence of a common motif of LRLLR in the majority of the peptides found from the original screen for SMTPs provided a starting point to assess if the LRLLR motif was necessary and sufficient for spontaneous translocation and
if the possible positioning of arginines produced motifs with enhanced translocation properties (Chapter 2). Understanding the minimal motif capable of spontaneous translocation across a lipid bilayer could yield design considerations for enhanced SMTPs which would further the repertoire of peptides in this underrepresented class of CPPs. Additional discovery of SMTPs are paramount for the expansion of drug delivery vehicles that can transport cargoes directly into the cell. Testing how SMTPs cross the lipid bilayer is equally important to understand further design considerations for additional SMTPs (Chapter 3). There is a current lack of understanding about the processes involved for direct energy-independent translocation of peptides across the bilayer. Further understanding of how SMTPs directly translocate across a lipid bilayer allows for characterizations necessary to identify other peptide sequences that may possess the same unique properties. The majority of cargoes tested with CPPs are biologically inert with the majority of cargoes consisting of fluorophores. Delivering useful impermeant cargoes that illicit a bioactive response is therefore more useful to the clinical setting. The limitations of delivering a bioactive cargo with the aid of SMTPs is explored in Chapter 4.

Novel high-throughput screens of peptide libraries can enable the discovery and characterization of additional peptide sequences that have the unique cell penetrating properties of SMTPs (energy-independent translocation without membrane disruption). Elucidating a CPP mechanism of entry prior to development of the peptide as a therapy is sufficient to understand how a potential cargo affects the physical-chemical properties of the peptide. As covered in Chapter 5, designing a peptide library screen capable of identifying peptide sequences that can cross a lipid bilayer without the attachment of a cargo would generate peptide sequences that are intrinsically cell permeable and more importantly possess the properties of SMTPs. Generation of peptide sequences that alone, without the influence of a cargo, directly translocate across a lipid
bilayer begins the process of understanding the limitations for cargo delivery with SMTPs. Therapeutic compounds with similar structure and properties could be classified as promising cargoes for SMTPs found from this screening approach while still maintaining a direct delivery across cell membranes. Conversely, a cargo-centric screen design also proposed in Chapter 5 can be performed. A specific therapeutic is associated with a myriad of peptides, screened, and mechanistic studies are performed after positive hits are found. Ultimately, characterization of additional SMTPs and CPPs with similar mechanisms of cellular entry can yield more novel therapeutics by expanding drug design strategies to include more compounds commonly avoided due to their unfavorable properties for permeability.
CHAPTER 2

A Common Motif from Spontaneous Membrane Translocating Peptides (SMTPs) for Directed Rational Design of Enhanced SMTPs.

Overview

Mechanism of entry is an important feature for a cell penetrating peptide (CPP) used for intracellular cargo delivery. Energy-dependent mechanisms of entry rely on cellular energetics for CPP uptake into the cell which involves some form of endocytosis\textsuperscript{51}. CPPs that rely on energy-dependent mechanisms of entry are faced with the additional problem of endosomal escape before being degraded by lysosomal fusion or further sequestration from the interior of the cell\textsuperscript{52}. Energy-independent mechanisms of entry or direct translocation across the cell membrane bypass the potentially degradative pathway associated with energy-dependent entry\textsuperscript{51}. The lack of sequence homology among CPPs makes rational design of an energy-independent spontaneous membrane translocating peptide (SMTP) a very difficult task\textsuperscript{53}. Efforts have been made to predict, \textit{in silico}, peptide sequences for their likeliness of possessing CPP properties, however, these predictive algorithms fall short at predicting the mechanism of entry of a proposed CPP\textsuperscript{54,55}. An ideal CPP for cargo delivery would utilize energy-independent mechanisms of entry to deliver intact cargo directly across the membrane without significant membrane perturbations to avoid general cytotoxicity.

Discovery of one family of peptides that rely on direct or spontaneous translocation across lipid bilayer membranes was accomplished by screening a peptide library that included several sequences of known CPPs\textsuperscript{56}. The peptide library consisted of over \textasciitilde10,000 unique members.
Library peptides were assayed for their ability to cross synthetic lipid bilayer membranes of vesicles without causing vesicle lysis. The screen provided several peptide sequences named after their mechanism of entry, **Spontaneous Membrane Translocating Peptides**, or SMTPs.

Interestingly, most of the peptides found from the screen for SMTPs had a common **LRLLR** motif in the same sequence position even though the library design allowed for 144 different amino acid sequences in that position and allowed for the **LRLLR** motif in several different positions. This **LRLLR** motif was also found in nature to interact with and partition into cell membranes as part of the S4 voltage sensing helix of voltage gated potassium channels\(^{43}\). A directed rational design strategy for improved SMTP was hypothesized to include the **LRLLR** motif given that this motif was found multiple times from the peptide library screen as well as in nature\(^{42,57}\). Having a minimal motif for spontaneous translocation would allow for rational design of an improved SMTP for instance by using the minimal motif as a cassette to be concatenated as needed to deliver a cargo.

The **LRLLR** motif and several arginine spacing variants of the motif were synthesized to test if the **LRLLR** motif was necessary and sufficient for spontaneous translocation and to test how the placement of arginines in the motif affected translocation (Table 1). Alteration of arginine spacing along the backbone of other CPPs was shown to influence the rate of peptide translocation into cells\(^{22}\). More recently, a “charge-distribution hypothesis” was proposed to explain the importance of cationic charge placement, particularly arginine, within a peptide sequence to lower the energy barrier for translocation\(^{58}\). Optimal placement of arginines can facilitate favorable peptide interactions with the interfacial region of the membrane by establishing hydrogen bonds between the guanidinium sidechain of arginine and the phosphates from phospholipid headgroups\(^{27,58,59}\). Further, optimal arginine placement within a peptide sequence can also lend to
<table>
<thead>
<tr>
<th>Peptide</th>
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<th>Symbol</th>
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<tbody>
<tr>
<td>TP2</td>
<td>PLIY-LRLLR-GQWC</td>
<td>X</td>
</tr>
<tr>
<td>ONEG</td>
<td>PLGR-PQLRR-GQWC</td>
<td>O</td>
</tr>
<tr>
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<td>LRLLR-WC</td>
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<tbody>
<tr>
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</tr>
<tr>
<td>LRLRL-WC</td>
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<tr>
<td>RLLLR-WC</td>
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<tr>
<td>RRRLL-WC</td>
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<tr>
<td>LLLLRR-WC</td>
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**Table 1**: A list of the arginine spacing motif variants with cationic arginines displayed in blue.

TP2 is a SMTP found from a peptide library screen. ONEG was also discovered in the same screen, however, it was a sequenced observed not to translocate and serves as a negative control for translocation. All peptides had a tryptophan (W) added for ease of quantification and a C-terminal cystine (C) for labeling with sulfhydryl reactive dyes (i.e. IANBD).
self-interactions by the formation of salt-bridges between the side chain of arginine and/or C-terminal carboxyl groups or other acidic residues\textsuperscript{58,60}. Therefore, alteration of arginine positioning around the highly conserved LRLLR motif could result in a motif with altered membrane translocation. Motif variants that show an enhanced translocation could serve as modular building blocks that once concatenated may yield additional SMTPs.

**Materials and Methods**

*Synthesis of LRLLR Motif Variants*

Peptides from Table 2.1 were synthesized on TentaGel S-Ram polystyrene resin (Rapp Polymere). FMOC-protected amino acids, were dissolved in DMF with N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate, (HBTU), hydroxybenzotriazole (HOBt) and diisopropylethylamine. Couplings were done at a 4–fold molar excess relative to the stated loading capacity of the resin, and were continued for 1 hour at room temperature. Fmoc removal was done with 30 % v/v piperidine in DMF for 15 minutes at room temperature. Ninhydrin colorimetric assays were performed after each coupling and Fmoc removal step to monitor completion. Peptide deprotection and cleavage from the resin was done with Reagent B (TFA/phenol/water/TIPS (88/5/5/2) % v/v) for 4 hours, starting at 0\textdegree C and warming to room temperature. Crude peptide was lyophilized from glacial acetic acid and purified via reverse phase HPLC. Peptide identity was verified with MALDI-TOF mass spectrometry. Labeling of peptides with nitrobenzoxadiazole (NBD) was achieved by dissolving purified peptide in DMF with 2 molar excess of NBD-maleimide for 2 hours at room temperature. Labeled peptides were then purified via HPLC using NBD fluorescence, resuspended in HFIP and concentrations determined using the extinction coefficient for the NBD fluorophore (19,500 M\(^{-1}\) cm\(^{-1}\)).
**Generation and Purification of Large Unilamellar Vesicles with Entrapped Chymotrypsin**

1-palmitoyl-2-oleoyl-3-sn-glycerophosphatidylcholine (POPC) lipids (Avanti Polar Lipids) were used to make large unilamellar vesicles (LUVs) by drying down a desired amount of lipids from their chloroform stock solutions under vacuum for at least 2 hours at room temperature. Once lipids were completely dried, a solution of 1 mg/mL TLCK-treated chymotrypsin (bovine, Sigma-Aldrich) was dissolved in buffer (10 mM HEPES, 40 mM NaCl, 1 mM EDTA, pH 7.4) to a desired concentration. The lipid and chymotrypsin solution was freeze-thawed 10 times using liquid nitrogen plunges followed by 10 passes through a pressurized lipid extruder equipped with a 100nm pore-sized membrane (Whatman). The resulting product contains chymotrypsin entrapped LUVs. The LUVs were then placed in a 100,000Da MWCO spin filter to rid the LUV preparation of free (un-trapped) chymotrypsin. Flow-through from the spin filters were collected while the LUVs were replenished with fresh buffer. Assaying the LUV prep for suitable removal of free chymotrypsin was performed on the flow-through collections by using a BODIPY-fluorescein labeled casein (EnzChek Kit, Life Technologies) which becomes highly fluorescent after protease degradation (Figure 2.1). Lipid concentrations were then determined by Stewart Assay\textsuperscript{61}.

**Translocation Experiments**

Details of a translocation experiment are illustrated in Figure 2.2A and are detailed by the following procedure: All NBD-labeled peptides were prepared in 100μL of buffer (10mM HEPES, 40mM NaCl, 1mM EDTA, pH 7.4) and analyzed on a reverse phase HPLC equipped with a C-18 column to acquire a retention time for whole and intact peptide based on NBD fluorescence (ex./em. 460/540nm). In addition, all NBD-labeled peptides were prepared in 100 μL of buffer with nM amount of chymotrypsin and analyzed on reverse phase HPLC to acquire a retention time
Figure 2.1 Chymotrypsin Entrapped LUV Purification Verification with EnzChek Assay

Results from an “EnzChek” assay for the presence of chymotrypsin. Casein, a milk protein, is labeled with fluorescent BODIPY dye on multiple amines (BODIPY-casein). The dye is significantly self-quenched until protease begins to degrade the casein resulting in an increase in BODIPY fluorescence. Chymotrypsin is first diluted to 1 mg/mL (“Before Separation” sample) and then entrapped in POPC large unilamellar vesicles (CM-LUVs). CM-LUVs are eluted through a size exclusion column to remove most external chymotrypsin. This is followed by placement of vesicles into a 100,000Da MWCO spin column for removal of residual enzyme. The subsequent flow-throughs are tested for the presence of chymotrypsin using the fluorescently labeled BODIPY-fluorescein casein until little change in BODIPY fluorescence is detect in the flow-through.
for cleaved NBD-labeled peptide based on NBD fluorescence of the C-terminal cleavage product (Tryp-Cys-NBD). LUVs with chymotrypsin entrapped that were purified of external chymotrypsin as outlined above were diluted to a concentration of 1 mM in 100 μL of buffer. Several samples of 1 mM purified LUVs with chymotrypsin entrapped were lysed with .1 % v/v reduced-Triton (Sigma-Aldrich) followed by incubation with α-1-antitrypsin (bovine, Sigma-Aldrich) at various concentrations for 30 minutes room temperature. These solutions were then added to separate v-bottomed HPLC vials each containing 2 μM of NBD-labeled peptide and incubated for at least 6 hours room temperature. Samples were then analyzed on a reverse phase HPLC equipped with a C-18 column to detect if peptide was cleaved by chymotrypsin at the C-terminal tryptophan by monitoring a shift in retention time due to the production of the NBD-labeled cleavage product. The samples that did not show a cleavage product (i.e. NBD-labeled peptide incubated with lysed vesicles and α-1-antitrypsin for 6 hours showing the identical retention time as NBD-labeled peptide alone) established an effective concentration of α-1-antitrypsin needed to inhibit all chymotrypsin activity present in 1 mM of purified LUVs with chymotrypsin entrapped and referred to as the blockage sample (Figure 2.2A). Translocation samples consisted of 1 mM purified LUVs with chymotrypsin entrapped that were incubated with the lowest concentration of α-1-antitrypsin found to inhibit all chymotrypsin activity in the blockage sample for 30 minutes at room temperature in a 1.5 mL Eppendorf tube. This solution was then added to a desired concentration of NBD-labeled peptide in a v-bottom HPLC vial for a desired incubation time and then analyzed on reverse phase HPLC and monitored for chymotrypsin cleavage of the NBD-labeled peptide. Translocation samples were designed to block any possible remaining chymotrypsin free in solution by the addition of an effective α-1-antitrypsin concentration while active chymotrypsin remained entrapped inside LUVs to cleave peptide that translocated across
the lipid bilayer of the LUV (Figure 2.2B). Cleavage samples were produced by lysing 1mM of purified LUVs with chymotrypsin entrapped with 0.1% v/v reduced-Triton in 100μL of buffer followed by addition to a v-bottom HPLC vial containing NBD-labeled peptide and incubated for 5 minutes. Cleavage samples were then analyzed on reverse phase HPLC to ensure that chymotrypsin remained active by detection of complete retention time shift of the NBD-labeled peptide.

*Measuring Translocation Rates*

Motif variants were incubated with 1mM of purified LUVs with entrapped chymotrypsin at various timepoints and peptide to lipid ratios and then analyzed on reverse phase HPLC equipped with a C-18 column to detect peptide cleaved by chymotrypsin at the c-terminal tryptophan. The % uncleaved peptide was determined for each timepoint by taking area under the curves from the HPLC chromatographs of the cleavage product and whole peptide by the following: ((Cleaved_{Area} / Whole_{Area}) * 100) (Figure 2.3A). Rates of translocation were calculated by fitting a one-phase decay equation to the % Uncleaved peptide as a function of incubation time (Figure 2.3B)

*Measuring Membrane Permeability*

A fluorophore (ANTS, ThermoFisher) and its quencher (DPX, ThermoFisher) at a molar ratio of 1:4, respectively in buffer (10mM HEPES, 40mM NaCl, 1mM EDTA, pH 7.4) was entrapped in LUVs by subsequent freeze-thaws using liquid nitrogen plunges. ANTS/DPX entrapped in LUVs were then placed on a size exclusion column (Sephadex-G75) to separate any external dye and quencher from the LUVs by collecting the faster migrating LUV fractions. Lipid concentrations of the fraction collected were then determined by Stewart Assay. ANTS/DPX entrapped in LUVs were then diluted in buffer and serial diluted in a 96-well plate followed by
A.  

<table>
<thead>
<tr>
<th></th>
<th>“Translocation” Sample</th>
<th>“Blockage” Sample</th>
<th>“Cleavage” Sample</th>
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</thead>
<tbody>
<tr>
<td>CM-LUV</td>
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</tr>
<tr>
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<td>+</td>
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<tr>
<td>Reduced Triton</td>
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</tbody>
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B.  

HPLC Detection

Peptide

Antitrypsin 46kDa

Cleaved Peptide

Chymotrypsin 25kDa

Large Unilamellar Vesicle 100nm
Figure 2.2 Outline of A Translocation Assay Using Chymotrypsin Entrapped LUVs

A. Outline of the samples needed to complete a measurement of translocation. Vials containing LUVs with entrapped chymotrypsin are incubated for 30 minutes with each scenario outlined. B. Schematic of a translocation experiment and detection of peptide cleavage on HPLC. This is actual raw data presented for an SMTP, TP2, translocating into chymotrypsin entrapped POPC-LUVs. The cleavage peak (RT = 13.08 min.) increases as a function of time. The % cleavage is calculated by taking the ratios of the area under the curve for cleaved peptide and whole peptide (RT = 15.77 min.).
addition of a fixed concentration of peptide to generate several peptide to lipid ratios for each peptide tested. Peptides were incubated with LUVs for 1 hour at room temperature and assayed on a plate reader (Bio-Tek Synergy II) to measure membrane permeation by monitoring an increase in ANTS fluorescence. Maximum membrane permeation was achieved by either the addition of .1 % reduced-Triton v/v or a known potent pore forming peptide, mellitin (Figure 2.4).

**Confocal Microscopy of Motif Variants, TP2 and ONEG**

CHO cells were seeded on 8-chamber glass coverslip slides (Labtek-II, NUNC) with 3x10⁴ cells/well in full media (DMEM, 10 % FBS, 1x NEAA and 1x antibiotic-antimycotic) and grown overnight in a 37°C incubator supplied with 5 % CO₂. Cells were then washed of full media with serum-free DMEM and incubated with serum-free DMEM that contained NBD-labeled peptides for 45 minutes in a 37°C incubator supplied with 5 % CO₂. Cells were then treated with 2 μg/mL of TAMRA-wheat germ agglutinin immediately before imaging on the microscope to ensure visualization of the cell membrane. All images were collected on a Leica SP2 confocal microscope (Figure 2.5).

**Circular Dichroism Measurements for the LRLLR Motif and Motif Variants**

NBD-labeled peptides could not be tested under conditions used for circular dichroism (CD) due to spectral interference caused by the presence of the NBD fluorophore. Therefore, all peptides studied using CD needed to be free of the NBD label and contain an alkylated cystine by reacting the cystine containing peptides with iodoacetamide (1:1 molar ratio in DMF and 1% v/v DIPEA, 1hr room temperature) followed by HPLC purification. Alkylation of the cystine prevents the formation of disulfide bridges and allows for spectral analysis of monomeric peptide. Alkylated peptides were diluted in buffer (10 mM Phosphate, 10 mM NaCl, pH 7.2) at a concentration of 30μM followed by an initial spectral analysis using 260-190nm scan at 1nm/sec intervals in a 1mm
quartz cuvette. Separate samples containing both 30 μM peptide and differing concentrations of POPC-LUVs in buffer (10 mM phosphate, 10 mM NaCl, pH 7.2) were analyzed on CD to achieve various peptide to lipid ratios. Ellipticity measurements were then converted to mean residue ellipticity by calculating the degree ellipticity per peptide bond (7 residues) and plotted (Figure 2.6).

Measuring Membrane Partitioning

POPC LUVs were prepared as previous described above in buffer (10 mM HEPES, 40 mM NaCl, 1 mM EDTA, pH 7.4). 10 mM of LUVs were added to one side of an equilibrium dialysis chamber (Harvard Apparatus) separated by a 100,000Da MWCO filter while 2 μM of a NBD-labeled motif variant was added to the other side. Dialysis chambers were placed on a rocker for at least 24 hours at room temperature. Samples were taken from each side of the filter and ran separately on HPLC to determine the mole fraction of peptide associated with lipids or remaining in bulk solution62 (Figure 2.7).

Measuring Translocation Rates of Concatenated Motif Variants

Two motif variants of the LRLLR motif were synthesized as concatemers using peptide synthesis methods described previously. The concatemers were labeled with IANBD and purified on HPLC. Translocation rates were determined as previously described (Figure 2.8).

Confocal Microscopy of the Concatenated Motif Variants

NBD-labeled concatemers of motif variants were incubated with CHO cells as previously described for the monomeric forms of the motif variants (Figure 2.9)

Results

Arginine Spacing of the LRLLR Motif Resulted in Variable Spontaneous Translocation Rates Across POPC-LUVs

Spatial rearrangement of arginines within the parent LRLLR motif resulted in variable
**Figure 2.3 Translocation of LRLLR Motif Variants into POPC-LUVs**

A. The results from a complete set of motif and motif variant peptides assayed for spontaneous translocation across chymotrypsin entrapped POPC-LUVs at a peptide to lipid ratio (P:L) = 100.

B. Plotted data using one-phase decay kinetics to display the rate of translocation for P:L of 1:100, 1:500 and 1:1000.
translocation rates across POPC bilayers of LUVs with entrapped chymotrypsin (Figure 2.3). Two additional peptides were included with the LRLLR motif and the arginine spacing variants. TP2, an SMTP found from the original screen by Marks, et.al., provided a positive control for spontaneous translocation. ONEG, a sequence also found from the original SMTP screen that was shown to neither translocate nor cause membrane permeability, provided a negative control for spontaneous translocation.

The LRLLR motif was observed to translocate across the membrane at similar rates to the parent peptide, TP2. A couple of the motif variants translocated with faster rates than that of the parent LRLLR motif (Figure 2.3B). RLRLL and LLRLR displayed increased translocation rates at a peptide to lipid ratio (P:L) of 1:100. All other motifs translocated with decreased rates with LRLRLR resulting in the slowest translocation rate at a P:L of 1:100. All motifs showed a substantial decrease in rates of translocation when the P:L was increased (decreasing peptide concentration). Interestingly, the opposite behavior was not observed with TP2. As the P:L ratio increased, the rate of translocation for TP2 increased.

**Motif Variants Did Not Result in Membrane Permeability**

Membrane permeabilization was measured to assess if the motif and motif variants previously observed to translocate across POPC-LUVs did so without a high degree of membrane disruption. Results that indicated a high degree of membrane permeabilization would most likely indicate a peptide with potentially pore-forming characteristics which would not correspond to the desired characteristics of SMTPs. Membrane disruption due to lipid bilayer defects caused by peptides can be measured by loading POPC LUVs with ANTS/DPX. If membrane disruption occurs, the ANTS will leak out and separate from its collisional quencher DPX resulting in increased ANTS fluorescence. Melittin, a potent pore forming peptide from Honey Bee venom,
Figure 2.4 Motif Variants Tested for Permeabilization of POPC-LUVs

Membrane permeabilization measured with entrapped ANTS/DPX POPC-LUVs. The % leakage is determined by the ratio of ANTS fluorescence of peptide treatment to 100 % leakage as determined by addition of detergent (Triton). Melittin is a potent pore former from Honey Bee venom and serves as a positive control for membrane permeabilization.
caused significant leakage and high ANTS fluorescence across most of the P:L tested, as expected (Figure 2.4). Motif variants, TP2 and ONEG incubated with LUVs entrapped with ANTS/DPX showed very little ANTS fluorescence, suggesting that the peptides do not cause significant membrane disruption and confirm that the translocation observed previously was not a result of membrane permeability.

*In vitro Translocation of Motif Variants Corresponded with Translocation Rates in POPC-LUVs*

CHO cells were incubated with two NBD-labeled motif variants that showed the highest rate of translocation, *RLRLL*, and the lowest rate of translocation, *LRLRL*, as observed in POPC-LUVs (Figure 2.3B). Also included were the NBD-labeled positive control for translocation, TP2, and the negative control for translocation, ONEG. TAMRA labeled wheat germ agglutinin was used to stain the exterior of the cell membrane to properly establish the correct plane under confocal conditions. The *RLRLL* motif variant which showed the highest rate of translocation across POPC-LUVs was observed diffuse as well as punctate in the cytosol of the treated cells after 45 minutes of incubation (Figure 2.5). TP2 treated cells showed diffuse cytosolic staining similar in appearance to that of the cells treated with the *RLRLL* motif variant. Conversely, the motif variant that showed the lowest rate of translocation in POPC-LUVs, *LRLRL*, was not present in the cytosol of treated cells and remained at the outer membrane like the results observed for the negative control for translocation, ONEG (Figure 2.5).
Figure 2.5 Confocal Images of LRLLR Motif Variants Treated CHO Cells

Confocal microscopy images in CHO cells treated with the motif variants that showed the highest rate of translocation, RLRLL and the lowest rate of translocation, LRLRL. TP2 and ONEG were used respectively as a positive and negative control for translocation. All peptides were labeled with a small fluorescent probe, NBD (green) and the cell membrane labeled with wheat germ agglutinin-TAMRA (red). Cells were treated with a final peptide concentration of 2 μM for 45 minutes at 37°C.
Circular Dichroism Measurements of the Motif and Motif Variants Did Not Result in Defined Secondary Structure When Lipid Bilayer was Present

The possibility of secondary structure formation was hypothesized to differ among the motif variants due to the change in position of the arginines which could lead to a conformational change in the presence of lipid membrane. Therefore, circular dichroism (CD) measurements were conducted on the motif and two motif variants in the presence of POPC-LUVs. The motif and the two motif variants did not possess a defined secondary structure in buffer alone as indicated by the mean residue ellipticity minima occurring at 205nm, indicative a peptide with random coil conformation (Figure 2.6). The addition of POPC bilayer did not result in a conformational change in any of the peptides analyzed as the minima representing random coil conformation did not change (Figure 2.6).

Partitioning Coefficients Do Not Correlate with Translocation Rates of Motif Variants

Partitioning into the interfacial domain of the membrane is the initial step required for a peptide to translocate across the lipid bilayer\textsuperscript{14}. Peptides that partition into the interfacial domain of the membrane may result in an increased translocation rate. Equilibrium dialysis can establish the preference a peptide has for lipid bilayer partitioning versus remaining in bulk solution. The motif variants were observed to have variable partitioning coefficients when POPC-LUVs were present, however, the peptides observed to have higher partitioning coefficients did not correlate to higher rates of translocation (Figure 2.7). The motif that showed the highest partitioning coefficient ($K_s = 4.10 \times 10^5 \pm 0.730 \times 10^5$ vs. TP2 $K_s = 0.502 \times 10^5 \pm 0.462 \times 10^5$), \textit{RLLLL}, resulted in one of the lowest translocation rates ($k_T = 0.149 \pm 0.054$ vs. \textit{RLRLL} $k_T = 1.47 \pm .293$). The peptides that translocated with the highest rates, \textit{RLRLL}, \textit{LRLLR}, and \textit{LRLLR} all had varying but similar partitioning coefficients ($K_s = 2.230 \times 10^5 \pm 0.814 \times 10^5$, $1.12 \times 10^5 \pm 0.489 \times 10^5$, and $2.57 \times 10^5$)
Figure 2.6 CD Measurements of LRLLR Motif Variants with POPC-LUVs

Circular dichroism performed on the motif and some motif variants. The spectra did not show any discernable secondary structure in buffer or in the presence of POPC-LUVs.
The diagram shows the relationship between the partition coefficient ($K_x$) and the translocation rate ($k_T$). Various symbols represent different values of $r$ and $p$, with $r = 0.1030$ and $p = 0.8083$ indicated.
**Figure 2.7 Translocation Rate vs. Partitioning Coefficient for the LRLLR Motif Variants**

Comparison between translocation rates with partitioning coefficients as determined by equilibrium dialysis and calculated for mole fraction bound to POPC-LUVs. The green point represents the *LRLLR* motif while the red dot represents TP2. No correlation was observed (Pearson $r = .1030$, $p > .05$).
Conversely, the peptides that translocated with the lowest rates, \( RRLL, RLLR \), and \( LRLR \) showed a much wider distribution of partitioning coefficients (\( K_x = 2.81 \times 10^5 \pm 0.250 \times 10^5, 4.10 \times 10^5 \pm 0.730 \times 10^5, \) and \( 0.622 \times 10^5 \pm 0.489 \times 10^5, \) respectively).

**Concatenation of Motif Variants Differed in Translocation Rates Versus Monomeric Motif Variants**

The motif variant shown to have the highest rate of translocation as a monomer, \( RLRLL \), was concatenated ([\( RLRLL \)\(_2\)]) to test if the longer peptide maintained similar translocation rates. Similar or higher rates of translocation between the monomeric and concatenated motif variant would prove some modularity exists to create SMTPs by repetitive addition of the highest translocating motif variant. The motif variant with the lowest translocation rate as a monomer, \( LRLRL \), was also concatenated ([\( LRLRL \)\(_2\)]) to test the opposite effect. Both concatenated peptides were labeled with NBD at the C-terminus and tested using POPC-LUVs with entrapped chymotrypsin with identical conditions as their monomeric forms to calculate the translocation rates (Figure 2.8). The concatenation of the \( RLRLL \) motif variant did not result in a peptide that had a similar or higher rate of translocation when compared to its monomeric form (\( k_T = 0.138 \pm .009 \) vs. monomeric \( k_T = 1.47 \pm .293 \)) at a P:L = 1:100. Concatenation of the \( LRLRL \) motif variant did result in a peptide that had a higher rate of translocation when compared to its monomeric form (\( k_T = 0.531 \pm .103 \) vs. monomeric \( k_T = .097 \pm .009 \)) at a P:L = 1:100. Both concatenated motif variant peptides had lower rates of translocation when compared to the positive control for translocation, TP2 (\( k_T = 0.869 \pm .023 \), Figure 2.8).

**In vitro Translocation of Concatenated Motif Variants**

Both NBD labeled concatenated motif variants showed both diffuse and punctate cytosolic staining of treated CHO cells as determined by confocal microscopy (Figure 2.9). The degree of translocation across the cell membrane was indistinguishable from the two concatenated motif
Figure 2.8 Translocation Rates for Concatenated LRLLR Motif Variants

Translocation rates for concatenated motif variants into POPC-LUVs at a P:L = 100.
Figure 2.9 Confocal Images of Concatenated LRLLR Motif Variants with CHO Cells

CHO cells treated with 2 μM of concatenations of the motif variants labeled with NBD (green) for 45 minutes at 37°C. The cell membrane was labeled with wheat germ agglutinin-TAMRA (red).
variants. Concatenating the motif variant observed to have the lowest translocation rate across POPC-LUVs and unable to translocate across cell membranes as a monomer, LRLRL, resulted in a larger peptide that was observed to translocate across the cell membrane.

**Discussion**

The LRLLR motif proved sufficient but not necessary for spontaneous membrane translocation as measured in translocation experiments (Figure 2.3B). Improvement of translocation rates for some of the arginine spacing variants of the LRLLR motif suggests that arginines can be arranged to provide enhanced translocation as hypothesized previous by the “charge-distribution hypothesis”\(^{58}\). Interestingly, a motif variant, RLRLL, that showed an increase in translocation rate relative to the parent motif, LRLLR, was not represented in the original screen for SMTPs\(^{56}\).

The decrease in translocation rates for motif variants at higher P:L ratios suggests that some degree of cooperativity may be necessary for translocation, possibly by self-interactions while translocating across the membrane. If self-interactions are necessary for the motif and motif variants to translocate, it is not due to some highly-organized structure upon membrane interactions as no defined secondary structure was apparent after CD analysis in the presence of motif variants and POPC-LUVs (Figure 2.6). TP2 was observed to have the opposite effect. Previously, it was hypothesized that TP2 translocates as a monomer in a loosely defined β-sheet structure\(^{42}\). The decrease in translocation rate when TP2 peptide concentration was increased suggests that a finite amount of peptide can translocate at one given time due to peptide occupying space within the hydrophobic core of the membrane and possibly increasing lateral pressure throughout the bilayer membrane. Ideally, a SMTP able to translocate across the membrane as a monomer effectively lowers the concentration of peptide needed to cross the membrane which is
a desired characteristic if SMTPs are used as delivery vehicles for therapeutic compounds. The necessity of higher concentration of peptide for increased rates of translocation as observed with the \textit{LRLLR} motif and motif variants does not relate to desired characteristic that TP2 possesses as a peptide able to spontaneously translocate across a lipid bilayer in monomeric form. Thus, using the motif or motif variants as delivery vehicles may require higher concentrations of peptide conjugate to achieve the same effect of an SMTP with similar translocation properties like that of TP2.

Translocation of a peptide across the lipid bilayer membrane requires that the peptide first partition from bulk solution into the interfacial region of the membrane. We hypothesized that a possible design consideration when rationally engineering an improved SMTP should involve selecting peptides that show high partitioning into bilayers because this should result in higher probability of translocation. Therefore, partitioning coefficients were calculated for the motif and all the arginine spacing variants in POPC LUVs and then compared to their translocation rates. Spatial rearrangements of the arginines did result in variable partitioning of the motif variants, however, no correlation existed between the motifs with the highest rates of translocation and the motifs with the highest partitioning coefficients (Figure 2.7). High partitioning coefficients do not necessarily result in peptides with higher translocation rates. One possible explanation could be that peptides with higher partitioning into bilayers may become unable to translocate further into the bilayer because their bilayer partitioning results in the most stable peptide-membrane interaction. Peptides become “stuck” at the membrane interface and do not undergo any further conformational changes or allow lipid reorganizations that could result in further partitioning into the bilayer and ultimately translocation.
The motif variant that showed an increase in translocation rate when compared to the parent \textit{LRLLR} motif was concatenated to test if any modularity of the motif variants exists for an improved SMTP. Motif variants with the highest rate of translocation could provide a cassette where by adding the motif as a modular repeat may result in a SMTP with improved translocation capabilities. As monomers, \textit{RLRLL} was shown to have the highest rate of translocation while \textit{LRLRL} was shown to have the lowest rate of translocation (Figure 2.3B). Concatenation of \textit{RLRLL}, \textit{[RLRLL]}\textsuperscript{2}, resulted in a decrease in translocation rate when compared to its monomeric form (Figure 2.8). Conversely, concatenation of \textit{LRLRL}, \textit{[LRLRL]}\textsuperscript{2}, resulted not only in an increase in translocation rate when compared to its monomeric form, but also an increase in translocation rate when compared to \textit{[RLRLL]}\textsuperscript{2}. Confocal microscopy of cells treated with the concatenated motif variants showed punctate and diffuse staining in the cytosol for both concatenated motif variants (Figure 2.9). Therefore, very little modularity exists to produce an improved SMTP from a monomer shown to translocate. Concatenation of the motifs undoubtedly alters the spatial pattern of the arginines different than that of the monomeric form. For instance, short range electrostatic interactions between the arginines and the C-terminus may be disrupted by lengthening the peptide and resulting in a differential translocation rate.

\textbf{Summary}

Rational design of an improved SMTP proved to be a difficult task despite the identification of a common motif found in several SMTPs previously discovered from a peptide library screen. Several design considerations were tested to yield an improved SMTP. First, the effect of arginine spacing around the parent motif, \textit{LRLLR}, yielded differential translocation rates suggesting that specific placements of arginines within the parent motif can improve translocation. Secondly, high partitioning coefficients did not correlate with high translocation rates for the peptides tested.
Finally, modularity, the ability to take a motif variant and use it as a building block for an improved SMTP, was not apparent. Future attempts to discover additional SMTPs should rely less on rational design and more on techniques described by synthetic molecular evolution\textsuperscript{3,44} where gain-of-function peptides are discovered by subsequent peptide library designs based on previous positive hits from a past screen of a peptide library.
CHAPTER 3

Mechanisms for Spontaneous Membrane Translocating Peptides

Overview

Several models have been proposed to explain the energy-independent mechanism of membrane translocation for CPPs (Figure 3.1). The initial model of direct, energy-independent translocation for a proposed CPP, penetratin, hypothesized the formation of inverted micelles within the lipid bilayers of cells\(^\text{64}\). Cationic residues of the CPP were proposed to bind negatively charged phospholipids present on the cell surface to form an invagination which encapsulates the surface-bound peptide followed by release into the cytosol\(^\text{65}\) (Figure 3.1A). However, the normal eukaryotic plasma membrane maintains an asymmetric distribution of negatively charged phospholipids located mostly on the inner-leaflet of the membrane\(^\text{15}\) while \(^{31}\text{P}-\text{NMR}\) structural studies and other assays that supported the inverted micelle model for direct translocation\(^\text{66}\) used synthetic membrane systems with a high amount of negatively charged phospholipids. A simpler “adaptive translocation” model was later proposed based on the observation that cationic peptides, particularly those containing arginine, partition from water into an octanol phase when incubated with neutral fatty acids\(^\text{27}\) (Figure 3.1C). The transient noncovalent interactions that allow arginine containing CPPs to enter hydrophobic environments of the lipid bilayer are proposed to be the result of the guanidinium group of arginine forming bidentate hydrogen bonding to the phosphates of the phospholipid head groups. This interaction enables chaperoning of the peptide across the bilayer by the bound phospholipids, possibly driven by membrane potential\(^\text{27,67}\). These interactions
**Figure 3.1** Proposed mechanisms for energy-independent translocation.

A. The “inverted micelle” model. B. The “transient pore” model. C. The “adaptive translocation” model.

*This figure was taken from the following publication:

are diminished when CPPs contain arginine variants with methylated guanidinium groups which resulted in less partitioning into hydrophobic environments\textsuperscript{27}. Other cationic residues such as lysine do not have the ability to form bidentate bonds with the bilayer interface which may explain why lysine substitution of arginines in CPPs decreases the translocation efficiency into cells\textsuperscript{68}. In both proposed models, the “inverted micelle” and “adaptive translocation” involve rearrangement of lipids in the bilayer to explain direct translocation while avoiding lipid rearrangements that would involve a significant disruption of the membrane and thus a potentially toxic event in cells. However, the observed mechanism of entry for several CPPs has been shown to be highly dependent on peptide concentration where increasing the peptide concentration appears to result in energy-independent direct entry into cells\textsuperscript{7,69}. This phenomenon is partially explained by the rapid accumulation of peptide on the surface of the cell with increasing peptide concentration. Accumulation on the membrane may trigger “inverted micelle”, “adaptive translocation” or it may lead to formation of membrane defects that cause thinning of the lipid bilayer with subsequent formation of transient pores which enable translocation of the peptide across the pore\textsuperscript{70}. The kinetics of this proposed “transient pore” model are measured using dye-efflux measurements from synthetic vesicles treated with CPPs\textsuperscript{71} and the open and close states of these pores ultimately dictate whether the peptide is toxic to the cell or not\textsuperscript{70}. Interestingly, neutron diffraction studies of TP2, a SMTP, in POPC bilayers showed that the membrane did not appear to be highly perturbed in agreement with a lack of dye efflux from lipid vesicles treated with TP2\textsuperscript{72}. Hence, the mechanism of energy-independent spontaneous translocation of TP2 may not involve the transient pore model.

More recent attempts to understand energy-independent entry of CPPs have relied on computational molecular dynamic simulations of bilayer-peptide interactions. TP2 was modeled
in two separate molecular dynamic simulations tasked with observing the spontaneous translocation across lipid bilayers. The earliest attempt used implicit slab membranes of certain thicknesses and placed TP2 in the bilayer core for each thickness simulated\textsuperscript{73}. The peptide’s tilt angle relative to membrane normal as a function of membrane depth was plotted along with free energy calculations (Figure 3.2B). TP2 was modeled to have a transition state that resembled a beta-harpin structure where its arginines played a crucial role forming salt bridges between the C-terminus and the other to a backbone carbonyl. The arginines also oriented the peptide toward both interfacial domains of the membrane in a snorkeling-type fashion (Figure 3.2A)\textsuperscript{73}. The most recent simulation of TP2 in bilayer membranes used a course-grained force field\textsuperscript{74}. Contrasting the previous simulation, the membrane was allowed to deform upon interaction with peptide. Three SMTPs including TP2 were modeled to all cause membrane deformations with TP2 having the deepest penetration into the lipid bilayer without the formation of a pore\textsuperscript{74}. The positioning of TP2’s arginines within in the *LRLLR* motif provided optimal interactions with the interfacial phosphates of the phospholipid headgroups during membrane deformations\textsuperscript{74}.

A definite mechanism of spontaneous translocation for SMTPs up to this point is still elusive. Given the present biophysical characterization\textsuperscript{72} and molecular simulations of TP2\textsuperscript{73,74}, spontaneous translocation is proposed to not include pore formation. However, membrane defects possibly like those involved with the “inverted micelle” and “adaptive translocation” models could hold true for SMTPs such as TP2. The use of a synthetic vesicles to measure the rates of spontaneous translocation for TP2, the *LRLLR* motif and motif variants was discussed in the previous chapter. In this chapter, synthetic vesicles will be used to elucidate the mechanism of spontaneous translocation.
Figure 3.2* Implicit Membrane Model of SMTP, TP2, Translocation

Results from an implicit membrane model simulation with an SMTP, TP2. A. The proposed transition state conformation of TP2 after several simulations of membrane thicknesses. Nitrogen is depicted in blue while oxygen is depicted in red. The peptide is oriented with the C-terminus facing up while the N-terminus is facing down. The guanidinum groups of arginine are depicted as oriented towards each membrane interface. B. A contour plot showing the average effective energy as a function of the peptide depth in the membrane (z) and tilt angle relative to the membrane normal (tilt). The red line denotes the lowest energy pathway taken by the peptide as it translocates deeper into the membrane. # indicates the lowest energy state as visualized to the left in A.

*This figure was taken from the following publication:

Materials and Methods

Generating Membrane Potential with Valinomycin

POPC vesicles were made as previously described in chapter 1 with the following exceptions: 1 mg/ml chymotrypsin was dissolved in buffer (10 mM HEPES, 128 mM KCl, 0.1 mM EDTA, KOH pH 7.4) and was entrapped in POPC LUVs (POPC-CVs) via freeze-thaws and extrusion as described previously. Purification of POPC-CVs were performed in a size exclusion column (Sephadex G-75) buffered with 10 mM HEPES, 128 NaCl, 0.1 mM EDTA, NaOH, pH 7.4. The fraction of POPC-LUVs with entrapped chymotrypsin ran through a column with buffer containing exclusively sodium were then further purified with spin column filters of 100,000 Da MWCO in sodium containing buffer until flow through contained undetectable chymotrypsin as determined by the EnzChek assay as described in Figure 2.1. After vesicle generation and purification, translocation experiments were carried out as previously in Figure 2.2 with the following exceptions: All experiments were conducted in buffer (10 mM HEPES, 128 mM NaCl, 0.1 mM EDTA, pH 7.4). After 30 min of incubating POPC-CV entrapped and 2 μM antitrypsin, 400 nM of valinomycin was added and incubated for 10 min to establish a membrane potential across the LUV bilayer (Figure 3.3). Peptide was then added to valinomycin treated vesicles or non-treated vesicles and analyzed on HPLC to measure translocation rates as described in Figure 2.2.

Induction of Membrane Disorder

The same POPC-LUVs with entrapped chymotrypsin that were used to generate a membrane potential (vesicles entrapped with K⁺) were used to measure the induction of membrane disorder with a potent pore forming peptide, alamethicin. Concentrations of alamethicin that would not induce membrane permeabilization first needed to be established. Translocation samples as
outlined in Figure 2.2 were prepared with various concentrations of alamethicin along with the NBD-labeled negative control peptide of translocation, ONEG, for 30 minutes (Figure 3.4A). Additional translocation samples were set up for the motif, motif variants and TP2 using a P:L of alamethicin:POPC observed to not allow pore formation (P:L = 1:6000) as determined by the lack of cleavage with ONEG in Figure 3.4A, followed by calculations for rate of translocation for each peptide tested with and without alamethicin treatment (Figure 3.4B). The effect of various P:L of TP2:POPC with and without concurrent alamethicin treatment on rates of translocation tested as outlined above where a P:L of alamethicin:POPC = 1:6000 was used (Figure 3.4C).

*Measurement of Peptide Induced Lipid Flip-Flop*

POPC vesicles were generated by freeze-thaws and then extruded through a Nuclepore polycarbonate membrane to achieve large unilamellar vesicles of 100nm in size in buffer (10 mM HEPES, 45 mM NaCl, 0.1 mM EDTA, pH 7.4). The outer leaflet of the POPC-LUVs were labeled with C6-NBD-PC (1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-y)amino]hexanoyl}-sn-glycero-3-phosphocholine) to create asymmetric POPC-LUVs with simple addition of 1mol% C6-NBD-PC prior to each experiment. Symmetrical vesicles were constructed by adding 1 mol% C6-NBD-PC to POPC during freeze-thaws and extrusion. Unlabeled peptide was then added to the asymmetric NBD-POPC-LUVs at a desired peptide to lipid (P:L) and incubated overnight at room temperature. The peptide-lipid mixture was then brought up in 100 μL of buffer and placed in a steady-state fluorometer to measure NBD fluorescence (ex. 470 nm, em. 540 nm) for 1 minute. During this time, a fresh stock of 0.6 M sodium dithionite was prepared by dissolving into ice cold buffer (10 mM TRIS, pH 10.5) for each sample measured. After the initial NBD fluorescence was measured, 45 mM final concentration of dithionite was added to the peptide-lipid mixture and NBD fluorescence was immediately monitored for 5 minutes (Figure 3.5A). The remaining NBD
fluorescence after dithionite quenching was used to calculate the total amount of NBD protected by dividing the initial NBD fluorescence by the NBD fluorescence after 5 minutes of dithionite quenching (Figure 3.5B).

**Translocation of Arginine-Variants of TP2**

Arginine variants of TP2 where the arginines of TP2 were replaced with norarginine or homoarginine were synthesized on resin using the protocol established in Table 2.1. Each arginine variant of TP2 was labeled with NBD on the C-terminal cystine and purified using HPLC. Translocation, blockage and cleavage samples as outlined in Figure 2.2 were set up with the arginine variants of TP2 using POPC-LUVs with chymotrypsin entrapped at a P:L = 1:100. Rates of translocation were calculated and compared to TP2 containing arginine (Figure 3.6).

**Confocal Microscopy of Arg9-Tamra**

Arg9 (an oligomer of nine arginines) was labeled on a C-terminal cystine with TAMRA-5-maleimide (ThermoFisher) and purified on HPLC. CHO cells were grown on 8-chamber coverslip slides at a cell count of 3.0 x 10⁴/well in full serum DMEM (10% FBS, 1x NEAA, 1x anti-anti) for 24 hours in a 37°C incubator supplied with 5% CO₂. Cells were washed with serum-free DMEM followed by treatment with serum-free DMEM containing 15 μg/mL dextran-AF488 (ThermoFisher) and various concentrations of Arg9-TAMRA for 1 hour in a 37°C incubator supplied with 5% CO₂. Cells were then imaged on a Leica SP2 confocal microscope (Figure 3.7).

**Results**

**Membrane Potential Does Not Promote Spontaneous Translocation of LRLLR motif and Motif Variants**

CPPs including SMTPs typically have a common feature of having an overall cationic charge³. A reported mechanism for spontaneous “adaptive” translocation of cationic CPPs rationalized the necessity of a draw or “sink” for peptides to cross the cell membrane once the
peptide has partitioned into the interfacial region of the membrane. An overall negative inside membrane potential present across the cell membrane exists in viable cells and has been proposed to promote translocation of cationic CPPs. Valinomycin is an ionophore that chelates potassium and carries it across lipid bilayers down a concentration gradient. Establishment of opposite concentration gradients of sodium and potassium across the membrane of POPC vesicles followed by treatment with valinomycin enables a membrane potential to form. This approach was used to test if a potential across a bilayer can drive or increases translocation of the motif, motif variants and an established SMTP, TP2. POPC vesicles with chymotrypsin entrapped were constructed in buffer containing only potassium chloride followed by exchange into a buffer containing only sodium chloride to establish a concentration gradient across the lipid bilayer. Valinomycin treatment of these POPC vesicles allowed for a net efflux of potassium out of the vesicle, down its concentration gradient to establish an electric potential across the bilayer (Figure 3.3, top). Peptides added to valinomycin treated vesicles did not result in pronounced increase in translocation rates across POPC bilayers for the LRLLR motif and motif variants (Figure 3.3). TP2 translocation rate moderately increased due to valinomycin treatment while the negative control for translocation, ONEG, showed no response.

Membrane Disorder Increases Spontaneous Translocation of All Peptides Tested

Alamethicin is a potent pore forming peptide from the fungus Trichoderma viride. At low concentrations, alamethicin is observed to lose pore-forming capabilities but remain membrane active by inducing lipid flip-flop within a lipid bilayer. The exchange of dye-labeled lipids across a bilayer was measured previously to occur at P:L < 1:1000 without pore formation. Therefore, using low concentrations of alamethicin below what would cause pore-formation but allow lipid flip-flop, would increase the amount of membrane disorder and would also possibly increase the
$k_T (\text{Hours}^{-1})$

- Valinomycin
+ Valinomycin

LRLRL-W
C
RLRLL-W
C
TP2
LLLRR-W
C
LLRLR-W
C
RRLLL-W
C
LRLLR-W
C
RLLLR-W
C
ONEG

Internal
128mM KCl
Chymotrypsin

Net Efflux

K$^+$

External
128mM NaCl
+ Valinomycin -
-
-
-
-
- - - -
-
-
Figure 3.3 Membrane Potential Affecting Translocation Rates into POPC-LUVs

The top of the figure depicts the generation of a membrane potential with valinomycin treated LUVs that had a chemical gradient established by exchange into a sodium containing buffer. Below are results from a translocation experiment on HPLC using POPC-LUVs with entrapped KCl and chymotrypsin treated with or without 400 nM of valinomycin prior to addition of peptide.
rate of translocation for membrane associated peptides. ONEG was used to ensure that a concentration of alamethicin which does not cause pore formation existed. Degradation of ONEG after 30 minutes of incubation with POPC-LUVs entrapped with chymotrypsin was not observed at P:L < 1:2000 of alamethicin (Figure 3.4A). A P:L = 1:6000 of alamethicin was then used to treat POPC LUVs entrapped with chymotrypsin along with sample peptide. Translocation rates increased for all peptides when alamethicin was present (Figure 3.4B). Interestingly, the motif variants that showed the highest partitioning coefficients, RLLLR (Figure 2.7), had the highest increased translocation rates with alamethicin treatment. RLLLR showed greater than a 7-fold increase in translocation rates with alamethicin treatment. Conversely, TP2, which had the lowest partitioning coefficient had a greater than 20-fold increase in translocation rates with alamethicin treatment. Concentration dependence for translocation of TP2 where increasing peptide concentration (decreasing P:L ratios) slowed translocation rates were reversed with alamethicin treatment (Figure 3.4C).

**Lipid Flip-Flop Measured During SMTP Translocation**

Spontaneous translocation of a charged peptide across the bilayer is thought to be highly dependent on the presence of arginine due to the bidentate interactions between arginine guanidinium side chain and the phosphate of phospholipid headgroups. Charge neutralization of the arginines can thus be satisfied by these interactions however, the mechanism(s) that enable spontaneous translocation are unclear. Possible mechanisms for spontaneous translocation of arginine containing peptides after interfacial partitioning involves either the formation of inverted micelles or lipid chaperoning of a neutral-complex of peptide and lipid(s) across the bilayer. The degree to which outer-leaflet lipids translocate across the bilayer during peptide translocation would be a good measure of lipid chaperoning that occurs during a spontaneous translocation.
Figure 3.4 Alamethicin-Induced Membrane Disorder Affecting Translocation Rates

A. Treatment of POPC-LUVs with entrapped chymotrypsin with various alamethicin concentrations (expressed as peptide to lipid ratios (P:L)) and a static amount of ONEG peptide for 30 minutes. This established the baseline of alamethicin concentration not able to create pores large enough for peptide to enter vesicles. ONEG was used because it is observed not to translocate. B. Translocation rates of peptides into POPC-LUVs entrapped with chymotrypsin treated with alamethicin P:L = 1:6000. C. Translocation rates of TP2 into POPC-LUVs entrapped with chymotrypsin at various P:L, with and without alamethicin P:L = 1:6000.
event. To measure this, a small fluorescent probe attached to the head group of a short-chain lipid tail, C6-NBD-POPC was used (Figure 3.5, top). This lipid spontaneously partitions into the outer-leaflet of lipid vesicles, and does not translocate measurably to the inner-leaflet in control samples without peptide. Thus this system can be used to test the possibility of a peptide-induced lipid chaperoning across the bilayer. Observing ≥ 90 % NBD quenching in samples without peptide after addition of sodium dithionite (“Blank” sample) verified that most of the C6-NBD-POPC lipids remained asymmetrically distributed on the outer leaflet of unperturbed POPC membrane (Figure 3.5B). POPC LUVs with symmetrical distribution of fluorescently labeled lipids (C6-NBD-POPC was present throughout the lipid preparation) were observed to have ~50 % of NBD fluorescence remaining after dithionite addition showing that sodium dithionite was not able to quench C6-NBD-PC protected on the inner leaflet of POPC LUV membranes as determined by the difference in % remaining of NBD fluorescence from symmetric versus asymmetric C6-NBD-POPC distribution. Asymmetric vesicles were treated overnight with peptides followed by addition of sodium dithionite to measure NBD protection and thus lipid flip-flop induced by peptide addition. TP2 showed the highest % of NBD protection followed by the LRLLR motif. Both were statistically different than that of asymmetric vesicles alone (“Blank” sample) using multiple comparison ANOVA (Figure 3.5B). All other motif variants and ONEG showed no difference in % NBD protection.

Arginine Sidechain Length Variants of TP2 Altered Translocation Rates

Previous molecular dynamic modeling TP2 translocation across an implicit lipid bilayer proposed a transition state involving the TP2 adopting a hairpin structure within the bilayer which causes both arginine residues oriented towards each of the interfacial regions of the bilayer (Figure 3.2). The orientation of the arginines of TP2 may have an influence on the rate of translocation
**A.**

Outer leaflet contains ~1% mol C6-NBD-POPC

**B.**

Bar graph showing % NBD-Protected over different conditions.

**Sodium Dithionite (Impermeant)**
Figure 3.5 Lipid Flip-flop Measured in POPC-LUVs with SMTP and Motif Variants

A. Schematic of the flip-flop assay. C6-NBD-POPC is added to the outer leaflet of POPC-LUVs at 1 mol% to produce asymmetric POPC-LUVs. Peptide is added and incubated with vesicles overnight. Dithionite, a membrane impermeant quencher of NBD fluorophore, is added and the fluorescence of NBD is monitored. NBD fluorescence remains if C6-NBD-POPC is protected from dithionite by remaining on the inner leaflet of an intact vesicle. C. Results from the flip-flop assay where peptide was added to asymmetric POPC-LUVs overnight followed by treatment of dithionite. The % NBD-Protected represents the ratio of fluorescence after 5 minutes of dithionite treatment to the initial NBD signal before dithionite treatment. TP2 and the LRLLR motif were found to have significantly protected NBD from dithionite quenching (Multiple comparison ANOVA, p ≤ .05, n = 4).
considering that the interfacial regions of the membrane contain the highest probability for charge neutralization of the guanidinium groups of the arginines via bidentate hydrogen bonding with phosphates of the phospholipid head groups. Orientation of the arginines of TP2 were altered by synthesizing two variants of TP2 where its arginines were replaced with two arginine homologs that differ in sidechain length. Homoarginine is a longer sidechain derivative of arginine because of the addition of an ε-carbon. Norarginine is a shorter sidechain derivative of arginine because of the removal of a sidechain methylene group. The lengthening and shortening of the guanidinium groups of arginine should result in differences in orientation of the groups with respect to the interfacial regions of the membrane. The homoarginine variant of TP2 will result in a version of TP2 that may have more interactions with the interfacial regions of the membrane during the proposed transition state from molecular dynamic simulations by the lengthening of arginine side chains whereas the norarginine variant of TP2 should result in the opposite effect. Replacement of TP2 arginines with either derivative decreased translocation rates into POPC-LUVs with chymotrypsin entrapped when compared to parent TP2 (Figure 3.6). In addition, there was no difference in translocation rate between the two arginine variants of TP2 when compared to each other.

Discussion

Membrane Potential does not Increase Translocation Rates of SMTPs

The “adaptive translocation” model for energy-independent spontaneous translocation proposes the presence of a membrane potential as the driving force for direct translocation across a lipid bilayer\textsuperscript{27}. This idea was tested using synthetic vesicles by first creating an electrochemical gradient across the bilayer with vesicle preparation conducted in buffer containing exclusively potassium cations followed by exchange into a buffer containing exclusively sodium cations.
**Figure 3.6 Translocation Rates for Arginine Variants of SMTP, TP2**

Results of a translocation rate for arginine variants of TP2 into chymotrypsin entrapped POPC-LUVs. The Arginine variants differ in side-chain lengths as depicted by their molecular structures on top and in the bars on the graph.
Valinomycin establishes a membrane potential by shuttling potassium ions across the membrane down the concentration gradient or a net efflux out of the vesicle. The reliance on a membrane potential for energy-independent CPP translocation has been observed with penetratin\textsuperscript{76} which would allow for the adaptive translocation model to describe its mechanism of action. However, we conclude that this model for energy-independent translocation may not apply to SMTPs such as TP2 or the motif and its variants due to little or no increase to translocation rates after the generation of a membrane potential with valinomycin. Recently, the idea that a membrane potential could be a driving force of energy-independent translocation has been questioned. CPPs with majority arginines in their sequences were shown to directly translocate across the cellular membrane completely independent of membrane potential\textsuperscript{80}. That work used patch-clamps across the membrane of cells and found that peptide treatment caused a hyperpolarization of the membrane and abolishing this hyperpolarization upon peptide treatment caused less direct translocation into cells\textsuperscript{80}. Further, other researchers have identified that Ca\textsuperscript{2+} influx accompanies CPP direct translocation, and thus resulting in a hyperpolarized membrane\textsuperscript{28}. Both studies have noted that direct translocation of CPPs into cells after hyperpolarization was heavily dependent on high (i.e. $\geq 10\mu M$) peptide concentration. Performing a similar assay where peptide concentration of a CPP with majority arginines labeled with an impermeant fluorophore, Arg9-TAMRA, was varied from 0.001$\mu M$ - 20$\mu M$ in the presence of 10kDa dextran-AF488 showed what appeared to be diffuse staining of the cytosol and thus indicative of energy-independent translocation into cells (Figure 3.7). However, Arg9-TAMRA was shown to colocalize intracellularly with diffuse dextran-AF488, indicating that the membrane was permeant enough to allow an influx of a high molecular weight molecule such as 10kDa dextran. This raises the concern of toxicity with the high concentration of highly cationic CPPs needed to achieve the desired result of energy-
**Figure 3.7 Confocal Images of Increasing Concentration of Arg9 with CHO Cells**

Confocal microscopy images of CHO cells treated with 0.01 mg/mL 10kDa dextran-AF488 (green) and various concentrations of Arg9-TAMRA (red) for 1 hour at 37°C. Punctate staining of Arg9-TAMRA colocalizing with dextran can be seen at lower concentrations (top row) of Arg9, indicating energy-dependent entry mechanisms. At higher concentrations (bottom row) of Arg9-TAMRA, diffuse staining is seen in the cytosol for both Arg9 and dextran, indicating membrane permeation of a 10kDa molecule. The highest concentration of Arg9-TAMRA (bottom right) shows membrane defects such as tubulations.
independent entry into cells. The lack of colocalization of TP2 and dextran demonstrates that it translocates into cells occurred without membrane disruption.

Increasing the amount of membrane disorder with concentrations of alamethicin shown to cause trans-bilayer translocation of lipids (lipid flip-flop) increased translocation rates of TP2, the *LRLLR* motif and motif variants (Figure 4B). These results suggest that, upon partitioning into the membrane, direct translocation may occur by peptide interacting with interfacial phosphates of the phospholipid head groups via arginines followed by a transient state where peptide is chaperoned across the hydrophobic core of the membrane. To further test this hypothesis, asymmetric vesicles were produced by labeling the outer leaflet of POPC-LUVs with fluorescently labeled lipids, C6NBD-PC, that are sensitive to quenching with an impermeant reducing agent, sodium dithionite. Incubation of such vesicles with TP2, and to a lesser extent the *LRLLR* motif, enabled protection of the fluorescently labeled lipids from quenching with sodium dithionite, meaning that they have induced lipids to translocate to the inner leaflet of the membrane of POPC-LUVs (Figure 3.5B). The motif variants of the *LRLLR* motif did not enable the same translocation of lipids as TP2 and the original motif. A possible mechanism of energy-independent translocation across the lipid bilayer for SMTPs may therefore involve the necessity to induce lipid flip-flop which would allow for a neutrally charged complex of peptide and lipid to traverse the hydrophobic environment of the bilayer core. Unique to TP2 was the correlation of translocation rates increasing with decreasing peptide concentration (Figure 3. 4C), suggesting that kinetics of translocation is governed by a finite amount of peptide able to translocate across the membrane at a given time. This effect was reversed by increasing the rate of lipid flip-flop with alamethicin (Figure 4C). Lipid flip-flop may lessen the likelihood of larger membrane defects (i.e. pore formation) during direct translocation and thus toxicity with SMTPs.
The lack of dependence of translocation on membrane potential and the induction of lipid flip-flop to increase spontaneous translocation of TP2 and the LRLLR motif variant across lipid bilayers highlights how the spacing of arginines may be required for translocation. However, the mechanism(s) responsible for optimal spontaneous translocation may be different for different spacings. Both TP2 and the LRLLR motif induced lipid flip-flop, whereas the other arginine spacing motif variants which had a relative increase in translocation rates did not. The published molecular dynamic studies of TP2 model the positioning of the arginines to be crucial for membrane interactions and partitioning. In particular, the implicit membrane model simulation proposed a transition intermediate of TP2 that necessitated the positioning of its arginines to both sides of the interfacial regions of the membrane. Enhancing this reaching or “snorkeling” of arginines towards interfacial regions of the membrane could improve translocation for TP2. To test this assertion based on the molecular dynamic study, two variants of TP2 with shorter or longer side-chain arginine residues (norarginine and homoarginine, respectively) were synthesized. The longer arginine side-chain variant of TP2, homoR-TP2, did not result in improved translocation rates across POPC-LUVs (Figure 3.6). As expected, the shorter arginine side-chain variant of TP2, norR-TP2, also did not increase translocation rates. Thus, the precise positioning of the arginines of TP2 apparently provide optimal interactions with the lipid bilayer for the most efficient translocation processes to occur, at least for a peptide with the LRLLR motif.

The most important reason to decipher a CPP’s mechanism of entry is to assess if the CPP can deliver a cargo free of sequestration and degradation in endosomes via energy-dependent endocytosis. If the end goal of developing CPPs is to use them as delivery therapeutics that would otherwise not cross a lipid bilayer then an ideal CPP would be one that avoids energy-dependent mechanisms over the widest peptide concentrations and conditions as possible. Studying how
CPPs translocate across lipid bilayers in an energy-independent fashion is therefore paramount for the progression of CPPs to be useful tools for therapeutic development. SMTPs, such as TP2, cross lipid bilayers in an energy-independent manner that possibly relies on specific interactions with the bilayer to induce flip-flop to cross the hydrophobic core. This behavior does seem to be dependent on concentration as increased translocation rates are observed with decreasing peptide concentration, suggesting that translocation can occur in a monomeric form, a very important property for applications of CPPs. The ability of TP2 and other SMTPs to maintain this behavior when interacting with membranes will be influenced by the attached cargo’s physical properties. Limitations of cargo delivery will always need to be assessed with CPPs acting as delivery vehicles, however, starting with peptide sequences characterized to be a spontaneous translocating peptide from their inception is an excellent starting point for an ideal CPP tasked with cargo delivery. The next chapter will cover efforts to test the limitations of cargo delivery with SMTPs. In particular, cargoes that elicit a bioactive response in cells.
CHAPTER 4

Cellular Delivery of Bioactive Cargoes with SMTPs

Overview

In the scientific literature, the majority of cargoes delivered by CPPs are fluorophores\textsuperscript{20}. While the use of fluorophores is convenient for ease of conjugation chemistries and optical properties, these cargoes do not represent the greatest utility that CPPs can offer as therapeutic or biotechnological tools. Bioactive molecules such as DNA, RNA, proteins, peptides and others are unable to cross the lipid bilayer due to their complex physical chemical properties such as large size, charges, polarity, etc. Therefore, the delivery of bioactive molecules as cargoes can display the full value offered by CPPs. Delivery of larger bioactive molecules is much desired in the clinical or laboratory setting due to the high degree of target specificity unlike many small molecules that passively cross the lipid bilayer and interact with numerous targets causing unwanted side-effects\textsuperscript{81}. The mechanism of entry of CPP-bioactive cargoes determines their therapeutic or biotechnological value. CPP-cargo conjugates that rely more frequently on energy-dependent mechanisms will subject the CPP-cargo complex to the segregated and degradative pathways associated with endosomal entrapment and lysosomal fusion\textsuperscript{50,52}. To avoid this scenario, cargo delivery with CPPs of other known mechanistic classification should be considered. SMTPs were found to cross lipid bilayers of synthetic vesicles and cell membranes via energy-independent mechanisms\textsuperscript{56}. Conjugating impermeant polar fluorescent cargoes to SMTPs allowed for their delivery into cells both under physiological and non-physiological conditions and without membrane permeabilization\textsuperscript{57}. An upper limitation on cargo size was determined when a SMTP, TP2, was conjugated to quantum dots did not gain access into the cytosol of cells\textsuperscript{57}. 
Here we tested the ability of TP2 to deliver two bioactive peptide cargoes. The diversity of potential bioactive cargoes is immense; however, an ideal cargo to test here is one that is cell impermeant and elicits a distinct biological response that is easily measured and observed. Phalloidin, a mushroom toxin from the death cap mushroom *Amanita phalloides*, is a polar cyclic heptapeptide (780Da.) that binds to filamentous actin (f-actin) to inhibit its depolymerization. Phalloidin, when conjugated to a fluorophore, is widely used in microscopy to image actin filaments, however, applying phalloidin requires that cells be fixed and permeabilized due to phalloidin being highly membrane impermeant. Since phalloidin is impermeable and has a well described interaction with, and effect on an intercellular target present in every eukaryotic cell type (i.e. f-actin), it was chosen as an ideal bioactive cargo for SMTP delivery. Furthermore, the cyclic structural class of peptides (including the crosslinked peptide structure of phalloidin) are beginning to attract significant attention in drug design. Therefore, a peptide that can successfully deliver phalloidin will likely be able to deliver other peptide drug molecules in this structural class.

Another bioactive cargo we tested for SMTP delivery is derived from a segment of the proapoptotic protein, BID. BID belongs to the BH3-only class of proapoptotic proteins that exists in an inactive cytosolic form and becomes active during an apoptotic signaling event either from extrinsic receptor-mediated activation or intrinsic mitochondrial membrane permeabilization. Activated BID exposes a helical BH3 portion that docks with cytosolic pro-apoptotic protein BAX to locate on the outer mitochondrial which initiates BAX homo-oligomerization into a stable pore in the mitochondrial membrane, release of cytochrome-c and induction of caspase-dependent apoptosis. Peptides derived from the BH3 domains of BID and related proapoptotic proteins are sufficient to activate BAX oligomerization and induce of apoptosis through mitochondrial membrane permeabilization when conjugated to CPPs. More recently, the structure of BAX
was crystalized with BH3 peptides derived from BID. The structures revealed that BH3-BID peptides caused conformational changes in BAX resulting in homodimerization and permeabilization of lipid bilayers\textsuperscript{90}. In addition, when the BH3-BID peptides were mutated at residues shown to interact at key sites within the BAX binding cleft, BAX was observed to not dimerize.

**Materials and Methods**

**Conjugation of Phalloidin to TP2**

Phalloidin with a lysine substitution at position 7 (Lys\textsuperscript{7}-phalloidin, BACHEM) was used as suitable cargo to conjugate to an available cysteine of TP2 by using a bifunctional linker, SMCC (ThermoFisher). A 4:1 ratio of phalloidin to SMCC by weight was dissolved in DMF followed by the addition of 1% DIPEA to react the succinimidyl-ester portion of the bifunctional SMCC linker with the amine present on Lys\textsuperscript{7} of phalloidin for 1 hour at room temperature. The reaction was monitored using UV absorbance (280nm) on reverse phase HPLC using a C-18 analytical column to ensure phalloidin conjugation to SMCC. The reaction mixture was then placed on vacuum to remove the DMF solvent. A 1:1 ratio of peptide (with available cystine) to phalloidin by weight was added to the dried SMCC-phalloidin reaction vessel by first dissolving peptide in HFIP. An equal amount of ddH\textsubscript{2}O v/v was added to the peptide-SMCC-phalloidin HFIP solution which allowed the maleimide portion of the SMCC bifunctional linker to react with the available cysteine of the peptide, TP2. The reaction was monitored on HPLC using tryptophan fluorescence (ex/em 285/340nm) until the reaction progressed to completion, typically 8 to 24 hours. The TP2-SMCC-phalloidin construct was purified using reverse phase HPLC equipped with a C-18 column. The mass of the construct was verified on MALDI-TOF mass spectroscopy. Fluorescent labeling of TP2-phalloidin was achieved by conjugating purified TP2-SMCC-phalloidin constructs with
NBD-Cl amine reactive dye. TP2-phalloidin-NBD was purified on HPLC and verified using MALDI-TOF mass spectroscopy.

**Pyrene-Actin Depolymerization Assay**

An actin polymerization biochemistry kit (Cytoskeleton Inc.) was used to assay the bioactivity of phalloidin conjugates. All experiments were performed in 96-well format and read on a fluorescent plate-reader (Biotek Synergy II). Actin-pyrene monomers formed filamentous actin by using the supplied polymerization buffer which caused pyrene epimers to fluoresce (ex/em 365/407nm). A serial dilution of phalloidin, TP2-phalloidin, D-form TP2d-phalloidin, or TP2 was added to the filamentous actin-pyrene as well as a no peptide control. A supplied depolymerization buffer was then added to samples and loss of fluorescence was monitored on the plate reader as a function of time. Rates of depolymerization were plotted for each sample by calculating linear regression and plotting the slopes. Calculated rates were then plotted, normalized and fit with a log (agonist) vs. normalized response equation (Figure 4.1).

**Alamar Blue Assay**

Several cell lines were grown in separate 96-well plates with DMEM growth media supplemented with 10% FBS, 1x anticytocic-antimicrobial, 1x non-essential amino acid and place in a 37°C incubator at 5% CO\textsubscript{2} to ~80% confluency. A serial dilution of peptide-SMCC-phalloidin or phalloidin in serum free media was added to cells and incubated at 37°C for a desired amount of time followed by the addition of alamar blue (ThermoFisher) using the manufacturer’s suggested conditions. Cells were measured in a plate-reader for alamar blue fluorescence (ex/em 570/585nm). % viability was calculated by the ratio of peptide treated cells to cells treated with no peptide control (Figure 4.2).
**Scratch Assays**

CHO or HEPG2 cells were grown to a confluent monolayer in DMEM growth media supplemented with 10% FBS, 1x antimycotic-antimicrobial, 1x non-essential amino acid and placed in a 37°C incubator at 5% CO₂. A 200 μL pipette tip was used to strike a scratch through the confluent monolayer of cells and then treated with serum free DMEM supplemented with peptide diluted at a desired concentration. Photos of the scratch were taken of the same field every 24 hours (Figure 4.3 A). Images were processed using ImageJ software (NIH.gov) to measure the width of the remaining scratch at each time point. Scratches were quantified by calculating using the following equation: \[
\% \text{ wound closure} = (1 - \frac{\text{Area}_{\text{final}}}{\text{Area}_{\text{initial}}}) \times 100
\] (Figure 4.3 B).

**Epifluorescent Imaging of Cells Treated With Peptide-Phalloidin or Phalloidin**

HepG2 cells were grown on a tissue culture treated 8-chamber microscope slide using a 3x10⁴ cells/well. Cells were either treated with 5 μM of TP2-phalloidin-NBD or phalloidin-TAMRA in serum-free DMEM for 3 hours at 37°C. Peptide treatments were then washed off cells 3 times using DPBS and fixated using 4% paraformaldehyde in DBPS for 20 minutes (“3hrs Pre-Fixation” samples). Cells were also fixated using 4% paraformaldehyde in DBPS for 20 minutes, permeabilized using 1% Triton-X in DBPS, and stained using 5 μM of TP2-phalloidin-NBD or phalloidin-TAMRA followed by DBPS washes (“Post-Fixation” samples). Cells were then preserved in mounting solution containing DAPI nuclear stain (Prolong Gold with DAPI, ThermoFisher) and imaged on a Nikon Ellipse fluorescent microscope (Figure 4.4).

**BH3 domain of BID Peptides**

BH3-BID (EDIIRNIARHLAQVGDSMDR-GG-WC-NH₂), TP2-BID (PLIYLRLLLRGQWC-GG-EDIIRNIARHLAQVGDSMDR-NH₂), BID-TP2 (EDIIRNIARHLAQVGDSMDR-GG-PLIYLRLLLRGQWC-NH₂), and mutant BID
(EDAARNIARHLAQVGSMDR-GG-WC-NH₂), BIDmu-TP2

(EDAARNIARHLAQVGSMDR-GG-PLIYLRLRGQWC-NH₂) were all commercially synthesized by BioSynthesis (Lewisville, Texas). Peptides were resuspended in DMSO and quantified using the extinction coefficients of each peptide to make concentrated stocks. Peptides were typically diluted in serum-free media with consideration to keep the final % DMSO (v/v) < 1 % for all final peptide concentrations tested.

**Caspase-3 Detection and Membrane Integrity Measurements with BH3-BID Peptides on Plate Reader**

Cells were grown in 96-well plates in DMEM growth media supplemented with 10 % FBS, 1x antimycotic-antimicrobial, 1x non-essential amino acid and placed in a 37°C incubator at 5 % CO₂ to ~80 % confluency. A serial dilution of BH3-BID-TP2, TP2, or BH3-BID peptides were diluted in serum free media and added to cells for a desired time and left in a 37°C incubator at 5 % CO₂. Staurosporine (ThermoFisher) was routinely used as a positive control for apoptosis in 0.3 – 1 μM final concentration ranges. Apoptosis was measured by adding 5 μM final concentration Cell-Event Caspase-3 detection reagent (ThermoFisher) for 30 minutes after peptide incubations. 96-well plates were then added to a plate-reader and read for FITC fluorescence (ex/em 488/525nm, Figure 4.5 A). A separate 96-well plate of cells was incubated with same peptide treatments needed for the apoptosis assays plus the addition of 1 μM final concentration SYTOX-green (ThermoFisher) to measure cell membrane integrity during peptide treatments. After a desired time of incubation, peptide and SYTOX were removed from the cells followed by a gentle wash with serum-free DMEM. The plate of cells was then added to the plate-reader and read for SYTOX-green fluorescence (ex/em 488/525nm, Figure 4.5 B). A time course assay to measure apoptosis using the Caspase-3 detection reagent was conducted using the same protocol as outlined above (Figure 4.5 C).
**Flow Cytometry**

HELA cells were incubated in 12-well dishes in DMEM growth media supplemented with 10 % FBS, 1x antmycotic-antimicrobial, 1x non-essential amino acid and placed in a 37°C incubator at 5 % CO₂. Peptides were diluted in serum-free DMEM media containing 5μM of either SYTOX-green or red and added to cells for a desired incubation time at 37°C. Caspase-3 detection reagent was added 30 minutes prior to a desired incubation time elapsed. The cells were then washed with serum-free media and harvested with a rubber policeman on ice using in chilled PBS and run on a flow cytometer (Figure 4.6 A). Annexin-V-AF546 was added to cells 15 minutes before a desired incubation time elapsed. Cells were washed with serum-free media to remove free annexin-V and harvested with a rubber policeman on ice using in chilled PBS. Samples were then run on a flow cytometer (BD LSR II, LSU-LCRC, Figure 4.6, B).

**Confocal Microscopy**

Cells were grown on 8-chamber coverslip slides (Nunc LabTek II) with 40,000 cells/well in DMEM growth media supplemented with 10 % FBS, 1x antmycotic-antimicrobial, 1x non-essential amino acid and placed in a 37°C incubator at 5 % CO₂. Cells were washed with serum free DMEM media prior to peptide treatments. Peptides were diluted in serum free DMEM media containing 5 μM of impermeant DNA dye, TOTO-3 (ThermoFisher), added to cells for 24 hours and placed in a 37°C incubator at 5 % CO₂. 1μM of Cell-Event Caspase 3 detection reagent was added to cells for 30-45 minutes prior to imaging. All images were captured on a Nikon A-1 Confocal microscope (Figure 4.7).

**Membrane Asymmetry Assayed with a Ratiometric Fluorescent Dye F2N12S**

Jurkat cells were initially grown in RPMI containing 10 % FBS and 1x antibiotic-antimycotic in a 37°C incubator supplied with 5 % CO₂. On the day of experiments, cells were
spun down and resuspended in 37°C serum and phenol red-free DMEM to yield a final cell density of 1x10^6 cells/mL. Peptides were added to 1.5 mL Eppendorf tubes from DMSO stocks to achieve a final concentration in 500 μL while maintaining a final DMSO concentration < 1 %. 500 μL of 1x10^6 cells/mL were added to each of the Eppendorf tubes containing peptide and allowed to incubate in a 37°C incubator supplied with 5% CO2 with the lids remaining opened for 4hours. Cells were then spun down in each tube at 1400rpm for 3 minutes to achieve a cell pellet followed by removal of the media containing peptide and resuspension in 37°C 500 μL 1xHBSS with MgCl and CaCl. Cells in HBSS were immediately diluted into a 1cm quartz cuvette containing 200nM F2N12S and assayed on a steady-state fluorometer (QuantaMaster 8000, Horiba Scientific) at 37°C for emission scans from 420nm-610nm using a 405nm excitation (Figure 4.8 A-D). Ratios of F2N12S emissions peaks at 530nm and 580nm were plotted from the previous emission scans (Figure 4.8 E).

Results

Part I: SMTP-phalloidin

Phalloidin-peptide Conjugates Maintain Bioactivity

Purified actin monomers can self-assemble when exposed to appropriate salt conditions. Actin labeled with pyrene moieties allows pyrene to “stack” on top of one another when actin begins to polymerize, resulting in fluorescent epimers that report real-time rates of g-actin polymerization into f-actin. F-actin treated with peptides followed by exchange in depolymerization buffer resulted in delayed depolymerization of actin as measured by pyrene epimer fluorescence loss over time (Figure 4.1). Phalloidin treatments maintained f-actin after depolymerization conditions as expected. Similar IC50s for actin depolymerization were calculated for phalloidin and the two TP2-phalloidin conjugates. TP2 treatment alone showed
**Figure 4.1 IC50 for Actin Depolymerization with SMTP-Phalloidin Constructs**

Plot of the normalized rates and calculated IC50 for actin depolymerization with phalloidin (red) alone or conjugated to a SMTP, TP2. Both L (TP2-Phal., green) and D (TP2d-Phal., blue) forms of TP2 were conjugated to phalloidin. TP2 without phalloidin (black) was used as a control.
some effect at high concentrations (> 15 μM), however, its calculated IC50 for depolymerization was 4 times that of phalloidin and either of the TP2-phalloidin conjugates and may be an artifact. Ultimately, conjugating phalloidin to TP2, either L or D form, did not diminish the bioactivity of phalloidin given that similar IC50 values were calculated.

Cytotoxicity Measurements with SMTP-Phalloidin Conjugates

Resazurin (Alamar Blue) was used to measure cell viability after phalloidin or TP2-phalloidin treatments. Viable cells convert the cell permeant resazurin dye to a highly fluorescent molecule if a reductive environment is maintained within the cells mitochondria. Melp5, a potent pore forming peptide, was used as a positive control for cytotoxicity. Cells treated with Melp5 displayed decreased resazurin fluorescence relative to no treatment control (Figure 4.2). Several cell types were then assayed for TP2-phalloidin induced cytotoxicity. Phalloidin treatments alone did not result in cytotoxicity of cells assayed as expected due to it not being cell permeant. Most of the cells tested were also tolerant of TP2-phalloidin treatments except for the human CCLP cell line (Figure 4.2). The D-form of TP2-phalloidin was also tested with human HEPG2 cells, however, this did not result in significant cytotoxicity.

Treatment of Cells with Bioactive TP2d-Phalloidin Causes Delay in Cell Migration

A scratch (or wound) assay was used to measure the effect of TP2-phalloidin treatments on cell migration. Dynamic reorganization of the actin cytoskeleton allows cells to migrate over surfaces. Disruption of the polymerization and/or depolymerization of actin by delivery of phalloidin to the cytosol of cells would result in a retardation of cell motility. HepG2 and CHO cells were grown to a confluent monolayer followed by the creation of a wound channel. Cells are expected to actively migrate into the voided space of the wound channel. Cells treated with phalloidin alone migrated into the wound channel after 24-48 hours (Figure 4.3 A).
Figure 4.2 Alamar Blue Cell Viability Assay with SMTP-Phalloidin Treated Cells

Alamar Blue viability assays on a panel of human cell lines (left column) and non-human cell lines (right column) treated with phalloidin or TP2-phalloidin for 72 hours (n = 3). Melp5, a potent pore forming peptide, was used to treat CHO cells for 1 hour (lower right) as a positive control for cytotoxicity. CCLP (pancreatic cancer cell line) was the only cell line that responded differently to TP2-phalloidin conjugate (middle left).
This result was expected due to phalloidin being impermeant. TP2-phalloidin and the D-form of TP2 conjugated to phalloidin, TP2d-phalloidin, showed a difference in their ability to retard cell migration into the created wound channel. TP2d-phalloidin treated cells were unable to migrate into the wound channel indicating that actin depolymerization is inhibited. L-amino acid TP2-phalloidin had a smaller effect on migration in treated cells after 48 hours of treatment as determined by calculating the % wound closure (Figure 4.3 B).

Microscopy of Fluorescent Dye-Labeled TP2-phalloidin-NBD

F-actin visualization in HepG2 cells was performed by treating fixed and permeabilized cells with a fluorescently-labeled phalloidin-TAMRA (Figure 4.4). TP2-phalloidin labelled with the fluorophore, NBD, also showed similar f-actin labeling as phalloidin-TAMRA after treating fixed and permeabilized cells. Live cells that were treated with phalloidin-TAMRA for 3 hours at 37°C followed by several washes and then fixed did not show f-actin staining, indicating that the fluorescently-labeled phalloidin was unable to enter cells. Conversely, TP2-phalloidin-NBD showed f-actin staining after live cells were treated for 3 hours at 37°C, washed and fixed. F-actin visualization in cells after TP2-phalloidin-NBD treatment under physiological conditions indicated that phalloidin conjugated to TP2 entered the cells while maintaining binding to its intracellular target. TP2-phalloidin was most likely not taken up by endocytosis because no other TP2-cargo conjugates are uptaken by endocytosis. Furthermore, the conjugate would not have the opportunity to bind with cytosolic f-actin if segregated to an endosome. Also, no punctate staining was observed in the cytosol, indicating that TP2-phalloidin-NBD did not enter the cell through an endocytic process.
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HepG2

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HepG2

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**Figure 4.3 Cell Migration Assays with SMTP-Phalloidin Treated Cells**

A. Scratch assays performed with HepG2 and CHO cells. Cells were grown to a confluent monolayer and then a wound channel was created by using the end of a pipette tip followed by peptide treatments up to 48 hours. B. The % wound closure was calculated by measuring the initial area of the wound channel before peptide treatment to the area of the wound channel after 48hrs. Treatments were performed with 20 μM of peptide. TP2d-phalloidin treatments showed retardation in wound closure in both cell types tested when compared to phalloidin treatments alone (ANOVA, n = 3).
Post-Fixation

3hrs Pre-Fixation

Phalloidin TAMRA

TP2-Phalloidin NBD
Figure 4.4 Epifluorescent Images of Cells Treated with SMTP-Phalloidin-NBD

HepG2 cells were either fixated, permeabilized and treated with 5 μM of peptides (“Post-Fixation” column) or incubated with 5 μM of peptides for 3 hours, washed and then fixated (“3 hrs Pre-Fixation” column). Phalloidin-TAMRA (red) stained f-actin when cells were permeabilized (upper left) but was not present in cells that were washed of the phalloidin-TAMRA treatment then fixated (upper right). TP2-phalloidin-NBD was observed to stain f-actin in both scenarios. Cell nuclei were stained DAPI (blue) after fixation.
Part II: SMTP-BID

*Induction of Apoptosis with SMTPs conjugated to BH3 domain of BID as Measured on a Plate-Reader*

Cells grown in a 96-well format were incubated with the BH3 domain of BID alone (Bid-BH3) or linked to an SMTP, TP2 and assayed for BH3-BID induced apoptosis using a caspase-3 detection reagent (Figure 4.5 A). Staurosporine, an indiscriminate inhibitor of kinases from *Streptomyces staurosporeus*, was used as a positive control of a membrane permeant inducer of apoptosis. The caspase-3 detection reagent consists of a membrane permeant tetrapeptide with a canonical caspase-3 cleavage sequence (DEVD, O-methyl) linked to a DNA intercalating dye unable to bind to dsDNA and not fluorescent while attached to the tetrapeptide. If caspase-3 is active in the cell, the tetrapeptide is recognized by the protease, digested and the DNA intercalating dye is free to bind to dsDNA which results in a highly fluorescent complex and indicates caspase-dependent apoptosis. Staurosporine treated cells showed a robust activation of caspase-3 in as little as 2 hours (Figure 4.5 A and C) and served as a suitable positive control for caspase-3 induced apoptosis. BH3-BID or TP2 alone did not result in caspase-3 activity over the concentrations tested (Figure 4.5 A). BID-BH3 conjugated to either the C-terminus (TP2-Bid) or the N-terminus (Bid-TP2) of TP2 showed caspase-3 activity > 5 μM at 4 hour treatments (Figure 4.5 A). Cells were also treated with peptides in the presence of a cell-impermeant DNA intercalating dye, SYTOX-green, for 4 hours to measure possible membrane disruption caused by peptide treatment (Figure 4.5 B). Melp5, a potent pore forming peptide, showed the highest degree of SYTOX fluorescence as expected. Bid-BH3 either alone or conjugated to TP2 did not result in SYTOX-green entry. A time course monitoring caspase-3 activation over 24 hour showed cells treated with 10 μM TP2-Bid peptides resulted in similar levels caspase-3 activation (Figure 4.5 C).
Figure 4.5 Plate Reader Detection of Caspase-3 in Cells Treated with SMTP-BID

A. Hela cells treated with a 1:1 serial dilution of peptides starting with 10 μM (left bar in each group) for 4 hours at 37°C and then measured for caspase-3 activation on a plate reader (2x n = 3). Staurosporine and Melp5 were only used at one concentration respectively at 1 μM and 5 μM (2x n = 3). B. Hela cells treated with a serial dilution of peptides starting with 10 μM (left bar in each group) and SYTOX green for 4 hours at 37°C. Staurosporine and Melp5 were used at one concentration respectively at 1 μM and 5 μM (n = 3). C. A time course of Hela cells treated with peptides at 10 μM and then measured for caspase-3 activation (n = 3).
Flow-cytometry Analysis of Cells Treated with SMTPs conjugated with BH3-BID Cargoes

Flow cytometry was also used to detect apoptosis induced by peptide delivery of a BH3-BID peptide conjugated to TP2 as well as membrane integrity (Figure 4.6). Membrane integrity was tested by incubating cells with concurrent treatments of SYTOX-red and peptide for 2 hours. The peptide concentrations tested did not result in many cells staining positive for SYTOX-red when compared cells with no treatment (“Blank”) control, indicating that the cell impermeant DNA dye was not able to cross the membrane due to membrane disruption caused by peptide treatments (Figure 4.6 A). Staurosporine was observed to have the highest percentage of cells that were caspase-3 positive as well as minimal positivity with SYTOX which is indicative of a membrane permeant small molecule inducer of apoptosis (Figure 4.6 A). Overall, the caspase-3 positive cells resulting from peptide treatment were similar to the plate reader data (Figure 4.5 A) where cells treated with the highest concentration of TP2-BID resulted in more caspase-3 activity while not incorporating SYTOX. Both TP2 and BH3-BID peptide treatments gave minimal caspase-3 activity and SYTOX incorporation.

Annexin-V staining was used to measure the early-stages of apoptosis caused by the loss of membrane symmetry with presentation of annexin-V binding partner, phosphatidylserine, on the surface of the cell rather than remain in the inner leaflet of the membrane (Figure 4.6 B). Cells were concurrently treated with peptide and SYTOX-green for 1 hour followed by removal of the media and a 3-hour recovery period. Cells that showed increase positivity of both annexin-V and SYTOX are most likely apoptotic due to membrane disruption as SYTOX incorporation was present only at the time of peptide addition and annexin-V staining was performed under conditions without peptide or SYTOX. Conversely, cells that show low SYTOX positivity and high annexin-V positivity are less likely apoptotic due to membrane disruption. The results with
Figure 4.6 Flow Cytometry of Cells Treated with SMTP-BID

A. Flow cytometry detection of caspase-3 activity and SYTOX-red incorporation Hela cells. Cells were treated for 2 hours at various peptide concentrations and a fixed concentration of SYTOX-red followed by late addition of cell-event caspase detection reagent. B. Flow cytometry data from Hela cells that were treated with various peptide concentrations and a fixed concentration of SYTOX-green for 1 hour. The media with peptide and SYTOX-green was removed and the cells recovered for 3 hours followed by late addition of annexin-V.
annexin-V from Figure 4.6 B were not ideal as the no treatment control (“Blank”) cells stained positive for annexin-V. This could be a result of two phenomena. The first and most likely scenario, high annexin-V staining in the no treatment control could be the result of inadequate removal of free annexin-V prior to damage caused by Hela cell harvesting with a cell scraper. This would allow any free annexin-V to stain cells stressed by the harvesting to bind to phosphatidylserine due to cell stress or annexin-V gaining access to the interior of a damaged cell caused by scrapping. The second and less likely scenario would be an overabundance of phosphatidylserine intrinsically present on the surface of Hela cells. These phenomena can be further investigated by live-cell microscopy imaging which would not require the need to scrape cells and should resolve if large amounts of phosphatidylserine are present on the surface of the cells used in this assay. Further, the concern of annexin-V artifacts can be avoided in the future by using a cell line that grows in suspension and does not require mechanical or enzymatic separation from a plate to harvest the cells for flow cytometry.

Confocal Microscopy Imaging of Cells Treated with SMTPs conjugated with BH3-BID Cargoes

Apoptosis was imaged using the caspase-3 detection reagent (green channel) in Hela cells that were treated with and without peptides for 24 hours. The cell impermeant DNA intercalating dye TOTO-3 (purple channel) was used to report on membrane integrity during peptide treatments. Incorporation of TOTO-3 without the detection of caspase-3 would indicate that peptide treatments caused membrane disruption rather than the induction of caspase-dependent apoptosis due to successful delivery of the BID peptide cargo. Staurosporine treatment of Hela cells was performed at a shorter 4 hour treatment to provide a positive control of apoptosis (Figure 4.7 B). Caspase-3 staining was more pronounced with staurosporine treated Hela cells when compared to control cells (Figure 4.7 A). TOTO-3 staining colocalized with caspase-3 positive cells, indicating that
A. TOTO-3 | Caspase-3 | Merge

B. TOTO-3 | Caspase-3 | Merge

C. TOTO-3 | Caspase-3 | Merge

D. TOTO-3 | Caspase-3 | Merge

E. TOTO-3 | Caspase-3 | Merge
Figure 4.7 Confocal Images of Cells Treated with SMTP-BID

A. Hela cells treated without peptide for 24 hours at 37°C. B. HELA cells treated with 500 nM staurosporine for 4 hours. C. Hela cells treated with 10 μM of BID alone for 24 hours. D. HELA cells treated with 10 μM of TP2-BID for 24 hours. E. Hela cells treated with 10 μM of BID-TP2 for 24 hours. All treatments were performed in serum-free media with 5 μM TOTO-3 (purple channel) present for the entire duration of treatment. Caspase-3 reagent (green channel) was added to each treatment 45 minutes prior to imaging on confocal.
staurosporine induced apoptosis independent of membrane permeabilization. Hela cells treated for
24 hours with the BH3 domain of BID alone (Figure 4.7 C) had similar caspase-3 and TOTO-3
positive cells as compared to control. BID cargo conjugated to the C-terminus of TP2 (Figure 4.7
D) showed an increase of caspase-3 positive cells when compared to control with the BID cargo
conjugated to the N-terminus of TP2 (Figure 4.7 E) showing more caspase-3 positive cells relative
to each other.

Membrane Asymmetry Loss after SMTP-BID Treatment of Jurkat Cells

Membrane asymmetry is vital for proper cell biology and loss of asymmetry is a hallmark
of early stages of apoptosis and necrosis\textsuperscript{95}. Anionic lipids are presented to the outer-leaflet of the
cell membrane during an apoptotic event for macrophage clearance, however these lipid species
can also be used for detection and quantification of apoptosis\textsuperscript{96}. A membrane probe, F2N12S, was
recently developed to sense the changes in surface charge of the outer-leaflet of the membrane
after anionic lipids are presented by a shift in the molecules emission spectra via an excited-state
intramolecular proton transfer (ESIPT)\textsuperscript{95}. The ratio between the two emission bands in response
to the change in surface charge establishes a quantitative measurement of membrane asymmetry
loss that is self-correcting and not subject to the false positives of other cell-surface membrane
probes of apoptosis such as the widely used annexin-V reagents. Thus, F2N12S provides a means
to detect the early stages of apoptosis in cells after treatment with SMTPs conjugated to
proapoptotic peptides derived from the BH3 domain of BID. Including TP2 conjugated to a mutant
BID peptide cargo shown by Czabotar et. al\textsuperscript{90} to not activate BAX oligomerization and thus
hypothesized to not cause apoptosis should serve as an additional negative control. Planktonic
Jurkat cells were treated with TP2 conjugated to BID or mutant BID (BIDMu), BID alone, BIDMu
alone or TP2 alone. The emission spectra of F2N12S in cell only controls showed a broad emission
A.

B.

C.

D.

E.

Cells Only
Staurosporine
BID
BID-TP2
BID-Mu
BID-Mu-TP2
TP2

Fluorescent Intensity

4000000
2000000
0

0
5000000
3000000
1000000
0
5000000
3000000
1000000
0

450 500 550 600 650
λ (nm)

450 500 550 600 650
λ (nm)

450 500 550 600 650
λ (nm)

450 500 550 600 650
λ (nm)

530/580 nm

500 nM Stauro

500 nM TP2

500 nM BID-Mu

500 nM BID-Mu-TP2

500 nM TP2

500 nM BID

500 nM BID-TP2

[peptide] μM

0 5 10 15 20 25
**Figure 4.8 Loss of Membrane Asymmetry in Jurkat Cells After SMTP-BID Treatments**

5x10^5 Jurkat cells treated with A. 1 μM B. 5 μM C. 10 μM or D. 20 μM of peptide (n = 3). The left column shows emission spectra of F2N12S after 4 hours of peptide treatment. E. The ratio of emission at 530nm and 580nm for F2N12S in cells treated with peptide. Staurosporine at 500 nM was used as a positive control for apoptosis (average 530/580nm, solid red line, n = 14).
peak at 530nm followed by a sharp peak at 580nm (Figure 4.8 A-D). Staurosporine treated cells provided the positive control for the assay with F2N12S emission showing a rise in 530nm emission peak that matched the 580nm emission peak, indicating a significant change in charge at the membrane surface. Either BID or the BIDMu peptides alone were not able to illicit a change in F2N12S emission at any concentration tested indicating that the peptide cargoes alone were not sufficient to induce a loss in membrane asymmetry and promote apoptosis. TP2 alone did not result in robust F2N12S emission increase at 530nm when the concentration was increased. The BID cargo conjugated to the N-terminus of TP2 (BID-TP2) showed an increase in F2N12S emission at 530nm at concentrations greater than 5 μM. BIDMu-TP2 also showed an increase in F2N12S emission at 530nm at the highest concentration tested (20 μM).

Discussion

The conjugation of membrane impermeant bioactive peptides to CPPs followed by successful delivery furthers highlights the utility of CPPs as therapeutically useful tools for drug design. Conjugation of phalloidin to TP2 is an ideal proof of principle for delivery of a membrane impermeant bioactive molecule. Cyclic peptides like that of phalloidin are of additional interest for therapeutic applications because they are resistant to proteolytic degradation while maintaining a large-stable conformation for target specificity that small molecules lack. Several cyclic peptide drugs are used in clinic and may be afforded greater therapeutic properties by conjugation to SMTPs. Conjugating TP2 to phalloidin did not diminish phalloidin’s bioactivity as peptide conjugates were able to bind to f-actin which resulted in a lack of depolymerization after desalting conditions (Figure 4.1). The lack of toxicity of the conjugate was initially surprising, however, given the stochiometric association between phalloidin and its intracellular target, f-actin, is 1:1, one would need to deliver a large amount of phalloidin to possibly promote toxicity. Also, the
actin network of certain cell types might differ enough to possibly cause a variability in the amount of phalloidin needed to cause a toxic event as the results for cell toxicity suggest (Figure 4.2). Regardless of the lack of cytotoxicity for the SMTP-phalloidin conjugates, enough phalloidin to promote a change in cell migration was observed (Figure 4.3). Wound migration was slowed in the two cell types treated with SMTP-phalloidin conjugates showing that the cargo was delivered. The effects of phalloidin were varied in the two cell types as well, further suggesting that the response of phalloidin may be cell-type specific. Delivery of different peptide cargoes of a similar size and orientation (i.e. cyclic) may be possible with SMTPs like TP2.

Delivery of the BH3 domain of BID was hypothesized to enable its interaction with other pro-apoptotic proteins such as BAX, resulting in BAX activation, permeabilization of the mitochondrial membrane and activation of apoptotic caspases. However, the strategy to synthesize the BH3 domain of proapoptotic protein BID with an SMTP, TP2, did not result in robust induction of caspase-dependent apoptosis. Given that a proposed mechanism of translocation of SMTPs like TP2 may involve peptide-induced lipid flip-flop for spontaneous translocating to occur\(^98\), a cargo attached through the backbone of the peptide sequence could thwart such mechanism and event from occurring efficiently by altering necessary conformational changes for translation. Other strategies to conjugate a peptide cargo such as the BH3 domain of BID should include conjugation of the peptide cargo with an available residue side chain of the SMTP such as a disulfide bridge between cysteines of the cargo peptide and the SMTP. This conjugation strategy would result in a peptide cargo that, if delivered free of endocytosis and lysosomal degradation, would be released from the peptide by the reduction of the sulfhydryl bond. Loss in membrane asymmetry, an early indicator of apoptosis, was detected with BID peptide cargo attached to TP2 (Figure 4.8). The significance of this finding may be the result of the cargo being attached to a membrane active
peptide, however, TP2 alone and the mutant BID peptide cargo attached to TP2 (BIDMu-TP2) did not result in the same magnitude of membrane asymmetry loss (Figure 4.8 E).

Both the possibilities and the limitations for bioactive peptide cargo delivery are realized with SMTPs. Linear peptide cargoes can adopt additional secondary structure while partitioning into a changing environment such as from bulk solution to the interfacial and hydrophobic core of a membrane which can further complicate their potential use as therapeutic bioactive cargoes. Bioactive cyclic peptides versus linear peptides may prove to be better bioactive cargoes for linear SMTPs due to less conformational variation for cyclic peptides before and during a translocation event. Ultimately, designing a peptide library screen tasked with monitoring the spontaneous and energy-independent delivery of one specific cargo for one specific intracellular target may be the most prudent option that can produce a CPP therapeutic in a high-throughput setting. The next chapter will propose a possible design for a peptide library screens centered on the delivery of one specific bioactive cargo as well as a peptide library screen for peptides that cross the lipid bilayer without influence from an attached cargo.
CHAPTER 5

Designing Future Peptide Library Screens for SMTP Discovery

Overview

One critical issue with using CPPs as delivery vehicles for potentially useful therapeutic cargoes is that the cargo itself contributes its own set of physical properties that can modulate the translocation abilities of the CPP to which it is conjugated. Fluorescent-molecules are commonly used polar cargoes for CPPs, however, the multitude of different fluorophores results in a gamut of cargo molecules with different physical chemical properties which have been shown to influence cell uptake of CPPs\(^8\). Further, cargoes such as peptides, peptide nucleic acids, oligonucleotides and other macromolecules are certain to have dramatic effects on uptake and delivery. One possible solution to cargo-dependent delivery would be to use established combinatorial chemistry and screening techniques to screen peptide libraries for delivery of particular cargoes. In this scenario, “designer” peptide sequences can be discovered to deliver a single cargo for a single therapy. This approach may give the desired outcome of delivering a specific cargo, but little information is generated to help us understand why and how such peptide sequences are able to deliver a specific cargo. What would better serve the CPP research field would be development of techniques using high-throughput discovery of peptide sequences that cross lipid bilayers without the reliance on some sort of reporter cargo like fluorophores. Without the presence of a cargo to change the physical chemistry of the peptide, researchers can begin to find experimentally derived peptide sequences that have innate cell-penetrating abilities. From these discoveries, trends associated with amino acid composition, position, length etc. can be compiled and used to construct peptides with optimal translocation properties prior to combining
the peptide with a cargo. Either solution outlined above can mitigate the problem of cargoes influencing the translocation properties of the attached CPP, however, which one is better depends on the question being asked by the researcher. Here some preliminary experiments to design such screens will be discussed.

A possible cargo-centric peptide library screen for energy-independent delivery of a peptoid version of DNA, called Peptide Nucleic Acids (PNAs), can be designed by entrapping dsDNA or ssDNA and cell impermeant DNA intercalating fluorescent dye in synthetic vesicles (Figure 5.1). PNAs are DNA mimics in which the backbone consisting of pentose linked together by phosphodiester bonds found in DNA are replaced with an uncharged peptoid backbone\textsuperscript{99}. The purine (adenine (A), guanine (G)) and pyrimidine (cytosine (C), thymine (T)) bases are then attached to the peptoid backbone of PNAs with methylene carbonyl linkages resulting in proper spatial organization for PNA/DNA basepairing (Figure 5.2). PNA/DNA complexes are more thermodynamically stable than DNA/DNA complexes as determined by higher dissociating melting temperatures for PNA/DNA complexes especially in low salt conditions\textsuperscript{100}. Strand invasion of dsDNA by PNA occurs with several binding modes with the majority of binding modes occurring between homopyrimidine PNA with homopurine dsDNA\textsuperscript{101,102}. PNAs are a particularly useful cargo to test with a peptide library because the PNA monomers can be synthesized with the same FMOC-chemistry used for solid supported peptide synthesis (SSPS). Previous studies indicated that PNAs synthesized continuously at the N-terminus of CPPs (i.e. the last addition when using SSPS) were more apt at delivering PNAs into cells versus stable covalent linkages involving side chains\textsuperscript{103}. Therefore, peptide library design using a single PNA sequence as a cargo attached to every peptide library member after completion of peptide synthesis is feasible.
Peptide Library Member with Complementary PNA Cargo

Successful PNA delivery = Loss of SYTOX Signal

DNA Oligos + Comp. PNA

SYTOX Green

Large Unilamellar Vesicles

dsDNA Oligos + SYTOX Green
Figure 5.1 Schematic of Synthetic Vesicle Screen for a PNA-Cargo Library

Schematic depicting a peptide library screen for peptide sequences that can spontaneously deliver a PNA cargo. All library members would be covalently attached with a PNA cargo that is complementary to one of the strands of the DNA oligos. dsDNA oligos are complexed with an impermeant DNA intercalating dye (in this case SYTOX-green), becoming fluorescent and then entrapped inside large unilamellar vesicles (LUVs). Successful delivery of PNA cargo will result in PNA strand invasion, disruption of dsDNA, and decrease SYTOX-green fluorescence. Measurements can be done in a 96-well format and detected on a plate reader.
Figure 5.2* PNA and DNA Watson-Crick Base Pairing

Depiction of the complementary basepairing between PNAs and DNAs. PNAs maintain the correct spatial organization of typical purine and pyrimidine bases while void of the typical highly charged pentose-phosphodiester linked backbone present in DNA.

*This figure was taken from:

A possible cargo-free peptide library screen for energy-independent delivery might be achievable using click chemistry. Screening a peptide library for spontaneous translocating peptides without the need of a cargo can eliminate the contributions of the cargo itself to the ability of the peptide to cross the lipid bilayer and enable us to easily test the sequence function relationships in translocation. The previous peptide library screen for SMTPs contained library members with a hydrophobic 7-Amino-4-methylcoumarin (AMC) cargo. The contribution of the AMC cargo to the ability of positive library members to translocate across lipid bilayers is unknown, however, TP2-AMC has a faster rate of translocation across POPC bilayers than TP2-NBD (Figure 5.3) possibly by the increased hydrophobicity of the AMC fluorophore cargo. Thus, developing a screen for a peptide library less influenced by an attached cargo gives more weight to the actual physical chemical contributions of the peptide sequence to translocate. Incorporation of an azide group as a “cargo”, which is inconsequential in size, charge, and polarity compared to fluorophore cargoes, in a “click-chemistry” scheme can allow for a screen of a peptide library less influenced by the cargo. Click-chemistry is a term used to describe the cycloaddition of an azide containing compound with an available alkyne to form a stable 1,2,3-triazole (Figure 5.4). The reaction rate of the cycloaddition is slow, however the addition of a copper (Cu I) catalyst allows the formation of a cyclic conjugated product on the order of minutes. Unfortunately, Cu I is unstable in aqueous solution and easily oxidizes into a catalytically inactive Cu II which can further degrade biological molecules, including reactions occurring with cells. Fortunately, the need for a copper catalyst to promote cycloadditions of azide-alkyne containing molecules is overcome by utilizing a “copper-free” click chemistry scheme with strain-promoted azide-alkyne cycloadditions (SPAAC). The SPAAC reaction is spontaneously driven by utilizing a highly strained cyclooctyne (the alkyne), thus eliminating the
**Figure 5.3 Translocation Rates for TP2 with Different Fluorescent Cargoes**

Translocation rates for TP2 with either a 7-Amino-4-methylcoumarin (AMC) or NBD fluorescent cargo in POPC-LUVs at a P:L = 1:500. The AMC cargo was present on all library members in the original SMTP library screen and represents the version of TP2-cargo with the highest rates of translocation.
**Figure 5.4** Click-Chemistry Reaction Schemes

A.* Reaction scheme outlining the cycloaddition of an azide (blue) with an alkyne (red) group without and with a copper catalyst. B. Reaction scheme outlining the copper-free Strain-Associated Alkyne Azide Cycloaddition (SPAAC).

*This figure was taken from:

need for a copper catalyst and all the possible toxicity associated with having copper present with biomolecules¹⁰⁶ (Figure 5.4 B).

Highly reactive cyclooctynes are commercially available and come conjugated to a wide variety of molecules, including several fluorophores. Azides can be incorporated in peptides with ease, most notably FMOC protected variants of lysine with an azide group substituting the side chain primary amine group. Incorporation of lysine-azide at the C-terminal position during a peptide library synthesis would ensure that all library members contain an azide compound and still maintain the N-terminus for downstream sequencing via Edman degradation. The commercially available compounds containing azides is also vast and includes a wide array of azide-fluorophores. A possible design of a library screen utilizing the SPAAC reaction with peptide library members all containing the small and stable azide group as a “cargo” to report on spontaneous translocation is outlined in Figure 5.5. The scheme of the screen relies on FRET pairing between an azide-fluorophore acceptor added alongside azide containing peptide library members to vesicles entrapped with a cyclooctyne-fluorophore donor. If an azide containing peptide library member can spontaneously cross the lipid bilayer of the vesicle without perturbing the membrane, then a SPAAC reaction between the peptide and cyclooctyne-fluorophore proceeds, blocking the possible FRET pairing between the cyclooctyne-fluorophore donor and azide-fluorophore acceptor (“Scenario 2” in Figure 5.5). Monitoring donor emission loss can quantify the efficiency of translocation of library members with a maintenance of donor emission due to peptide-cyclooctyne conjugates representing a positive result translocation event. This essentially cargo-less peptide library screen for spontaneous translocating peptides can establish a means of discovering SMTPs where all the physical chemical interactions needed for translocation are represented by the actual peptide sequence.
External

Library Member (Azide)

$\text{N}_3$-Fluorophore

DBCO-Fluorophore (ALKYNE)

Pore Formation

$\text{N}_3$-Fluorophore

$\text{N}_3$-Fluorophore

$\text{N}_3$-Fluorophore

Loss of Donor Emission

Scenario 1

Maintain Donor Emission

Scenario 2

Loss of Donor Emission

Scenario 3

Lyse

Lyse
Figure 5.5 Schematic of a Synthetic Vesicle Screen for a Cargo-free Library

Schematic of a copper-free click-chemistry screen design for SMTPs performed in large unilamellar vesicles (blue circles). The design calls for entrapment of an cyclooctyne containing fluorophore (DBCO-flurophore) in LUVs followed by the addition of an external azide containing fluorophore capable of FRET pairing with the chosen entrapped fluorophore as a donor-acceptor pair, respectively. Library members each containing an azide group are also added to the LUVs with entrapped alkyne-fluorophore. Three scenarios present with the present design. In “Scenario 1”, peptides that elicit considerable membrane disruption will allow external azide-fluorophore to cross the bilayer of the vesicle and react with the entrapped alkyne fluorophore, resulting in FRET pairing and loss of donor emission. “Scenario 2” depicts peptides that translocate across the bilayer without membrane disruption, react with the alkyne fluorophore and therefore the azide-fluorophore remains unable to react with the entrapped alkyne-fluorophore. This result can be further verified by lysing the vesicles and still report a maintenance of donor emission followed by HPLC verification of labeled peptide with the entrapped fluorophore. “Scenario 3” is the result of a library member that does not translocate nor causes significant membrane perturbation. Once these vesicles are lysed, the external azide-fluorophore would be able to access the entrapped alkyne fluorophore, react and result in a FRET paring and loss of donor emission.
Materials and Methods

PNA Displacement Assays

DNA oligonucleotides were ordered (Life Technologies), resuspended in DNase-free water, and concentrations measured on a spectrophotometer using UV absorbance at 260nm (Nano-Drop). PNA NH$_2$-CCTCTTACCTCAGTTACA-CONH$_2$ was ordered (PNA Bio) and concentrated in DMSO. Where dsDNA was needed, equimolar amounts of complementary oligos were mixed in a buffer (10 mM HEPES, 45 mM NaCl, 1 mM EDTA, pH 7.4) and allowed to anneal at room temperature for a minimum of 30 minutes. SYTOX was diluted from a concentrated stock of 5 mM in DMSO (ThermoFisher) at 1:1000 in buffer to 5 μM SYTOX for PNA/DNA displacement samples. DNA oligos depicted in Figure 5.6 A were diluted to the desired final concentrations in buffer containing 5 μM SYTOX in 96-well plates followed by measurement of SYTOX fluorescence (ex/em 504/523nm) on a plate reader (BioTek Synergy II). PNA was then added at the desired final concentration followed by continual measurement of SYTOX fluorescence over time (Figure 5.6 A). The same experimental procedure outlined above was carried out using a buffer containing either 45 mM of salt or no salt at all and either full length “dsDNA” oligos with a complementary strand to the PNA sequence 5’-TGTAACCTGAGGTAAGG or just this complementary strand referred to as “ssDNA” (Figure 5.6 B). Electrophoresis was performed by forming a polyacrylamide gel to include dsDNA with one complementary strand to the PNA sequence (“dsDNA” sample), dsDNA plus PNA, the complementary strand to the PNA sequence (“ssDNA” sample), and the complementary strand to the PNA sequence plus PNA (“ssDNA + PNA” sample) in buffer containing either 45 mM salt or no salt at all (Figure 5.6 B, bottom). Samples were incubated in buffer overnight before
electrophoresis was performed. The gel was imaged for the presence of dsDNA using ethidium bromide staining and imaged under UV light.

A homopyrimidine PNA, NH$_2$-CCCTTTTTTCCC-CONH$_2$, was synthesized on SRAM resin using peptide synthesis techniques used to synthesize, purify and verify the peptides from Table 2.1. Both the homopurine DNA oligo complementary to the homopyrimidine PNA and a homopyrimidine DNA oligo were ordered (Life Technologies), diluted in DNase-free water, followed by concentrations determination using UV absorbance at 260nm. Either the homopurine DNA oligo complementary to the PNA (“Comp. ssDNA” sample) or the non-complementary homopyrimidine DNA oligo (“Noncomp. ssDNA” sample) was diluted in buffer (10 mM HEPES, 45 mM NaCl, 1 mM EDTA, pH 7.4) containing 5 μM SYTOX-green and with or without addition of homopyrimidine PNA in a 96-well plate. SYTOX-green fluorescence was monitored over time using a plate reader (Figure 5.7 A). The % Sytox Signal lost was calculated from comparing the ratio of SYTOX-green signal with and without PNA treatment for either the homopurine DNA oligo (“Complementary DNA” sample) or the homopyrimidine DNA oligo (“Noncomplementary DNA” sample, Figure 5.7 B). A large volume of 2.5 μM of homopurine and homopyrimidine DNA oligos were allowed to anneal in buffer containing 5 μM SYTOX-green at room temperature for 1 hour. This dsDNA solution in buffer containing SYTOX-green was further diluted in buffer containing 5 μM SYTOX-green to create multiple samples with final concentrations of dsDNA ranging from 0.1-2.5 μM in a 96-well plate. Each dilution of dsDNA received either 2.5 μM of homopyrimidine PNA or no PNA followed by monitoring of loss of SYTOX-green fluorescence using a plate reader (Figure 5.7 C).

2.5 μM of ssDNA oligos listed in the table (Figure 5.7 D) were diluted in buffer (10 mM HEPES, 45 mM NaCl, 1 mM EDTA, pH 7.4) containing 5 μM SYTOX-green and allowed to
anneal at room temperature for 1 hour in a 96-well plate. The dsDNA oligos were then treated with and without 7.5 μM homopyrimidine PNA and incubated for 24 hours. SYTOX-green fluorescence was then measured using a plate reader (Figure 5.7 D).

**Entrapping dsDNA and SYTOX in Lipid Vesicles**

Enough 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti) lipids were dried under vacuum from a chloroform stock for at least 2 hours to make 1.2 mL of 20 mM lipids. SYTOX-green was diluted from a concentrated stock of 5 mM in DMSO (ThermoFisher) at 1:1000 in 1.2 mL buffer (10 mM HEPES, 45 mM NaCl, 1 mM EDTA, pH 7.4). Homopurine ssDNA (5’-GGG AAA AAA GGG) oligo complementary to the homopyrimidine PNA sequence (NH2-CCC TTT TTT CCC) was added to the 1.2 mL of 5 μM SYTOX-green solution at a final concentration of 100 μM. POPC lipids were then resuspended with the SYTOX-ssDNA solution and subjected to 10 freeze thaw cycles by liquid N2 plunges. After freeze thaws, the lipid solution was extruded through a 0.1 μm nucleopore membrane filter using a compressed nitrogen lipid extruder apparatus. The extruded POPC-LUVs were then placed into a 100,000Da MWCO spin filter at 4°C to rid the external solution of any SYTOX-green and ssDNA that did not become entrapped. The compartment that contained the POPC-LUVs was continually replenished with fresh buffer containing no SYTOX-green or ssDNA. Flow through was monitored for SYTOX-green fluorescence to ensure all SYTOX-green and ssDNA was entrapped in POPC-LUVs and not free in solution (Figure 5.8 A). After purification, lipid concentrations were determined by performing a Stewart Assay107.

Melp5, a potent pore forming peptide, was either added or not added to POPC-LUVs with ssDNA and SYTOX-green entrapped (“PC(ssDNA)” sample), POPC-LUVs, and buffer alone followed by addition of either 2,4, or 8 μM of homopyrimidine PNA for 30 minutes (Figure 5.8 B,
left). The same experiment was performed except only 8μM of homopyrimidine PNA was used for a 24 hour treatment (Figure 5.8, right).

**FRET measurements with Copper-free Click Chemistry Fluorophore**

Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC) reagents were purchased (Jena Bioscience) and resuspended in DMSO to a stock concentration of 2mg/mL and aliquoted. All experiments were performed in 96-well plate format and measured for donor emission (fluorescein, ex/em 496/518nm) on a plate reader (BioTek Synergy II). A typical experiment would require the serial dilution of one of the SPAAC reagents, followed by the addition of another reactive SPAAC reagent in DBPS 1x. This allowed for multiple molar ratios of the reactants to be tested at once. Donor emission was measured over time after addition of an azide-cyclooctyne fluorophore pair (Figure 5.9).

**Synthesis of an Azide-containing Peptide**

Tentagel Megabead resin (RAPP Polymere) was used to synthesize an azide-containing peptide. A UV-cleavable photo-linker was first added to the resin in order to simulate real peptide library conditions. The synthesis of a model azide-containing peptide was then performed with the following sequence: NH$_2$-C-K-K-K-K(N$_3$). Azido-lysine (K(N$_3$)-FMOC was purchased (Chemtech) and underwent typical coupling conditions for peptide synthesis. Azide-containing peptide was then cleaved from resin by UV treatment for at least 4 hours and resuspended with DCM, placed in a separate glass vial, dried under N$_2$ stream, and resuspended in ddH$_2$O. Stocks of peptide in ddH$_2$O were then used in assays (Figure 5.10).

**Entrapping Copper-free Click Chemistry Fluorophore into Lipid Vesicles**

Enough DBCO-Fluor was added to POPC lipids in chloroform to have a final concentration of DBCO-Fluor of 20 μM. Both DBCO-Fluor and POPC lipids were dried under vacuum for at
least 4 hours followed by resuspension with DPBS 1x. The lipid-cyclooctyne-fluorophore solution was then subjected to 10 freeze thaw cycles by liquid N\textsubscript{2} plunes followed by extrusion through a 0.1 \textmu M nucleopore membrane filter. The POPC-LUVs entrapped with DBCO-Fluor were then placed in a 100,000Da MWCO spin filter at 4°C. The flow through was collected and fresh buffer was continually added to the compartment of the filter that contained the POPC-LUVs. Flow through was monitored for fluorescein fluorescence to ensure that no free DBCO-Fluor was present. After purification, lipid concentrations were determined using Stewart assay.

POPC-LUVs with DBCO-Fluor entrapped were diluted in 1x DPBS at a concentration of 1 mM in a 96 well plate. A serial dilution of acceptor fluorophore able to undergo SPAAC with entrapped DBCO-Fluor, Cy3-azide (N\textsubscript{3}-Cy3), was either added or not to POPC-LUVs with DBCO-Fluor entrapped and monitored for donor emission loss on a plate reader (Figure 5.11 A). POPC-LUVs with DBCO-Fluor entrapped were diluted in 1x DPBS at a concentration of 1mM in a 96 well plate but before the addition of Cy3-Azide, POPC-LUVs with DBCO-Fluor entrapped were treated with 0.1% reduced Triton-X (Figure 5.11 B).

Results

PNA Displacement of Short and Full-Length dsDNA Oligonucleotides

Initial experiments to test the ability of PNA to disrupt dsDNA as monitored by loss of SYTOX fluorescence were performed with a PNA sequence containing a mixture of purine and pyrimidine nucleobases. The PNA sequence corresponds to another project ongoing in the lab at the time. Short oligonucleotides (7 b.p.) highlighted in color where complementary to the PNA sequence (dsDNA 1 and dsDNA 2) were used to form dsDNA complexes as detected by increased SYTOX fluorescence (Figure 5.6 A). Each of the complementary oligonucleotide contained a strand that was antisense to the PNA sequence and thus able to bind to the PNA if present. These
A. Sytox Fluorescence

- Sytox Only
- 1µM PNA
- 1µM PNA + 1µM ssDNA
- 1µM dsDNA 1
- 1µM PNA + dsDNA 1
- 1µM dsDNA 2
- 1µM PNA + dsDNA 2

B. 45mM NaCl vs No Salt

- 45mM NaCl
- No Salt

<table>
<thead>
<tr>
<th>dsDNA</th>
<th>ssDNA</th>
<th>dsDNA + PNA</th>
<th>ssDNA + PNA</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
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PNA
\[
\text{NH}_2\text{CCT CTT ACC TCA GTT ACA -CONH}_2
\]

dsDNA 1
\[
5'\text{TAA GAG G -3'}
\]
\[
5'\text{CCT CTT A -3'}
\]
dsDNA 2
\[
5'\text{GAG GTA AG -3'}
\]
\[
5'\text{CTT ACC TG -3'}
\]
Figure 5.6 dsDNA Strand Displacement with PNAs

A. Short (8 b.p.) DNA oligonucleotides each with a strand anti-sense to the PNA. The strands of the DNA oligonucleotides anti-sense to the PNA sequence are highlighted in red and/or green. Strand displacement was measured by the decrease in SYTOX fluorescence. B. PNA was added to 2.5 μM of either full-length (18 b.p.) dsDNA or a ssDNA anti-sense (complementary) to the PNA sequence and measured for SYTOX fluorescence. Electrophoresis was performed on equimolar PNA incubations with either dsDNA or ssDNA complementary to the PNA sequence in buffer with and without salt. “1” and “2” above the wells denote replicates. The gel was imaged with an ethidium bromide stain to detect the presence of dsDNA.
DNA oligonucleotides were designed to have minimal opportunities to self-anneal after PNA strand invasion to avoid residual SYTOX fluorescence. SYTOX only in buffer resulted in very low fluorescence as expected due to no dsDNA present. PNA alone did not result in significant SYTOX fluorescence suggesting that self-annealing of PNA does not report with SYTOX fluorescence. A full-length oligonucleotide anti-sense to the PNA sequence was added equimolar to the PNA sequence (“PNA + ssDNA sample”). The PNA/DNA hybrid did not result in SYTOX fluorescence. Addition of equimolar PNA to the short (8 b.p.) dsDNA oligos resulted in a decrease in SYTOX fluorescence indicating that PNA disrupted dsDNA complexes (Figure 5.6 A). To increase the signal to noise ratio, longer complementary oligonucleotides (18 b.p.) were tested with PNA additions (Figure 5.6 B). Both salt added and no salt conditions were tested to possibly enhance PNA strand invasion by destabilizing the annealing dsDNA due to the absence of cationic counterions. The longer oligos comprised of purines and pyrimidines are predicted to self-anneal, allowing for the formation of hairpin DNA regardless of a complementary DNA strand being present which can also report with SYTOX fluorescence. Under salted conditions (45 mM), PNA was not able to displace the longer dsDNA oligos as the SYTOX signal did not change significantly. However, ssDNA SYTOX fluorescence decreased when complementary PNA was added in both salt added and no salt conditions. No salt conditions greatly reduced the SYTOX fluorescence of dsDNA when compared to salt added conditions. PNA addition to dsDNA under no salt condition showed a variable decrease in SYTOX fluorescence, suggesting that PNA can cause strand invasion of dsDNA under optimal salt conditions as previously reported. Electrophoresis was used to image the displacement of dsDNA with PNA treatment (Figure 5.6 B., bottom) with ethidium bromide (EtBr). Like the SYTOX florescence detection, EtBr staining of PNA treatment of dsDNA in salt added conditions resulted in no dsDNA disruption. However,
no salt conditions allowed for most of the dsDNA to be disrupted upon PNA treatment with faint staining of dsDNA.

A simple homopyrimidine PNA (NH$_2$-CCC TTT TTT CCC) was synthesized to better control for strand invasion of homopurine ssDNA (5’-GGG AAA AAA GGG) (Figure 5.7 A). Interestingly, the complementary ssDNA containing homopurines showed SYTOX fluorescence despite the inability to self-annel or presence of a complementary oligo to for dsDNA formation. Addition of PNA to the complementary ssDNA resulted in a loss of SYTOX fluorescence. Sequential addition of more PNA was added to the same samples and monitored over the order of minutes resulted in even more loss of SYTOX fluorescence with a molar ratio of 3:1 PNA:ssDNA giving the maximal decrease of SYTOX fluorescence. An equal volume of ddH$_2$O was added to samples containing only ssDNA as a control for the additional volume added to samples due to PNA treatments. Addition of PNA to the noncomplementary homopyrimidine ssDNA oligo did not result in any change of SYTOX fluorescence. The % Sytox Signal was calculated based on the relative drop in SYTOX fluorescence after PNA treatments over the SYTOX fluorescence without PNA treatments (Figure 5.7 B). These results indicate that PNA was able to bind a complementary ssDNA resulting in a displacement of SYTOX and a decrease in SYTOX fluorescence. The same experiment was performed with the formation of dsDNA by annealing the two homopyrimidine/purine DNA oligos at various concentrations, followed by addition of 2.5μM of PNA. SYTOX fluorescence did not decrease at any molar ratio of PNA:dsDNA tested (Figure 5.7 C). A few oligos were design to promote PNA strand invasion of dsDNA with either a mismatched base in the middle of its sequence or the removal of G/C content from either the 5’ or 3’ end of the complementary strand. A 3:1 molar ratio of PNA to oligos was again used to measure the decrease
A. 

B. 

C. 

D.
Figure 5.7 Homopyrimidine PNA Strand Displacement of dsDNA and ssDNA

A. Table shows the homopyrimidine PNA sequence along with a complementary ssDNA oligo sequence comprised of homopurines and a noncomplementary ssDNA oligo sequence comprised of homopyrimidines. The addition of PNA to the complementary homopurine ssDNA resulted in a decrease in SYTOX fluorescence. B. The % sytox signal lost as a result of PNA treatment of homopurine ssDNA. C. 2.5 μM of homopyrimidine PNA was added to dsDNA made by annealing the homopurine/pyrimidine oligos from the table above. There was no loss of SYTOX fluorescence for multiple molar ratios of PNA:dsDNA. D. 24 hour incubation of dsDNA with design considerations (in table to the right) to promote PNA strand invasion. PNA was added to dsDNA at a molar ratio of 3:1. SYTOX fluorescence showed a decrease with smaller and less G/C content dsDNA oligos.
in SYTOX fluorescence. The mismatched oligo did not result much decrease of SYTOX fluorescence when treated with PNA, however the decrease in length and or G/C content of the complementary DNA strand for the PNA resulted in a decrease in SYTOX fluorescence (Figure 5.7 D).

*Entrapping ssDNA and SYTOX in POPC-LUVs*

Based on the previous results outlined in Figure 5.7, a homopurine ssDNA sequence (5’-GGG AAA AAA GGG) and SYTOX were entrapped in POPC large unilamellar vesicles (POPC-LUVs) to test the feasibility of using PNA displacement of SYTOX as a possible peptide library screen design for SMTPs capable of delivering PNA cargoes. Verification of ssDNA and SYTOX entrapment was accomplished by measuring flowthroughs from molecular weight cut-off purification of extruded lipids for SYTOX fluorescence (Figure 5.8 A). Flowthroughs were additionally tested by adding an excess of SYTOX (“+SYTOX” group). Purified POPC LUVs entrapped with ssDNA and SYTOX (PC(ssDNA)) were then used for PNA treatments (Figure 5.8 B). PNA was added at various concentrations for 30 minutes and 24-hour incubations. PC(ssDNA) vesicles did not show a drop in SYTOX fluorescence with any of the PNA treatments tested, indicating that the PNA is unable to cross the vesicle bilayer. A potent pore forming peptide, Melp5, was added to vesicles for PNA to access the entrapped ssDNA. A loss in SYTOX fluorescence was observed with Melp5 treatment of PC(ssDNA) vesicles. 24 hour incubations showed further decrease in SYTOX fluorescence with Melp5 treated PC(ssDNA) vesicles. In summary, ssDNA with SYTOX can be entrapped in LUVs and PNA alone cannot cross the lipid bilayer unless the bilayer is disrupted (i.e. Melp5 treatments) or, presumably, unless the PNA is transported across the membrane by a translocating peptide.
**Figure 5.8 SYTOX-ssDNA Entrapped POPC-LUVs Purification Verification**

A. Results from testing flowthroughs from POPC-LUVs entrapped with ssDNA and SYTOX that were placed in a MWCO spin column. Flowthroughs were tested for the presence of residual (un-trapped) ssDNA by adding SYTOX (“+Sytox” group). B. After external solution was verified to not contain ssDNA or SYTOX, PNA was added at various concentrations to either buffer with 5 μM SYTOX, POPC-LUVs (POPC) with 5 μM SYTOX, or POPC-LUVs entrapped with complementary ssDNA and SYTOX (PC(ssDNA)).
FRET Measurements of Copper-free Click Chemistry Fluorophores

Förster Resonance Energy Transfer (FRET) of commercially available copper-free click chemistry fluorophores was tested. FITC and Cy3 fluorophores were chosen for their ideal FRET spectral properties, where FITC serves as the donor fluorophore and Cy3 serves as the acceptor fluorophore\textsuperscript{109} (Figure 5.9 A). FITC and CY3 fluorophores both come commercially available as either azide or cyclooctyne (DBCO) containing reagents which allows donor/acceptor pairs to be switched based on the clickable moieties present on the fluorophore. A loss of donor emission can be measured if FRET pairing occurs due to SPAAC reactions between azide and cyclooctyne fluorophores. DBCO-Cy3 (acceptor fluorophore) was serially diluted with 200 nM Azide-fluorescein (donor-fluorophore). A loss in donor emission was observed over time when the acceptor fluorophore was in molar excess (Figure 5.9 B). Cy3-maleimide (a sulfhydryl reactive moiety) was used in place of DBCO-Cy3 to test if any non-specific interactions between the fluorophores produce the same drop in donor emission observed previously (Figure 5.9 C). No donor emission loss was observed when the same molar ratios of fluorophores were used. Therefore, donor emission loss is associated with the SPAAC reaction between the two fluorophores and not due to any non-specific interactions between fluorophores.

Various scenarios outlined in Figure 5.5 that could result from using these copper-free click chemistry fluorophores as part of a peptide library screen for cargo-less spontaneous translocation of azide-containing peptides were tested. “Scenario 2” depicts a peptide library member that crosses the lipid bilayer of vesicles to undergo SPAAC reaction with the entrapped DBCO-fluorophore while not perturbing the membrane to allow external azide-fluorophore from entering the vesicle to also undergo SPAAC reaction with the entrapped DBCO-fluorophore (Figure 5.5, “Scenario 2”). This would be considered a positive event of spontaneous translocation because
A. Time (Min) 528/20 nM E m.

B. Time (Min)

C. Time (Min)
**Figure 5.9 FRET Measurements of SPAAC Fluorescent Reagents**

A. Spectral analysis plotting excitation (dotted lines) and emission (solid filled) wavelengths of the donor (FITC/Fluorescein, yellow) and acceptor (Cy3, blue) fluorophores (*Graph made using biolegend.com spectral analyzer) B. A serial dilution of acceptor fluorophore cyclooctyne (DBCO)-Cy3 was added to 200 nM of donor fluorophore azide-fluorescein (Azide-Fluor) and monitored for donor emission loss (i.e. fluorescein emission (528nm)) over time. Donor emission of Azide-Fluor was measured over the same time period without addition of DBCO-Cy3 (upper right). C. Cy3-maleimide was used in place of DBCO-Cy3 to measure any non-specific contribution of CY3 to the results found in B. No loss in donor emission was observed with Cy3-maleimide treatments.
DBCO-fluorophore would react with the azide-containing peptide library member sequestered in the vesicle and thus a maintenance of DBCO-fluorophore emission. A model azide-containing peptide was synthesized with a variant of lysine that has an azide group substituting the side chain primary amine group. The azido-lysine peptide (NH$_2$-CK$_4$K-Azide) was incubated with a serial dilution of donor fluorophore, DBCO-fluorescein (DBCO-Fluor), to undergo SPAAC reactions for 24 hours followed by the addition of acceptor fluorophore, Azide-Cy3 (Figure 5.10 A). Donor emission was monitored (i.e. DBCO-Fluor emission) overtime after the addition of Azide-Cy3. All samples that did not contain a preincubation of azide-containing peptide saw a loss in donor emission. Conversely, DBCO-Fluor preincubated with azide-containing peptide saw a maintenance of donor emission for all of the molar ratios of donor:acceptor tested. Azide-containing peptide was therefore determined to react with the DBCO-Fluor thereby inhibiting FRET to occur with the acceptor fluorophore, Azide-Cy3. “Scenario 1 and 3” from Figure 5.5 depict what would happen if a loss of donor emission occurred after peptide treatment either by peptide-induced membrane perturbation (Figure 5.5 “Scenario 1”) or by totally membrane inactive peptides neither translocating nor causing membrane perturbations (Figure 5 “Scenario 3”). Azide-containing peptide was added to a serial dilution of acceptor fluorophore, Azide-Cy3, followed by addition of donor fluorophore, DBCO-Fluor (Figure 5.10 B). Donor fluorescence was then monitored over time, resulting in all samples showing a loss in donor emission. The presence of both azide-containing peptide did not outcompete Azide-Cy3 from reacting with DBCO-Fluor, indicating that if azide containing peptide is present at same timescales as azide-fluorophore, SPAAC between the fluorophores would proceed resulting in donor emission loss.

**Entrapping Copper-free Click Chemistry Fluorophore into Lipid Vesicles**

Several attempts to entrap cyclooctyne containing fluorophores were performed. 1mM POPC-
Figure 5.10 FRET Measurements of SPAAC Fluorescent Reagents with Azide-Peptide

A. Serial dilution of DBCO-fluorescein (DBCO-Fluor) was incubated with (black) and without (gray) peptide containing an azido-lysine (NH₂-CK₄K-Azide) for 24 hours followed by next day addition of 7 μM of Azide-Cy3 to each well. Donor emission was then measured over time. This experiment is modeled to test “Scenario 2” of the copper-free click chemistry screen (Figure 5).

B. 1.5 μM of DBCO-Fluor was added to a serial dilution of Azide-Fluor and azido-lysine peptide followed by measuring donor emission loss over time. This experiment is modeled to test “Scenario 1” and “Scenario 3” of the copper-free click chemistry screen (Figure 5).
LUVs entrapped with the donor fluorophore, DBCO-Fluor, were added to a serial dilution of the acceptor fluorophore, Azide-Cy3, or to buffer alone (Figure 5.11 A). Steady donor emission loss was apparent over the timepoints measured. The donor emission in vesicles alone did not change over time. Reduced Triton-X was used to treat 1mM of POPC-LUVs entrapped with DBCO-Fluor to disrupt the membrane and allow full access of the donor fluorophore with the acceptor fluorophore (Figure 5.11 B). Reduced Triton-X treated POPC-LUVs with entrapped DBCO-Fluor did result in a faster loss of donor emission when compared to untreated POPC-LUVs (Figure 5.11 B vs. A). In summary, it appears that the acceptor fluorophore, Azide-Cy3, can access the entrapped donor fluorophore, DBCO-Flour. In adequate entrapment of the donor fluorophore in POPC-LUVs may have been ruled out because purification of these vesicles utilized MWCO spin filters which yielded flow through that did not contain the donor fluorophore.
A.

B.
Figure 5.11 SPAAC Fluorescent Reagents Entrapped POPC-LUVs

A. 1 mM of POPC-LUVs entrapped with DBCO-Fluor added to a serial dilution of Azide-Cy3 (N3-Cy3, black dots) or to buffer alone (black “X”s). B. 1 mM of POPC-LUVs entrapped with DBCO-Fluor and treated with reduced 0.1 % Triton-X followed by addition to a serial dilution of Azide-Cy3 or to buffer alone.
Discussion

The effects a cargo can have on the translocation efficiency of a CPP should not be overlooked. Designing peptide library screens which consider the total physical chemical contribution of the cargo can be accomplished with a cargo-centric emphasis by conjugating one cargo to every peptide library member and then screening for spontaneous translocation across a synthetic lipid bilayer. Conversely, implementing a cargo-less peptide library screen that selects peptide sequences which possess the physical chemical properties for spontaneous translocation across synthetic lipid bilayer. These peptide sequences can further inform if any discernable patterns exist for spontaneous peptide translocation.

A cargo-centric screen was proposed to include delivery of peptide nucleic acids (PNAs) in part because of the ease of incorporation into already established peptide synthesis techniques, but also to design a screen capable of finding peptide sequences that can spontaneously deliver a potentially bioactive PNA sequence. It is assumed that the design of this screen would allow any DNA target sequence to be entrapped inside lipid vesicles along with a fluorescent DNA dye such as SYTOX. All peptide library members would then be synthesized with the complementary PNA sequence for the entrapped DNA target sequence. The sum of the PNA cargo and peptide physical chemical properties can then be screened for spontaneous translocation of the PNA cargo into lipid vesicles as determined by a loss in fluorescent DNA dye signal as PNA binds tightly to the target DNA sequence.

The feasibility of the cargo-centric screen for PNA cargoes was assessed (Figure 5.1). Design considerations for the proposed DNA target sequence to entrap inside lipid vesicles should consider the ionic strength of the buffer system used as well as the length of DNA target sequence needing to be entrapped. When DNA oligos were shorter in length to the complementary PNA
sequence or contained less G/C content, better displacement of DNA intercalating dye, SYTOX, was observed (Figure 5.6 A and Figure 5.7 D). ssDNA does fluoresce with SYTOX and does remain entrapped inside POPC-LUVs (Figure 5.8) and therefore can be used to design a cargo-centric peptide library screen for the spontaneous delivery of PNA cargo. The use of dsDNA would be more advantageous in this screen design because the fluorescent properties of most DNA intercalating dyes are greatly enhanced with dsDNA rather than ssDNA. PNA strand invasion of a proposed dsDNA target sequence to entrap would need to be studied in some detail. It is known that the mechanism of PNA strand invasion is reliant on the ability to destabilize or open the dsDNA, either by designed mismatches or changing the ionic strength of the buffer. Indeed, the incorporation of one mismatch in the middle of a target dsDNA oligo did result in improved PNA strand invasion as determined by loss in SYTOX signal (Figure 5.7 D). PNAs are known to triplex with a homopurine target within a dsDNA to promote strand invasion, however, this significantly hampers the library screen design if all PNA cargoes need to be homopyrimidines.

A cargo-less screen, able to find peptide sequences that spontaneously cross a lipid bilayer independent of cargo, is equally important for the study of sequence-function relationships. Utilization of biorthogonal copper-free click chemistry fluorophores was proposed to design a screen of a peptide library whose library members only need the single addition of a modified amino acid that contains a small biologically inert hydrophilic azide (N$_3^+$) group (Figure 5.4 and 5.5). Strain-Promoted Alkyne-Azide Cycloaddition (SPAAC) is a useful biorthogonal conjugation scheme that has a myriad of commercially available azide-containing reagents and cyclooctyne-containing reagents. This reaction was thought to produce a FRET pairing between two reactive donor/acceptor fluorophores, one azide-fluorophore with one cyclooctyne-fluorophore. By segregating the cyclooctyne reactive fluorophore inside a lipid-vesicle, peptide-library members
all containing an azide group would be screened for their ability to translocate across the lipid bilayer with nothing more than their azide “cargo” and undergo SPAAC with the cyclooctyne-fluorophore. This reaction between the library member and the cyclooctyne-fluorophore would block possible FRET pairing of an azide-fluorophore resulting in a maintenance of donor-fluorophore emission even after vesicle disruption. This scenario described would be indicative of a positive observance of a spontaneous translocating peptide free of the effects from the physical chemical properties of a conjugated cargo.

The use of fluorescein and Cy3 as a donor-acceptor pair for FRET measurements was used to test azide-cyclooctyne SPAAC reactions. The donor-acceptor pairs were tested as either an azide or a cyclooctyne with both conditions showing SPAAC induced FRET (Figure 5.9 and 5.10). A short peptide containing an azido-lysine blocked SPAAC induced FRET by first reacting with the cyclooctyne-fluorophore (Figure 5.10 A). Entrapment of the cyclooctyne (DBCO-Fluor) in POPC-LUVs proved to be straightforward, however, addition of the azide-fluorophore showed SPAAC induced FRET regardless membrane integrity (Figure 5.11). We hypothesize that the large hydrophobic DBCO group either disrupts membranes directly or can translocate across, at least transiently. This presents a problem with the initial design of the library screen (Figure 5.5). An external azide-fluorophore is needed to undergo SPAAC induced FRET pairing to assess if the peptide library members are causing membrane disruption or having no affect at all (“Scenario 1 and 3” in Figure 5.5). Future design considerations need to maintain exclusion of the two SPAAC reagents, possibly by design of a DBCO-containing fluorophore with more membrane impermeant properties such as an azido-lysine variant of ONEG, an observed negative for spontaneous translocation originally discovered in the first peptide library screen for SMTPs56. The spectral overlap of these two fluorophores is sufficient to produce FRET and the R₀ for this fluorophore
pair was definitively calculated to be 56 Å. As the authors of the previous study which calculated this $R_0$ value note, the distance needed to produce FRET between fluorescein and Cy3 is large relative to other donor-acceptor pairs. This is yet another consideration for the design of this proposed library screen involving SPAAC induced FRET. In summary, the principle of using copper-free click chemistry to design a screen for a peptide library where no cargo is needed for monitoring spontaneous membrane translocating peptides is possible. Additional research needs to be conducted in order to find a set of conditions where lipid vesicles can appropriately separate SPAAC reagents.
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