Expanding the Applications of Mitochondrial Targeted Delivery Peptides

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Abstract

Mitochondria play a critical role in the generation of cellular energy, the organization of metabolic pathways, and regulation of programmed cell death. The study of these processes is complicated by the impermeability of the mitochondrial membrane, particularly the inner membrane, to chemical agents designed to probe the biochemistry of mitochondria. Previous research conducted in the Kelley lab has led to the development of peptide-based vectors to overcome these limitations. These mitochondria penetrating peptides (MPPs) are capable of transduction through the cell and mitochondrial membranes, concentrating within the mitochondrial matrix with high specificity. MPPs have been used to localize a number of small molecule probes and therapeutics allowing for the study of biological processes which would have previously been impossible.

With the basic principles of MPP based mitochondrial study established, herein data is presented on the expansion of the breadth of compounds, organisms, and molecular targets which can be manipulated using MPP conjugates. We describe the development of a releasable linker for MPP conjugation to bioactive cargo in order to release compounds from MPPs following localization in the mitochondrial matrix. This
strategy allows for the use of compounds whose activity would otherwise be impacted by MPP conjugation. The co-targeting of bacteria by MPPs based on the physiochemical similarities of bacteria to mitochondria is also investigated. The chemical properties required by MPPs for bacterial and mitochondrial co-targeting as well as methods to control relative affinity towards these targets are presented in order to allow for preferential control of delivery. Finally, initial investigations into the use of MPPs paired with a new class of \textit{in cellulo} RNA secondary structure probe are presented. The combination of a secondary structure probe and an MPP could potentially lead to new avenues towards understanding the \textit{in vivo} structure of key structural mitochondrial RNAs such as rRNAs and tRNAs and the biochemical basis in which disease-causing mutations affect their function. Taken together, these findings expand the potential uses for MPPs for biochemical research as well as potential therapeutic applications.
Acknowledgments

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List of Abbreviations

ALS – Amyotrophic lateral sclerosis
AMP – Antimicrobial peptide
AMPK – AMP-activated protein kinase
ANT – Adenine nucleotide translocator
APAF1 – Apoptotic protease activating factor 1
ATP – Adenosine triphosphate
BID – BH3 interacting-domain death agonist
CDI – Carbonyldiimidazole
CPP – Cell penetrating peptide
DHFR – Dihydrofolate reductase
DNA – Deoxyribonucleic acid
DTT – Dithiothreitol
ER – Endoplasmic reticulum
ETC – Electron transport chain
FADH₂ – Dihydroflavine adenine dinucleotide
FAI – 2-methyl-3-furoic acid imidazolide
FITC - Fluorescein isothiocyanate
GTP – Guanosine-5′-triphosphate

HSP90 – Heat shock protein 90

LC3 – Microtubule-associated proteins 1A/1B light chain 3A

MELAS – Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes

MERFF – Myoclonic epilepsy with ragged-red fibers

MIA complex – Mitochondrial intermembrane space assembly complex

MOMP – Mitochondrial outer membrane permeabilization

MPP – Mitochondrial penetrating peptide

mtRNAP – Mitochondrial single-subunit RNA polymerase

MTS – Mitochondrial targeting sequence

MUTYH – MutY DNA glycosylase

NADH – Nicotinamide adenine dinucleotide

NMR – Nuclear magnetic resonance spectroscopy

N-terminal – Amino terminal

PAM complex – Presequence translocase-associated motor complex

PEG – Polyethylene glycol

PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PINK1 – PTEN-induced putative kinase 1

RAD23A – UV excision repair protein RAD23 homolog A

RNA – Ribonucleic acid
ROS – Reactive oxygen species

rRNA – Ribosomal ribonucleic acid

SAM complex – Sorting and assembly machinery complex

SHAPE – Selective 2’-hydroxyl acylation analyzed by primer extension

SIRT 1 – NAD-dependent deacetylase sirtuin-1

TAMRA – Carboxytetramethylrhodamine

TCA cycle – Tricarboxylic acid cycle

TCEP – Tris(2-carboxyethyl)phosphine

TFAM – Mitochondrial transcription factor A

TIM – Translocase of the inner membrane

TOM – Translocase of the outer membrane

TPP – Triphenylphosphonium

TRAP-1 – TNF receptor-associated protein 1

tRNA – Transfer ribonucleic acid

VDAC – Voltage-dependent anion channel

XRCC4 – X-ray repair cross-complementing protein 4
1.1 Overview of Mitochondrial Biology

Since the genesis of complex life, evolution has selected for the compartmentalization of cellular function into organelles, subcellular bodies designed to organize and enhance the myriad of cellular processes required to sustain life. Understanding the individual actions of these organelles is the basis of understanding the cell itself, and even the seemingly simplest organelles exhibit intricate complexity upon closer study. Perhaps the most complex of the organelles in terms of function, structure, and organization is the mitochondrion. Mitochondria are best known as the primary site of aerobic energy production; however, their function goes far beyond that of providing cellular power even in the most general terms. They also play a central role in the internal control of cell life and death, are both a central receiver and producer of cellular signals and serve as the physical platform for many of the key metabolic processes of the cell.

As the scientific understanding of the mechanisms underpinning cellular homeostasis, disease states, and pharmacological interactions becomes clearer the key role mitochondria play in many of these biological processes has become increasingly well established. The diversity in mitochondrial function originates from the exceptionally complex machinery that drives mitochondrial biology. While significant advancements have been made in understanding the general mechanisms of mitochondrial function, many aspects these pathways remain unknown due to the difficulty in studying these organelles.

Mitochondrial biology and the nature of the interactions they make with the other components of the cell are both diverse and unique compared to other organelles (Figure 1.1). Mitochondria are the only animal cell organelle besides the nucleus which contains genomic material, in addition to a unique set of proteins for DNA replication, transcription, and translation. This arose from the fact that mitochondria are
Figure 1.1 – Summary of major mitochondrial processes. A) Mitochondrial proteins are imported through the TIM/TOM translocase complexes based on the recognition of a mitochondrial targeting sequence in the imported protein and/or a chaperone which binds to it. B) Mitochondrial DNA replication and transcription is controlled through the levels and activity of transcription factors. Various retrograde and anterograde signaling processes between mitochondria and the nucleus control the overall activity and number of mitochondria through this process. C) Mitochondrial DNA exists in a multi-copy state and encodes 13 mRNAs for components of the ETC machinery, as well as 22 tRNAs and 2 rRNAs. Replication is conducted by mitochondrial polymerase γ. D) Translation of the mitochondrial mRNAs occurs independently of the rest of the cell with mitochondrial specific ribosomes. E) The electron transport chain is the major producer of cellular energy as ATP and is the process which generates the potential difference across the mitochondrial inner membrane. F) Dysfunctional mitochondria are cleared through the action of mitophagy, in which membrane signals are recognized by the autophagic machinery leading to mitochondrial digestion and recycling.
the result of a prehistoric fusion between an ancient eukaryotic ancestor and a symbiotic α-proteobacteria\textsuperscript{1}. The extracellular origin of mitochondria is particularly obvious when looking closer at the biochemical properties of mitochondrial replication\textsuperscript{2}. The structure and organization of the components of the mitochondrial machinery differ significantly from their cytosolic counterparts, reflecting their external origin\textsuperscript{3}. The mitochondrial DNA polymerase Pol\textsuperscript{γ} and RNA polymerase mtRNAP both closely resemble those found in T7 bacteriophages rather than eukaryotic or bacterial species\textsuperscript{4, 5}. It is likely these genes were contained within the original proteobacterial endosymbiont as a result of phage infection and DNA recombination\textsuperscript{6}, although the possibility of subsequent acquisition is also possible. The mitochondrial translation machinery more closely resembles the expected eubacterial structure\textsuperscript{7, 8}, however, there are significant differences in the structure of the mitochondrial machinery from their bacterial and viral analogs, arising from the evolutionary divergence mitochondria have experienced over the course of evolution.

The majority of genes encoding mitochondrial proteins have undergone gene transfer to the nucleus, a phenomenon believed to have arisen as functional redundancy between nuclear and mitochondrial proteins selected for their elimination in mitochondria and consolidation in the nucleus\textsuperscript{9}. In terms of genetic organization, the mitochondrial DNA encodes 13 key components of the electron transport chain as well as an exclusive set of 22 mitochondrial tRNA and 2 rRNAs, components essential and required in high volume for mitochondrial function. A number of theories that account for the evident evolutionary pressure towards nuclear gene transfer have been proposed, such as the lower nuclear mutation rate and the competitive replication benefits of a smaller mitochondrial genome, however, the relative impact of each theory is as yet unclear\textsuperscript{10}. Therefore, most of the ~1000 proteins involved in mitochondrial function are encoded in the nucleus and must be imported into mitochondria\textsuperscript{11}.

A number of molecular mechanisms are used for protein import into the mitochondrial periplasmic space and matrix. Most commonly, proteins destined for mitochondria contain mitochondrial targeting pre-sequences in the N-terminal region which act as a recognition site for cytosolic chaperones and other specific targeting factors that facilitate delivery to mitochondria\textsuperscript{12}. Mitochondrial surface receptors then
recognize the pre-sequence/chaperone complex and facilitate the transfer of the precursor through the ATP dependent outer membrane translocase complex TOM\textsuperscript{13}. In the intermembrane space, the precursors bind to the Tim9/Tim10 complex, stabilizing the precursor and in some cases guiding them to subsequent processing complexes\textsuperscript{14, 15}. From this point, a number of other events occur depending on the final location of the precursor protein, dictated by the molecular sorting signals contained at the N-terminus and/or internally within the peptide sequence. Proteins destined for the outer mitochondrial membrane are inserted by the SAM complex\textsuperscript{16}, those destined for the intermembrane space are stabilized and released by the MIA complex\textsuperscript{17}, and those destined for the inner membrane are inserted by the TIM22 complex\textsuperscript{18}. Mitochondrial matrix proteins must go through a second inner membrane translocase complex. An intramembrane domain of the TOM complex recognizes the matrix targeting pre-sequence and delivers the precursor to Tim50\textsuperscript{19}, which guides the precursor through the membrane channel Tim23\textsuperscript{20}. The translocation of the precursor is then driven by the binding of the ATP-dependent motor complex PAM\textsuperscript{21}. In all cases, cleavable pre-sequences are cleaved by mitochondrial peptidases releasing the mature peptide. While this is the general scheme of mitochondrial protein import, many precursor specific variations which use alternative pathways or forgo certain aspects of the import machinery have been noted, and aspects of protein import regulation and signaling are still being researched to this day\textsuperscript{22}.

The total volume of mitochondria in the cell is tightly regulated to maintain cellular energy homeostasis. While mitochondrial proliferation in the cell is independent of the proliferation of the cell itself, it is highly regulated through signaling mechanisms between the nucleus and mitochondria\textsuperscript{23}. Regulation of mitochondrial biogenesis is controlled through PGC-1α, a nuclear transcriptional coactivator which controls the expression of a number of genes involved in mitochondrial proliferation\textsuperscript{24}. PGC-1α expression and activity are in turn controlled by a number of other pathways which sense cellular energy load, oxidative stress, and even physical factors such as temperature\textsuperscript{25}. The need for increased mitochondrial biogenesis is sensed through these pathways, and increased expression of genes encoding mitochondrial proteins in the nucleus is initiated by PGC-1α. These genes also include TFAM, a nuclear
encoded mitochondrial transcription factor which promotes the replication and transcription of the mitochondrial DNA\textsuperscript{26, 27}. Mitochondrial autophagy forms the counterpart to biogenesis and acts to eliminate damaged or unneeded mitochondria in a controlled manner. Mitochondria are marked for degradation by the accumulation of surface markers such as PINK1, a mitochondrial-associated kinase which is normally imported and degraded by mitochondrial proteases\textsuperscript{28, 29}. Mitochondrial damage inhibits the degradation of PINK1 which then recruits the E3 ubiquitin ligase parkin, resulting in the ubiquitination of several mitochondrial membrane proteins. These surface markers recruit LC3, a membrane associated protein which is a key component of autophagic membranes\textsuperscript{30}. This interaction directs the encapsulation of mitochondria in an autophagosome which then fuses with lysosomes leading to degradation and recycling of mitochondria. The interplay between mitobiogenesis and mitophagy controls the overall level of mitochondria in the cell, dictated by the cellular need for energy, metabolites, and other products of mitochondrial function.

1.2 Mitochondrial Metabolic Function

The prototypical function of mitochondria revolves around its role as the main producer of cellular energy in the form of ATP. Mitochondria house and organize the oxidative phosphorylation machinery, five protein complexes which catalyze the production of ATP. Briefly, the first four complexes comprise the electron transport chain which are responsible for the conversion of energy through the oxidation of NADH and succinate to generate a proton gradient between the mitochondrial matrix and intermembrane space\textsuperscript{31}. Oxidation of these energetic intermediates liberates electrons which are shuttled through quinoid and transition metal centers of sequentially decreasing reductive potential contained within the complexes, as well as the small molecule coenzyme Q and the protein cytochrome C, electron shuttles which carry electrons to complexes III and IV respectively. The energy of the flow of electrons through the chain is harnessed by complexes I, III, and IV to drive protons into the intermembrane space, generating an electrochemical potential which is then used by complex V (also known as ATP synthase) to drive ATP production.
Mitochondria also house the biochemical pathways that generate the energetic intermediates used as substrates for the electron transport chain. The tricarboxylic cycle is an 8-enzyme cyclic pathway that integrates the metabolic intermediates of carbohydrates, lipids, and amino acids into the generation of NADH and FADH$_2$ which are then used directly or integrated into the electron transport chain$^{32, 33}$. Various other pathways exist to convert metabolic energy sources to intermediates of the tricarboxylic cycle, such as glycolysis for carbohydrates$^{34}$, fatty acid oxidation for lipids$^{35}$, and the transaminase/urea cycle for amino acids$^{36}$. Many components of the metabolic machinery of energy generation are localized either to the mitochondrial matrix or inner membrane as in the ETC, tricarboxylic cycle, and fatty acid oxidation. Additionally, most of these catabolic processes can also either be directly or indirectly reversed$^{37}$, and many of the intermediates generated in the TCA cycle are also substrates for anabolic pathways. Therefore, mitochondria are a center of both cellular catabolism and anabolism, and at least indirectly participates in most biochemical processes in the cell.

1.3 Alternative Cellular Roles of Mitochondria: Control of Cell Signaling

Mitochondria are generally understood to act as energy-producing organelles within the cell, housing the machinery for oxidative phosphorylation and producing the majority of ATP used by other cellular processes. However, they are also inextricably involved in a plethora of cellular signaling processes from regulation of cell growth to control of cell death. In a therapeutic context, the role of mitochondria in the regulation of apoptosis is of a particular interest, as mitochondria may present an ideal target for the elimination of cancer cells$^{38, 39}$. Mitochondria are the central hub of apoptosis in the cell, both containing and acting as a scaffold for many components of the apoptotic machinery. The initiation of apoptosis has been found to follow two main pathways, and external pathway which is initiated by ligand-receptor signaling on the cell membrane, and an intrinsic pathway in which mitochondria act directly as the site and initiator of apoptosis. In the intrinsic pathway, the apoptotic cascade is initiated permeabilization of the mitochondrial outer membrane, leading to the release of mitochondrial proapoptotic factors. This process termed the mitochondrial outer membrane permeability transition or MOMP is itself controlled and induced by a number of factors including oxidative
damage of mitochondria, DNA damage in the nucleus, ER stress, the activation of certain kinases and transcription factors, and a multitude of other processes\textsuperscript{40, 41}.

Most pathways converge on the activation of BH3-only proteins which can bind and activate pro-apoptotic effector proteins, Bax and Bak, directly and by inhibiting the activity of anti-apoptotic Bcl-2 proteins\textsuperscript{42}. BH3-only binding induces mitochondrial localization of Bax and oligomerization of both Bax and Bak on the mitochondrial surface\textsuperscript{43, 44}, forming a toroidal pore on the outer membrane surface\textsuperscript{44} and inducing the release of cytochrome C into the intermembrane space. Formation of the outer membrane pore seems to also induce the formation of an associated inner membrane permeability pore which leads to mitochondrial depolarization, swelling, and possible further loss of outer membrane integrity\textsuperscript{45, 46}. Current evidence points to dimers of complex V as the core constituent of the pore\textsuperscript{47}, and a number of proteins have been implicated in the formation of this permeability complex, including ANT, VDAC, and Cyclophilin D. However, it is clear that this process is not always necessary for the induction of apoptosis as knockdown experiments of these individual components failed to prevent apoptosis\textsuperscript{48-50}, making it difficult to elucidate the exact mechanism of this process.

The end result of outer membrane permeabilization is the release of cytochrome C into the cytoplasm where it then binds APAF1, inducing its oligomerization\textsuperscript{51}. The complex then recruits caspase 9, a member of a family of cysteine proteases which require proteolytic cleavage for activation. APAF1 proteolytically activates caspase 9 completing the formation of the apoptosome, the major apoptotic initiation complex in the intrinsic pathway\textsuperscript{51}. The apoptosome then proceeds to activate executioner caspase 3, a caspase which then activates other caspases, proteolytic, and other degratory factors which execute the progression of cell death and digestion of the cellular components\textsuperscript{51}. In the extrinsic pathway, plasma membrane receptors are activated by ligand binding which recruits and activate caspase 8\textsuperscript{52}, which can act directly on caspase 3 to initiate apoptosis. However, caspase 8 also cleaves the protein BID into tBID, which then activates Bax and the mitochondrial apoptotic pathway\textsuperscript{53}. Therefore, mitochondria play a key role in both pathways of apoptosis.
Asides from the control of apoptosis, mitochondria play a role in a number of other signaling mechanisms. Perhaps the most intuitive contribution of mitochondria towards cellular signaling is through the generation of reactive oxygen species or ROS, chemically active intermediates which are generated as a side product of electron release from the ETC. Cellular signals which affect the integrity and efficiency of mitochondria can increase the production of mitochondrial ROS which can act as the initiator of a signaling response. For example, hypoxia decreases the amount of oxygen available as a terminal acceptor of the electron transport chain increases the release of electrons from complex III and generates increased ROS\textsuperscript{54}. Increased ROS stabilizes HIF1-α in the cytoplasm, a constitutively expressed but normally rapidly degraded transcription factor which induces the cellular hypoxia response\textsuperscript{55}. HIF1-α stabilization then induces the expression of genes involved in short and long-term responses such as glucose metabolism, cell proliferation, angiogenesis, and erythropoiesis.

Mitochondrial ROS generation also plays a signaling role in neuronal activity and behavioral response, immune function, and ageing\textsuperscript{56-58}. Mitochondria also rely on other effectors in order to elucidate changes in the cell including calcium ion concentrations, membrane polarity, and protein factors\textsuperscript{59}. The understanding of the role of mitochondria in cell signaling is an active area of research, and numerous novel pathways of mitochondrial retrograde signaling are being investigated\textsuperscript{60}. A complete understanding of mitochondrial dynamics is a necessity for more effective therapies which involve mitochondria.

1.4 Mitochondrial Dysfunction and Disease: Mitochondria as a Therapeutic Target

Due to their key role in the metabolic and energetic homeostasis of the cell and in apoptotic cell death, mitochondria have garnered intense academic and industrial interest as a therapeutic target. A growing field of research has centered around the understanding of primary mitochondrial diseases, those caused by mutations in mitochondrial genes or nuclear encoded mitochondrial targeted proteins which result in functional abnormalities (Table 1.1). Mitochondrial diseases are highly heterogeneous
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<td>• mt tRNA$^\text{Lys}$ 8344A&gt;G</td>
<td>• Lactic acidosis</td>
<td>68, 68</td>
</tr>
<tr>
<td></td>
<td>• Other mtDNA mutations, primarily tRNA</td>
<td>• Myoclonus</td>
<td>70, 71</td>
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<tr>
<td></td>
<td></td>
<td>• Seizures</td>
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<td></td>
<td></td>
<td>• Cerebellar ataxia</td>
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<td>• Myopathy</td>
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<tr>
<td>Syndrome/Condition</td>
<td>Key Characteristics</td>
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<td>--------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------</td>
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<tr>
<td><strong>Leber's hereditary optic neuropathy</strong></td>
<td>- mt ND1 3460G&gt;A&lt;br&gt;- mt ND4 1778G&gt;A&lt;br&gt;- mt ND6 14484T&gt;C&lt;br&gt;- Other mtDNA mutations of complex I&lt;br&gt;- Ragged-red fibers (Muscle cells)&lt;br&gt;- Central vision loss&lt;br&gt;- Microangiopathy&lt;br&gt;- Dystonia</td>
<td></td>
<td></td>
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<tr>
<td><strong>Leigh Syndrome</strong></td>
<td>- Myriad mutations of nuclear and mtDNA which inhibit mitochondrial energy generation&lt;br&gt;- Hypotonia&lt;br&gt;- Spasticity&lt;br&gt;- Movement disorders&lt;br&gt;- Cerebellar ataxia&lt;br&gt;- Peripheral neuropathy&lt;br&gt;- Cardiomyopathy</td>
<td></td>
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<tr>
<td><strong>Pearson Syndrome</strong></td>
<td>- Large scale mtDNA deletions&lt;br&gt;- proximal myopathy&lt;br&gt;- seizures&lt;br&gt;- ataxia&lt;br&gt;- Skin lesions&lt;br&gt;- Proximal renal tubular acidosis</td>
<td></td>
<td></td>
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<tr>
<td><strong>Neuropathy, ataxia, retinitis pigmentosa (NARP)/Maternally inherited Leigh syndrome (MILS)</strong></td>
<td>- mt ATP6 8993T&gt;G/C&lt;br&gt;- Neuropathy&lt;br&gt;- Cerebellar Ataxia&lt;br&gt;- Retinitis pigmentosa&lt;br&gt;- Neurological Lesions (MILS)</td>
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in their clinical phenotypes, with symptoms ranging from neurological abnormalities to diabetes to single organ failures\textsuperscript{77}. Recent estimates of disease prevalence has pointed to 1 in 5000 individuals suffering from a genetically linked mitochondrial disease,\textsuperscript{78} however despite the relative frequency of mitochondrial diseases little progress has been made in the development of effective treatments. Most current treatment options are largely based around relieving specific symptoms or attempting to prevent disease progression\textsuperscript{79}, and no clinically available treatment currently addressed the root genetic cause.

Treatment options currently under development include delivery of nucleic acids and gene manipulation in order to replace dysfunctional genes, delivery of functional protein replacements either directly or through transient gene delivery, targeting the downstream pathologies of the disease using small molecule, and stem cell therapy\textsuperscript{58}. Interestingly, due to the multicopy nature of mitochondrial DNA, disease causing mutations are almost always heteroplasmic, with both mutant and native populations of mitochondrial genomes being present within an individual cell. Additionally, most mtDNA diseases only present symptoms when a threshold concentration of mutant DNA has been reached\textsuperscript{80}. Therefore, genetic mtDNA diseases may actually be simpler to solve as compared to nuclear DNA as the relative amount of mutant mtDNA as a whole can be targeting. Strategies to either specifically remove mutant genomes or to decrease their relative replication efficiency are being investigated towards these ends\textsuperscript{81}.

Mitochondria also appear to play a key role in a number of other pathologies in which they are not the primary cause. Notably, mitochondrial dysfunction has been implicated as a major contributor to the pathophysiology of a variety of neurodegenerative disorders, including Alzheimer’s, Parkinson’s, ALS, and Huntington’s disease\textsuperscript{82}. Taking Alzheimer’s as an example, mitochondria in neuronal cells progressing through the disease have been noted to accumulate mtDNA damage\textsuperscript{83}, have reduced mtDNA copy number, exhibit general dysfunction\textsuperscript{84}, and exhibit abnormal mitochondrial dynamics\textsuperscript{85}. As past efforts in clearing the buildup of amyloid plaques, the most physiologically obvious symptoms of Alzheimer’s, have proven to be ineffective in providing significant reversal in the progression of the disease, alternative pathways
such as repairing mitochondrial damage are now being investigated with more interest\(^8\).  

Mitochondrial ROS production as a result of electron transport chain dysfunction has been targeted by a number of nonspecific and mitochondrially targeted antioxidants for the treatment of Alzheimer’s\(^{87}\), Parkinson’s\(^{88}\), and other neurodegenerative diseases. Pharmacological intervention to directly increase mitochondrial volume in diseased cells have also been investigated as a method of improving brain function. Genetic modulation of cultured cells to enhance mitobiogenesis has proven successful protecting against toxicity in models of neurodegenerative diseases\(^{89}\), and small molecule activators of pro-mitobiogenesis pathways such as AMPK\(^{90}\) and SIRT1\(^{91}\) have also replicated these effects. Looking beyond neurodegeneration, modulation of mitochondrial function is also currently under investigation for type II diabetes and even the general maladies associated with aging\(^{92}\). Continued investigation into the role of mitochondria in disease may better validate their impact on a host of pathologies and yield a number of effective strategies in diseases which have previously proven difficult to target.

Due to their role as the organizational center for the intrinsic apoptotic pathway, mitochondrially targeted therapies have also been a major area of investigation in chemotherapeutic development. Cancer cells exhibit generalizable abnormalities in mitochondrial function\(^{93}\) and in mitochondrial apoptotic signaling\(^{94}\). Compared to normal cells, cancer cells are often in a state of mitochondrial and cellular stress which would normally result in the activation of the apoptotic pathway, however, is maintained through inhibition of some aspect of the innate apoptotic machinery\(^{94, 95}\). Since the elucidation of the innate apoptotic pathway a plethora of pharmacological strategies to activate apoptosis in cancer cells have been investigated, either by inducing additional mitochondrial damage in order to overcome apoptotic inhibition or by alleviating the inhibition itself. This research has yielded a number of successful chemotherapeutics, however many varieties of cancer remain difficult to treat and it is clear that additional work remains\(^{96}\).
A newer field of research gaining traction is the direct chemical targeting of mitochondria using mitotropic agents, compounds which are taken up specifically by mitochondria\textsuperscript{97}. Due to metabolic changes in mitochondria such as the Warburg effect and dysfunction in the coupling of the proton motive force to ATP generation, mitochondria typically have higher transmembrane potential as compared to normal cells, with transmembrane potential being associated with aggressiveness and invasiveness of the cancer\textsuperscript{98}. This has been used to target cationic molecules, nanoparticles, and peptides to mitochondria in order to more effectively and specifically deliver chemotherapeutic agents to their site of action\textsuperscript{97}. Novel mitotropic compounds, or nonspecific compounds which have been retargeted using mitotropic vectors, have proven effective in targeting a number of varieties of cancer. However, this field is relatively young in chemotherapeutic development, and additional research into targeted therapeutics could yield therapeutic strategies with increased efficacy and breadth in the types of oncologic diseases which can be treated with these agents.

1.5 Therapeutic Targeting of Mitochondria with Mitotropic Vectors

Despite intense academic and industrial interest in studying the role of mitochondria in cellular homeostasis and disease, many processes remain poorly understood. In addition to the challenges of studying a specific subcellular compartment, mitochondria are a particularly difficult target for the chemical and genetic manipulation strategies typically used to probe cellular processes. This is due in large part to the impermeability of the mitochondrial membrane structure to external agents. Mitochondria are separated from the cytoplasm by a unique dual membrane structure, composed of the inner and outer mitochondrial membrane. The outer membrane is relatively permeable due to the presence of a family of porin proteins, also called VDACs, which allow the free diffusion of molecules up to \( \sim 8000 \) Da in size\textsuperscript{99}. This makes processes which occur on the outer mitochondrial membrane or in the intermembrane space relatively accessible, however not without the innate challenges of specificity in organelle specific targeting. The mitochondrial inner membrane, however, lacks these membrane channels and is particularly impermeable to most small molecules and macromolecular species. Some efforts in targeting cargos to
mitochondria have taken advantage of the innate mitochondrial transport machinery by fusing MTS to proteins\textsuperscript{100} or nucleic acid derivatives\textsuperscript{101}. However, the structural sensitivity of the mitochondrial import machinery may make this strategy untenable to molecules which are significantly different in structure to biological macromolecules such as nanoparticles or certain small molecules. In addition, MTS fusions usually still require a trans-cytoplasmic membrane vector, complicating the design of the system.

Alternatively, while the mitochondrial membrane architecture severely limits small molecule targeting of the mitochondrial matrix, some molecules are able to diffuse through the membrane. Most mitochondrial permeable molecules contain delocalized positive charge which electrostatically attracts them to the negative potential of the matrix. This relationship is defined by the Nernst equation, which states that given that the membrane potential across the mitochondrial membrane is typically around 120-180 mV in human cells, cationic species should localize to 100 to 1000-fold in the mitochondrial matrix\textsuperscript{102}. However, the extent and rate of localization are also affected by the ability of the compound to pass through the hydrophobic membrane. Delocalization of the positive charge reduces the energetic barrier of passing a changed species through the hydrophobic environment of a cellular membrane. This combined with a general hydrophobicity allows for penetration of the inner mitochondrial membrane and access to the matrix. This strategy has been used to develop a number of molecules designed to probe physical properties of mitochondria, particularly fluorescent reporters of mitochondrial morphology, polarity, redox status, metal species, and other indicators of mitochondrial health or function\textsuperscript{103}. However, redesigning compounds which rely on a defined chemical structure for target binding or biological function to include the physical properties required for mitochondrial localization is difficult to impossible for many classes of molecules. In this case, rather than modifying the original compound, a conjugate between a strongly mitotropic and biologically inert vector and a bioactive cargo molecule can induce mitochondrial localization without the need for significant modification of the original structure (Table 1.2).

A number of targeting vectors have been designed with this function in mind, each with their own strengths and weaknesses. The simplest method involves direct
Table 1.2 – Compounds which have been targeted to mitochondria and their prospective therapeutic applications

<table>
<thead>
<tr>
<th>Target Application</th>
<th>Targeting System</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotherapeutics</td>
<td>MPPs</td>
<td>Doxorubicin\textsuperscript{104}, Chlorambucil\textsuperscript{105}, Cisplatin\textsuperscript{106}, Luminespib\textsuperscript{107}</td>
</tr>
<tr>
<td></td>
<td>TPP</td>
<td>Metformin\textsuperscript{108}, Doxorubicin\textsuperscript{109}, Geldanamycin\textsuperscript{110}, Chlorambucil\textsuperscript{111}, Dichloroacetate\textsuperscript{112}, Curcumin\textsuperscript{113}</td>
</tr>
<tr>
<td></td>
<td>Nanocarriers</td>
<td>Paclitaxel\textsuperscript{114}, Epirubicin\textsuperscript{115}, Doxorubicin\textsuperscript{116}, Topotecan\textsuperscript{117}, Lonidamine\textsuperscript{118}, Daunorubicin/Quinacrine</td>
</tr>
<tr>
<td>Neurodegenerative and aging diseases</td>
<td>Bicyclic guanidine oligomers</td>
<td>Geldanamycin\textsuperscript{110}</td>
</tr>
<tr>
<td></td>
<td>TPP</td>
<td>Vitamin E\textsuperscript{122, 123}, Idebenone\textsuperscript{124, 125}, Resveratrol\textsuperscript{126}, Vitamin C\textsuperscript{127}, Vitamin E\textsuperscript{123}, CoQ10\textsuperscript{125}, Apocynin\textsuperscript{128}, Dinitrophenol\textsuperscript{129}</td>
</tr>
<tr>
<td></td>
<td>Amphiphilic polyproline</td>
<td>Dimethyl tyrosine\textsuperscript{130}</td>
</tr>
<tr>
<td></td>
<td>Nanocarriers</td>
<td>Curcumin\textsuperscript{119, 131}, CoQ10\textsuperscript{132}, Ferulic acid\textsuperscript{133}, Thymoquinone\textsuperscript{134}, Chrysin\textsuperscript{135}</td>
</tr>
<tr>
<td>Obesity and diabetes (excluding antioxidants listed for neurodegenerate)</td>
<td>TPP</td>
<td>Plastoquinone\textsuperscript{136}</td>
</tr>
<tr>
<td></td>
<td>Nanocarrier</td>
<td>Dinitrophenol\textsuperscript{119}</td>
</tr>
<tr>
<td>Mitochondrial genetic disease</td>
<td>Nanocarriers</td>
<td>mtND4 containing plasmid\textsuperscript{137}</td>
</tr>
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conjugation of a bioactive molecule to a mitotropic dye, allowing for innate confirmation of mitochondrial localization. This method has been successful in localizing a number of photosensitizers to mitochondria with the goal of inducing mitochondrial-dependent apoptosis in cancer cells. However, most dyes have some innate effect on mitochondrial biology due to their photo reactivity which may be generally undesirable. Therefore, non-bioactive mitotropics are more commonly used in mitochondrial drug targeting (Figure 1.2). There have been a plethora of mitotropic compounds developed around the guanidinium ion, including amphiphatic helical peptide derivatives, guanidinium decorated sugar scaffolds, and bicyclic guanidine oligomers. Due to the biocompatibility of guanidinium, these compounds can induce localization of a cargo molecule without impacting mitochondrial function. However, the concentration of positively charged vectors into the mitochondrial matrix does eventually cause non-specific toxicity, and the relatively high size and molecular charge of these compounds may limit the ratio of localization to toxicity.

Another small molecule vector, triphenylphosphonium, showed excellent localization with significantly diminished mitochondrial toxicity, with cell viability being unaffected into high micromolar concentrations. This combined with the ability of TPP to concentrate cargoes into the mitochondrial matrix to physiologically relevant levels at low or sub micromolar concentrations has made it a popular choice in mitochondrially targeted drug conjugates. However, due to its monoionic nature, TPP is unusable with compounds that exist in an anionic state at physiological conditions as conjugation would result in neutralization of the net positive charge required to target the membrane potential of mitochondria.

An alternative strategy for mitochondrial delivery is the use of mitochondrially targeted polymeric nanoparticles for the intramitochondrial release of encapsulated cargoes. These complexes utilize polymers studded with mitotroptic groups such as triphenylphosphonium to drive their mitochondrial uptake. However, further studies are required to confirm whether these polymers are taken into the mitochondrial matrix or whether they release their contents proximal to mitochondria rely on higher local concentration to drive uptake into the matrix. This strategy has also been utilized for targeting of lipid-based nanoparticles which mitochondrial membrane fusogenic activity
Figure 1.2 – Chemical structures of mitotropic vectors.
for delivery into the mitochondrial matrix\textsuperscript{142}. Liposome like particles can also be formed with lipid like small molecules such as dequalinium chloride, a compound composed of two delocalized positively charged centered connected by an extended alkyl chain. These particles have shown some promise in targeting both nucleic acids and small molecules to mitochondria\textsuperscript{143, 144}. However, they are limited in their delivery capacity by the relatively high toxicity, possibly caused by their membrane-fusion activity. Therefore, a niche exists for a mitotropic agent with high specificity, tunability, and flexibility in the chemical identity of the cargo while maintaining low cellular toxicity.

1.6 Development of Mitochondria Penetrating Peptides

Previous research conducted in the Kelley lab has led to the development of a peptide based mitochondrial delivery system, named mitochondrial penetrating peptides or MPPs. Peptide based mitotropic vectors had not been investigated previous to MPPs, however, we hypothesized that a peptide vector provides a biocompatible, stable, and tunable alternative to the mitochondrial vectors previously used. The inspiration for the design of MPPs came from the observation that while cationic cell penetrating peptides (CPPs) such as Tat and penetratin appear to be able to directly penetrate the cell membrane\textsuperscript{145, 146}, they fail to cross the mitochondrial inner membrane\textsuperscript{147}. Despite the fact that their highly cationic character suggests that they could localize at high concentrations in the mitochondrial matrix, CPPs typically lack the strong hydrophobic character shared by mitotropic compounds\textsuperscript{148} and therefore are unable to penetrate the mitochondrial inner membrane.

The initial development of MPPs utilized the basic residues frequently found in CPPs such as lysine and arginine and combined them with highly hydrophobic residues such as phenylalanine and the unnatural amino acid cyclohexylalanine\textsuperscript{149}. Cyclohexylalanine was found to be instrumental for efficient mitochondrial localization as peptides containing phenylalanine only were unable to cross the mitochondrial membrane likely due to their lower relative hydrophobicity. Hydrophobic and cationic residues were placed in an alternating pattern, a strategy confirmed to promote membrane penetration as diffusion of the positive charge across the molecule decreases the energetic penalty of the ionic groups when embedded in a membrane\textsuperscript{150}. 
The first generation peptides were synthesized with a mix of lysine and arginine, however, comparison to other mitotropics suggested that delocalized cations such as arginine are more effective at mitochondrial targeting\textsuperscript{151}. A comparison of a number of peptides containing different guanidinium and pyridinium based delocalized cations revealed that delocalization of all charges on MPPs greatly increases the specificity of the peptide towards mitochondria. Additionally, while pyridinium cations targeted mitochondria with high specificity, their overall uptake was decreased compared to guanidinium\textsuperscript{152}. Chirality of the cation was also found to play a role in the uptake of the MPP, with d-amino acids exhibiting increased uptake, although the specificity of localization remained unaffected. Therefore, subsequent peptides were designed around a standard MPP model composed of amino acid repeats of cyclohexylalanine and d-arginine, modified based on the needs of the specific compound.

Compared to other mitochondrial vectors, MPPs represent an intermediary between guanidinium oligomers and triphenylphosphonium, typically containing three repeats of arginine/cyclohexylalanine residues. The optimized placement of charge and hydrophobicity of MPPs may explain why they localize to mitochondria with lower molecular charge than is typical for other guanidinium based vectors\textsuperscript{140, 141}. The multiplicity of charge also allows for the localization of anionic cargo, as opposed to monoionic vectors such as TPP. Similarly to the other non-polymeric mitotrophic vectors, MPPs exhibit energy independent direct potential driven diffusion into mitochondria\textsuperscript{149}. Solid state NMR studies of unilamellar micelles designed to simulate mitochondria have pointed towards an electroporation mechanism of uptake\textsuperscript{153}. In this model, the MPPs were found to bind to the membrane at the C2 position of phosphatidylcholine and phosphatidylethanolamine lipids through the cyclohexyl alanine residues while the arginine side chains interacted ionically with the phospholipid headgroups (Figure 1.3). At higher concentrations, the positive charge of the MPPs neutralizes the negative charge of the phospholipids to the point where an electric field between the inner and outer leaflet of the membrane is generated. This destabilizes the membrane allowing the peptide to cross to the inner leaflet, gaining access to the mitochondrial matrix while not affecting overall membrane lamellarity. MPPs exhibit rapid potential dependent uptake, with uptake reaching near maxima within 30 minutes of treatment and maximal
Figure 1.3 – Electroporation model of MPP uptake through the inner mitochondrial membrane. A) MPPs bind to the acyl chains of membrane phospholipids through their hydrophobic residues, while the cationic residues participate in ionic interactions with the phosphate head groups. B) MPPs concentrate on the surface of the membrane. At a threshold point, the neutralization of the negative charge of the outer leaflet phospholipids by the clustered MPPs induces a potential difference across the bilayer. This causes the MPPs to form a transient toroidal pore allowing them to cross to the inner leaflet. C) MPPs on the inner membrane leaflet diffuse from the pore, restoring the membrane potential and allowing additional peptide to cross.
uptake at 90 – 120 minutes\textsuperscript{149}. The rapid uptake of MPPs paired with their high specificity makes them ideal delivery vectors for bioactive cargoes.

1.7 Applications of Mitochondrial Penetrating Peptides

The strong mitotropic properties of MPPs have been used to deliver a number of bioactive compounds to the mitochondrial matrix with a diverse set of chemical properties and mechanisms (Figure 1.4). Early studies with MPPs focused on the delivery of the DNA damaging compounds doxorubicin and chlorambucil to the mitochondrial matrix in an effort to investigate both their therapeutic properties and potential use as DNA damage probes. The MPP-doxorubicin conjugate mt-Dox was shown to induce mitochondrial specific DNA damage as opposed to the mix of mitochondrial and nuclear toxicity of doxorubicin alone\textsuperscript{104}. Mitochondrial localization of mt-Dox was also shown to overcome resistance in cancer cells overexpressing the cytoplasmic membrane drug efflux pump P-glycoprotein, a major contributor to doxorubicin resistance in cancer. The desirable pharmacological properties of mt-Dox also translated to mouse models of P-glycoprotein expressing tumors, where mt-Dox outperformed doxorubicin alone in reducing tumor growth\textsuperscript{154}.

Mt-Dox has also proven useful as a scientific tool to help answer questions about the pharmacological properties of doxorubicin itself. Clinical dosing of doxorubicin as a chemotherapeutic is limited by off target cardiotoxicity and is detrimental to the therapeutic window of the drug\textsuperscript{155}. The origin of this cardiotoxicity was previously hypothesized to be due to the uptake of doxorubicin by cardiomyocytes which, due to their increased reliance on mitochondrial activity, caused increased uptake and damage to mitochondrial DNA and decreased heart function. However, mt-Dox was found to be less toxic to cardiomyocytes as compared to an ovarian carcinoma cell line, while doxorubicin itself was toxic to both cell lines\textsuperscript{156}. In addition, cardiomyocytes treated with mt-Dox were found to exhibit some signs of toxicity directly after treatment but recover within 24 hours through an increase in mitobiogenesis. Doxorubicin treatment resulted in prolonged and time dependent mitochondrial toxicity, suggesting that clinical cardiotoxicity of doxorubicin is a nuclear damage associated phenotype. This was also reflected in mouse studies, where doxorubicin resulted in a pronounced decrease in
Figure 1.4 – Summary of previous mitochondrial penetrating peptide conjugates.
A) Structure of the prototypical mitochondrial penetrating peptide. The location of the cargo is symbolized with an X. B) Compounds delivered to mitochondria by MPPs previous to this work, and the pharmacological outcomes of MPP conjugation.
body weight and increased circulation markers of cardiotoxicity while mt-Dox treatment elicited no decrease in body weight and insignificant changes in circulation markers\textsuperscript{156}.

MPP conjugation of chlorambucil (mt-Cbl) also retained cargo activity and mitochondrial specific targeting. Mt-Cbl displayed up to a >30fold increase in activity against a panel of cancer cell lines as compared to chlorambucil alone\textsuperscript{105}. Mt-Cbl was also able shown to be effective against cell lines with resistance to chlorambucil due to overexpression of Bcl\textsubscript{x}l and glutathione S-transferase, a cytosolic enzyme responsible for the deactivation of chlorambucil. Interestingly, mt-Cbl was also found to transiently inhibit P-glycoprotein pumps directly in a dependent manner depending on the chlorambucil moiety, a phenomenon which was shown to be useful in increasing the uptake of other pharmacological compounds susceptible to Pgp efflux\textsuperscript{157}, making the compound useful as a potential synergistic therapeutic. Mitochondrial localization of mt-Cbl also appeared to change its chemotherapeutic mechanism from DNA damage induced apoptosis to a protein alkylation based necrotic mechanism, although mitochondrial DNA damage was shown to be present as well\textsuperscript{158}. Localization of high concentrations of mt-Cbl in mitochondria may have been sufficient in inducing enough direct alkylative damage to directly compromise mitochondrial and cellular integrity. Chemical modification of mt-Cbl to reduce its alkylating activity changed the mechanism of death from necrotic back into an apoptotic mechanism, highlighting the importance of reaction kinetics in the cell death mechanism induced by Cbl\textsuperscript{159}.

Mitochondrial targeting of a cisplatin derivative was also shown to be effective in targeting cancer cells\textsuperscript{106}. Both cisplatin and mt-cisplatin induced apoptotic death of cancer cells, however, mt-cisplatin elicited these effects exclusively through mitochondrial DNA damage. Similar to the other DNA damaging compounds, mitochondrial localization was successful in evading resistance mechanisms based on drug efflux and increased expression of nuclear DNA repair factors. From the results obtained with these three DNA damaging agents, it is clear that conjugation of pharmacologically active cargoes to MPPs can successfully and specifically target the mitochondrial matrix and evade cellular mechanisms to remove or deactivate their activity. In addition, the mt-Dox conjugate shows how mitochondrial localization can
help answer underlying questions of the interactions between the cargo and the mitochondrial biology.

With the success of mt-Dox as a mitochondrial probe, using pharmacologically active cargoes as probes for mitochondrial biology is a strategy that has been further investigated using MPPs. An MPP conjugate of thiazole orange, a fluorescent dye which is also known to induce oxidative damage as a byproduct of fluorescence\textsuperscript{160}, was developed as a specific DNA damage probe. The conjugate, named mt-Ox, was used to probe for genes which increase susceptibility to mitochondrial oxidative DNA damage when silenced and therefore may play a role in the repair of oxidative DNA damage in mitochondria. Mt-Dox was also included in the screen to investigate the response to DNA damage caused by double stranded breaks, a damage type specific to doxorubicin.

The screen returned a number of genes known to be involved in mitochondrial DNA damage repair such as mitochondrial polymerase γ (PolG), DNA ligase III, and MUTYH. Additionally, the screen identified a number of proteins previously unknown to be involved in mtDNA damage repair or mitochondria in general. For example, the known nuclear nucleotide excision repair protein RAD23A was identified in the screen and subsequently found to localize to mitochondria and preserve mtDNA integrity against oxidative damage. The double-stranded break repair protein XRCC4 was also identified by both mt-Ox and mt-Dox and was subsequently found to contribute to a previously unknown mitochondrial double stranded break repair pathway in mitochondria. The screen also pointed towards the recently characterized DNA polymerase θ as being involved in mtDNA damage, a protein involved in non-canonical break repair and translesion bypass in the nucleus. Interestingly, knockout of DNA polymerase θ not only affected mitochondrial integrity against oxidative damage, but also reduced mitochondrial respiration, transmembrane potential, and cellular viability at a basal level, indicating the protein is necessary for proper mitochondrial function. These results highlight how MPPs can be used with bioactive cargoes in order to probe for aspects of mitochondrial biology where previously access and specificity would have prevented the acquisition of meaningful results.
1.8 Thesis Objectives: Release of Cargoes from MPPs

While we have achieved significant success in developing novel mitochondrial directed chemical probes using the MPP platform, significant barriers still exist in the diversity of compounds which can be used with MPPs and mitochondrial vectors in general. Because chemical vectors must be covalently conjugated to the cargo, vectors risk affecting the activity of the cargo itself. Particularly, while the steric impact of vector conjugation can be mitigated by the inclusion of a long chain linker (alkyl, PEG linkers, etc.), compounds which have a particularly sensitive structure-function relationship such as small molecule ligands may still be deactivated by conjugation. Additionally, the chemical groups typically used for vector conjugation include carboxyl, hydroxyl, amines, and derivatives thereof. These groups are particularly important in small molecule ligands as they form hydrogen bonds with groups in the protein binding site. Therefore, small molecule ligands are particularly difficult cargos to work with using MPPs and other mitochondrial vectors. Indeed, the majority of the compounds we have found to be successful with MPP conjugation are compounds which induce a biological effect without structure specific binding, or by binding to larger macromolecular structures such as DNA which may be more forgiving with regards to molecular structure.

In order to expand the repertoire of compounds which can be used with MPPs, we investigated a method for compound release and regeneration in mitochondria following localization. Two previous studies have previously investigated mitochondrial compound release from TPP vectors, one using dichloroacetate\textsuperscript{112} and one using lipoic acid\textsuperscript{161} as their model cargo. Both compounds used the enzymatic cleavage of an ester linker by mitochondrial esterases for cargo release. The compounds were shown to successfully release active compound and elicit the expected mitochondrial effects, kinase inhibition and induction of apoptosis for dichloroacetate and protection from ROS related damage for lipoic acid. In addition, the release of lipoic acid from TPP was found to decrease the toxicity of the compound when compared to an unreleasable analogue\textsuperscript{161}. However, enzymatic cleavage of esters as a method for cargo release presents a number of problems in the adoption of this strategy to other systems. Firstly, only cargoes with a carboxylic acid or hydroxyl functional groups can be used with this
method. In addition, relying on enzymatic cleavage by esterases makes the linker more sensitive to steric effects around the linker as the enzyme must be able to access the ester bond. This may have been a non-issue to mitochondrially targeted DCA due to the fact that the cargo, in this case, was exceptionally small\textsuperscript{112}. In addition, the mitochondrially targeted lipoic acid conjugate was able to take advantage of the alkyl chain of lipoic acid in combination with an alkyl spacer on the TPP moiety to increase the accessibility of the cleavable ester\textsuperscript{161}. However, many pharmacological molecules tend to be larger, more complex, and lack extended alkyl chains, therefore ester conjugation of a mitotropic vector even with an extended linker on the vector side may negatively affect cleavage of the linker.

Some macromolecular assemblies such as liposomes and polymer based nanoparticles have been successful in releasing free compound in mitochondria, most notably Mito-Porter, a liposomal mitochondrially targeted transporter\textsuperscript{162} which has been used to deliver nucleic acids to mitochondria\textsuperscript{163}. However, the innate mitochondrial toxicity of the liposome-based particles may confound efforts in the development of chemical probes of mitochondria or the development of mitochondrial therapeutics. In addition, most mitochondrially targeted liposomes tend to release compound at both the inner and outer mitochondrial membranes\textsuperscript{162} and therefore tend to have decreased specificity for delivery to the mitochondrial matrix as compared to small molecule mitotropic vectors. Recent research suggests that some nanoparticles do have the capacity to directly transduce into the mitochondrial matrix\textsuperscript{119, 121}. However, these studies also used cargos with innate mitochondrial targeting, thereby making it difficult to determine if the mitochondrial effects observed were due to direct cargo release in mitochondria.

Due to the disadvantages of the current strategies for mitochondrial cargo release, we sought to develop a more generalizable and reliable method which could be used with a wider set of compounds and conditions. Ideally, the cargo system would also rely on a release mechanism which is ubiquitous, reliable, and relatively constant between cell types. Disulfide based linkers were selected as the ideal candidate for cargo release in mitochondria, as reducing agents to cleave the disulfide bond are present in all cells at concentrations which lie within an order of magnitude between cell
types\textsuperscript{164}. In addition, the lack of an enzymatic requirement for disulfide reduction decreases the likelihood steric properties of the cargo would affect cleavage of the linker.

However, similarly to the other release methods discussed, there is a potential for the linker to be cleaved prematurely in the cytoplasm prior to mitochondrial localization. In fact, disulfide linkers have been used ubiquitously in cytosolic delivery agents such as nanoparticles\textsuperscript{165, 166} and various drug conjugates\textsuperscript{167, 168} as reversible linkers, although little direct investigation on their intracellular cleavage kinetics has been done. While MPPs exhibit exceptionally fast mitochondrial uptake, an investigation into the stability and feasibility of disulfide linkers in the context of small molecule delivery is required. In addition, the necessity of a cargo-side thiol severely limits the diversity of compounds which could be used with such a system. Therefore, a method to attach a self-immolating thiol tag to the cargo molecule is required to expand the delivery potential of this system beyond thiol-containing molecules. The investigations we conducted into cellular disulfide stability and their use as a mitochondrial specific cleavable linker are presented in Chapter 4.

1.9 Thesis Objectives: Investigating the Bacterial Cross Targeting of MPPs

The uptake of MPPs into mitochondria is a result of their ability to cross biological membranes and to interact with a membrane potential. Therefore, MPPs should also be taken up into other compartments with similar physical properties. The common evolutionary origin between bacteria and mitochondria are reflected in the organization of the internal structure of both species, and their similar properties make bacteria a target of cationic compounds such as MPPs. Much like mitochondria, bacteria develop a significant electrochemical potential across their membrane in order to sustain bacterial respiration\textsuperscript{169}. Consequentially, many natural and synthetic antimicrobial therapeutics utilize this potential as a targeting strategy by including the cationic character in their structures, particularly those which are peptide based.

Peptide antimicrobials are a class of molecule found abundantly in nature typically found as part of the pathogen defense of a variety of bacterial, plant and animal
species\textsuperscript{170}. Since their discovery, a number of these compounds have been purified and modified for use as antimicrobials\textsuperscript{171}. Highly heterogeneous, they have been organized into a number of classes, the largest of which is the cationic antimicrobial peptides. These peptides take advantage of the negative membrane potential and overall negative character of most bacterial membranes to drive membrane accumulation and uptake\textsuperscript{172}. Generally, their mechanisms of action can be organized into the lytic and non-lytic mechanisms, although many compounds have been found to act through a combination of both mechanisms\textsuperscript{173}.

Lytic peptides bind to the bacterial membrane and induce the formation of ion channels or transmembrane pores, eventually leading to osmotic lysis of the bacteria\textsuperscript{174}. Structurally, these peptides frequently adopt an amphipathic \(\alpha\)-helical fold which allows them to bind to and destabilize the membrane, although \(\beta\)-sheet, loop, and extended motifs are also observed\textsuperscript{175}. Lytic peptides exhibit a number of desirable pharmacological properties, such as broad-spectrum activity, relatively low host toxicity, and low propensity for resistance development\textsuperscript{176}. However, their clinical use is complicated by difficulties in synthesis, cost, and limited stability due to proteolytic activity, especially when composed of natural amino acids. Non-lytic peptides have a highly diverse mode of action and can inhibit the bacterial replication machinery, RNA and protein synthesis, cell wall synthesis, the activity of key metabolic enzymes, and a number of other factors\textsuperscript{173}. Due to their more specific mechanisms of action, these peptides also exhibit low cellular toxicity, however also are more susceptible to bacterial resistance and share similar physical considerations as lytic peptides. In addition, most antimicrobial peptides do not appear to directly cross cell membranes in a passive manner, making targeting intracellular pathogens difficult.

The predisposition of bacteria to the uptake of cationic antimicrobial peptides also translates into other classes of cationic peptides as well, including both certain CPPs and MPPs as well. Some CPPs have been also found to exhibit some level of antimicrobial activity and vice versa\textsuperscript{177}. However, CPPs which have low direct antimicrobial activity have also been used as targeting vectors for bacterial delivery of antimicrobials. These compounds have been successful in increasing antimicrobial uptake\textsuperscript{178}, circumventing resistance mechanisms such as efflux pumps\textsuperscript{179}, and
increasing the ability to target bacteria with an intracellular life cycle\textsuperscript{180}. However, antimicrobial delivery with CPPs results in the concentration of the cargo molecules in the cytoplasm which may be problematic if the cargo has off target toxicity due to a cytosolic interaction.

As with CPPs, previous investigations into the bacterial targeting of the MPP platform has indicated that some MPPs can exhibit dual targeting properties. These dual targeting properties can be leveraged to add a number of beneficial properties to potential antibacterials even when compared to CPP vectors. The mitochondrial sequestration of excess antimicrobial conjugate can help prevent off target interactions between the appended cargo and host cytosolic components. Antimicrobials which are effective but otherwise unusable because of cytosolic toxicity can be sequestered in mitochondria if a mitochondrial analog of the target protein is not present. This strategy was found to increase the antimicrobial properties of methotrexate, a chemotherapeutic compound which antagonizes the activity of dihydrofolate reductase (DHFR)\textsuperscript{181}. Methotrexate has been found to induce death of both human and bacterial cells by shutting down the synthesis of purines and certain amino acids, however, the toxic effects of methotrexate on human cells makes it a poor candidate as an antibacterial\textsuperscript{182}. In human cells, DHFR is located in the cytoplasm, therefore MPP conjugation of methotrexate and mitochondrial localization effectively hides the compound from its mammalian cell target while also increasing delivery to bacteria. This strategy was found to increase the therapeutic window of methotrexate as an antibacterial up to four orders of magnitude and is an example of how bioactive retargeting can be used to change the properties and applications of a cargo molecule\textsuperscript{181}.

The innate cell penetrating properties of MPPs makes them effective against cytosolic intracellular bacteria which have proven to be a particularly challenging target for traditional antibiotics due to the difficulty of penetrating the added barrier of the cytosolic membrane. MPP conjugation ensures efficient targeting of these bacteria, allowing for the use of antimicrobial compounds which would previously be untenable for these bacterial species. Additionally, the tunability of the MPP platform also allows for a degree of preferential targeting of either mitochondrial or bacterial species. While the general physiochemical aspects of bacteria and mitochondria are similar,
differences in the chemical character of MPPs can lead to changes in the relative targeting of the two structures. This can be used to further tune the activity of the conjugate towards increased bacterial uptake, however, the degree and mechanisms for increasing bacterial targeting had yet to be investigated as of this work. In chapter 3, we investigated the degree to which modifying the two main determinants of MPP targeting, hydrophobicity, and cationic character, affect the relative targeting between bacteria and mitochondria in an intracellular infection model.

1.10 Thesis Objectives: Development of a New Class of Mitochondrial RNA Structural Probes

The mitochondrial DNA encodes the 22 mitochondrial tRNAs and the 12S and 16S ribosomal RNAs that are specific to mitochondria and essential for maintenance of mitochondrial energy production and proliferation. These molecules fold into distinct three-dimensional structures and from the recognition sites and catalytic centers which are critical for their function. Compared to their cytosolic homologs, the structure of the mitochondrial tRNAs diverges significantly due to their non-mammalian origin. While they generally share certain features of the canonical cloverleaf structure of the cytosolic tRNAs, their overall structures are significantly different and are organized into three different classes of non-canonical structures. The most distinct of these is the tRNA$^{\text{Ser(AGY)}}$, which lacks the entire D loop region and is the only tRNA classified as a type III-fold. The tRNA$^{\text{Ser(UCN)}}$ also has a distinct unique class I structure, with fewer residues in the D loop, extra loop, spacer region between the A and D stem, and an extended anticodon stem. Other mitochondrial tRNAs have been categorized with a class II structure, which lacks a canonical D to T loop interaction which is found in class 0 cytosolic tRNAs and contain some variability in their size and sequence. The mitochondrial tRNA also contained significantly limited nucleoside modification as compared to their cytosolic counterparts.

The mitochondrial tRNAs and rRNAs function as part of the translation machinery and are therefore indispensable for the proper maintenance of mitochondrial integrity. This is highlighted by the frequency of mitochondrial diseases caused by mutations in the genes encoding the mitochondrial structural RNAs. For example, MELAS is estimated to
be the most prevalent genetic mitochondrial diseases\textsuperscript{78}, and while a number of genetic mutations have been associated with MELAS, the 3243A>G point mutation in the tRNA\textsuperscript{Leu(UUR)} is responsible for over 80\% of MELAS cases\textsuperscript{184}. In fact, mutations in mitochondrial tRNA may be responsible for almost half of diseases associated with the mitochondrial genome and more than a third of mitochondrial disease as a whole\textsuperscript{78}. Similarly, mutations in the mitochondrial rRNA have been associated with both antibiotic-induced and non-syndromic hearing loss\textsuperscript{185}, and while the association of disease phenotypes with mitochondrial genetic mutations is still developing, epidemiologic studies suggest that mitochondrial rRNA mutation based pathologies may exist in a significantly higher prevalence than currently attributed\textsuperscript{186,187}. However, the association of disease phenotypes with mitochondrial genetics has lagged behind our understanding of diseases with a nuclear genetic basis. Associating diseases with mitochondrial genetics is complicated by the multicopy nature of the mitochondrial genome, and many patients exhibit some degree of heteroplasmy in mitochondrial disease-causing mutations. This is further complicated by the fact that the degree of heteroplasmy is tissue specific, and genetic analysis of tissues non-primary to the disease may further obfuscate the role of mitochondrial genetics in disease pathology\textsuperscript{188}. Because of this, new associations between the mitochondrial genome and disease are still being made to this day, and many questions about the molecular basis for these diseases remain unanswered. Therefore, novel methods of studying the structure-function relationship of the mitochondrial tRNA and rRNAs would be beneficial in advancing the understanding the molecular mechanisms of a significant portion of mitochondrial genetic diseases.

Currently, determination of RNA structure is most frequently accomplished through computational prediction or using \textit{in vitro} methods such as X-ray crystallography and chemical probing. However, these methods cannot fully replicate conditions representative of the cellular environment and cannot account for their effect on the theoretical structure. Both methods also struggle to accurately predict the binding mode of proteins to the RNA of interest and therefore may overlook phenomena that occur as a result of protein interactions. Recent advancements have been made, however, in RNA secondary structure determination by \textit{in cellulo} selective 2'-hydroxyl acylation analyzed by primer extension (RNA SHAPE)\textsuperscript{189}. This method was developed to conduct RNA
secondary structure probing in intact cells, thereby directly probing RNA structure in their native environment.

In general, RNA SHAPE uses chemical reagents which are selectively reactive with the 2'-hydroxyl group of the ribose group of the residue (Figure 1.5). The selectivity arises from the fact that the 2'-hydroxyl can be catalytically activated by the 3'-phosphodiester bond in certain conformations\textsuperscript{190}. RNA residues which are not involved in base pairing interactions can sample these conformations at random, allowing them to react with SHAPE reagents, while those involved in base pairing interactions typically, cannot. In certain rare cases, base pairing locks the nucleotide in a conformation where the 2'-hydroxyl is activated, leading to a hyper-reactive residue\textsuperscript{191}. At low concentrations, RNA molecules are covalently modified at a single position which is retained following RNA purification. The modification can be detected by 3'-5' primer extension reactions with a labeled primer, as the modified residues block reverse transcriptase progression which results in the generation of a set of extended primer fragments whose length is indicative of the position of RNA modification.

These results can then be used to either manually, or with computational assistance, determine the secondary structure of the RNA molecule. In addition, chemical reactivity of the 2' hydroxyls is dependent on steric availability, which can be blocked by proteins or other cofactors. Therefore, lack of SHAPE modification in loop regions which have no corresponding pairing region can act as signals for protein binding, allowing for information about higher order complexes to be collected. We hypothesized that \textit{in cellulo} SHAPE probing could be adapted for use with MPPs for probing mitochondrial tRNA and rRNA structure. We hypothesize that the \textit{in vivo} structures of these molecules differ significantly from the currently available \textit{in vitro} models and that this analysis could identify novel structural elements in these biologically significant RNAs. In addition, the ability to probe for the structural difference in cells harboring a mutation in these RNAs could be instrumental in determining the basis of mitochondrial disease and inform future therapeutic strategies. In chapter 5, we present our data in developing a mitochondrial SHAPE probe.
Figure 1.5 – Schematic representation of SHAPE structural probing. A) RNAs are treated with a SHAPE reagent (red circle) which can potentially modify any unpaired RNA within the molecule. Treatment concentrations are controlled so that most individual RNAs are modified only at a single residue and the position of modification determined by reverse transcriptase first strand extension arrest. Mutations or other perturbations which affect RNA structure are revealed as a difference in the pattern of modified residues. B) The 2’ hydroxyl of the ribose backbone can displace the activated SHAPE reagent only when the nucleophilicity is enhanced during certain conformations. Unpaired residues are flexible enough to sample these conformations and are irreversibly modified which blocks subsequent primer extension reactions.
Chapter 2

Delivery and Release of Small-Molecule Probes in Mitochondria Using Traceless Linkers

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2.1 Introduction

Mitochondria-penetrating peptides (MPPs) are specific targeting vectors for the localization of small molecules to the mitochondrial matrix. Mitochondrial targeting of small molecules has enabled the development of a number of potential therapeutics and chemical probes. However, the need for covalent conjugation of small molecules to MPPs can negatively affect the activity of the appended cargo against its cellular target. Here, we describe cleavable linkers designed for the traceless release of chemical cargo from MPPs following mitochondrial transit. The cleavage kinetics of a number of disulfides were investigated using a fluorescent reporter system in order to optimize linker stability for mitochondrial release. The stability of mono- and disubstituted disulfides was determined to be sufficient during transit through the cytosol while still allowing for the release of the cargo within 24 hours. This linker system successfully released the compound Luminespib, an HSP90 inhibitor, which was deactivated by direct MPP conjugation. The releasable conjugate regenerated Luminespib activity and induced mitochondrial phenotypes of HSP90 inhibition. This linker may prove useful in expanding the repertoire of small molecules that can be used with mitochondrial targeting vectors.

Mitochondria of mammalian cells are important energy producers and regulators of programmed cell death. Their function is critical for cellular health, and dysregulation of mitochondria has been connected with a variety of human diseases. The delivery of therapeutics or small molecule probes to this cellular organelle is challenging, however, because of the double-membrane structure of mitochondria that is very difficult to penetrate.

Over the last several years, a variety of molecular delivery systems that can transport cargo into mitochondria have been reported. We have developed mitochondria penetrating peptides (MPPs), which are mitochondrial localization vectors that directly target small molecules to the mitochondrial matrix. The use of MPPs for mitochondrial small molecule targeting has proven useful for the development of new probes for mitochondrial biology and investigating drug activities within mitochondria with organellar specificity. However, all of the MPP
conjugates generated and studied to date feature covalent and uncleavable linkers, and therefore the peptide remains attached to molecular cargo. While this approach has produced several interesting compounds with drug-like properties and significant levels of activity for a variety of probes, the presence of the delivery vehicle after transport to mitochondria is a limitation of MPPs and many other mitochondrial delivery vectors. Several existing examples of cargo release in mitochondria have focused on taking advantage of enzymatic cleavage of a labile ester linker.\textsuperscript{112, 161} However, linkers that rely on enzymatic cleavage are particularly sensitive to sterics around the cleavage site. In addition, enzyme expression can vary by cell type, environment, and metabolic status, which could make cleavage kinetics inconsistent. Therefore, we hypothesized that a linker cleaved by endogenous chemical agents could be more suitable for mitochondrial small-molecule targeting and release.

2.2 Results

2.2.1 Investigating Disulfide Stability in Cells

We chose disulfides as a basis for a releasable linker because reducing agents, glutathione in particular, are ubiquitous in the cell but relatively scarce in the external environment.\textsuperscript{196} Disulfides have been used successfully in a number of peptide-based cytosolic delivery agents.\textsuperscript{180, 197} However, as relative concentrations of glutathione in the mitochondrial matrix and the cytoplasm are similar,\textsuperscript{198} the stability of a disulfide-based linker as it passes through the cytoplasm must be tested to identify a structure with an optimal delivery and release profile.

A reporter system for linker stability (Figure 2.1) was developed by combining a MPP-conjugated fluorophore (Y) with a fluorescence quencher (X) linked by a disulfide bond. The selection of the MPP was based on prior studies identifying peptides with high levels of cellular uptake and mitochondrial localization, and low levels of cytotoxicity. Proximity-based quenching of the fluorescence occurs when the linker is intact but is disrupted upon disulfide cleavage, leading to a fluorescence turn-on signal. We tested three linkers featuring thiols with differing levels of substitution that would
Figure 2.1 – Overview of linkers tested for mitochondrial delivery and release of molecular cargo. Reporter conjugates contained the TAMRA fluorophore \([Y]\) paired to a BHQ-2 quencher \([X]\) through disulfide linkers. Compound 1 features an uncleavable linker, while compounds 2, 3, and 4 contained unsubstituted, monosubstituted, and disubstituted disulfides, respectively. \(Z\) represents the structure of the mitochondria-penetrating peptide selected for this study. The arginines are incorporated into the peptide as unnatural d-amino acids, and the cyclohexylalanines are l-amino acids.
modulate intracellular stability to identify a structure that would maximize delivery of small molecule cargo. Three reporter conjugates featuring the different linkages (Compounds 2-4, Figure 1) were synthesized and compared to an uncleavable control (Compound 1).

We investigated the in vitro cleavage of the compounds to assess their relative stabilities (Figure 2.2 A). The fluorescence of the compounds in buffered solution in the presence of dithiothreitol was monitored over a period of 2.5 hours. Compound 1 did not exhibit any increases in fluorescence, as expected from the inclusion of an uncleavable linker in this conjugate. Compound 4, which included a disubstituted carbon proximal to the disulfide, exhibited the slowest cleavage kinetics. Compounds 2 and 3, bearing unsubstituted and monosubstituted carbons next to the disulfide, respectively, exhibited faster cleavage kinetics, with compound 2 being cleaved with 10 minutes and compound 3 requiring approximately 45 minutes.

When fluorescence recovery correlated with linker cleavage was monitored in cellulo, a similar trend was observed albeit with longer release times (Figure 2.2 B). Cells were incubated with the compounds and the fluorescence of cell lysates was monitored over 48 hours. Compound 2 exhibited the fastest fluorescence recovery kinetics with saturation being reached in about 20 hours. However, this compound also exhibited measurable levels (10%) of cleavage directly following treatment, suggesting rapid cytosolic cleavage making it less attractive for mitochondrial delivery applications. The mono- and disubstituted conjugates exhibited lower levels of initial cleavage and reached saturation over ~ 48 hours.

The time-dependence of linker cleavage in live cells was also confirmed visually using fluorescence microscopy. In experiments where all imaging conditions were held constant over the time course, all three disulfide-containing reporters exhibited a time dependent increase in fluorescence over time as opposed to the uncleavable control (Figure 2.3). These results indicate that all three of these disulfides can be used for mitochondrial delivery, depending on the desired cleavage kinetics. The monosubstituted linker was prioritized as a platform for further development as it had low preincubation cleavage while still releasing the majority of its cargo within 24 hours.
Figure 2.2. – *In vitro and in cellulo cleavage kinetics.* (A) Fluorescence recovery of the reporter conjugates in PBS incubated with 0.5 mM DTT. (B) Fluorescence recovery of the indicated reporter conjugates in K562 cells. K562s were treated with the corresponding reporter then lysed. Lysates were split and the fluorescence of one sample was normalized to a second sample which was treated for 10 minutes with 25 mM TCEP as a fully cleaved control.
Figure 2.3 – Time-dependent images of reporter conjugates in living cells.
Fluorescence microscopy of cells treated with the reporter conjugates over time. Image acquisition settings were maintained between compounds and time points. Scale bar represents 20 µm.
The extent of mitochondrial localization for the three disulfide-linked compounds was also assessed (Figure 2.4). When compared to a known mitochondrial stain (Mitotracker deep red), all three of the disulfide-linked compounds exhibited high levels of colocalization. The extent of colocalization was assessed quantitatively through the calculation of Pearson’s correlation coefficients, and the values were above 0.75 for all three conjugates.

2.2.2 Activity of a Mitochondrial Targeted Releasable HSP90 Inhibitor

To showcase the ability of this linker chemistry to release cargo into the mitochondrial matrix, the HSP90 inhibitor Luminespib was used as a test cargo. HSP90 inhibitors have attracted intense pharmacological interest due to their chemotherapeutic properties and their lack of toxicity to non-cancer cells.200 A number of HSP90 inhibitors have been developed in recent years targeting the cytoplasmic HSP90 pools of cancer cells. Inhibition of cytoplasmic HSP90 has been previously shown to cause the arrest of cancer cell growth by antagonizing the stabilizing effect of HSP90 on signaling proteins involved in cancer cell growth and survival.201 However, induction of cell death by cytoplasmic HSP90 inhibition has been found to be inconsistent, with some compounds inducing cell death in some cell lines and growth arrest in others.202 This has led to difficulties in the clinical application of HSP90 inhibitors, especially as single agents.203 Recent studies exploring HSP90 inhibitors delivered to the mitochondrial matrix via cationic vectors have suggested that inhibition of mitochondrial HSP90 and TRAP-1, a mitochondrial analogue, can more consistently and rapidly induce cell death via induction of apoptosis.110, 204 However, IC50 values for the best characterized mitochondrial HSP90 inhibitors are relatively high (∼10 µM), indicating that cationic vectors may not lead to optimal efficacy.

We selected the HSP90 inhibitor Luminespib as a candidate for our traceless linker approach. This compound has not previously been tested for mitochondrial activity because the functional groups that could be used for conjugation of a delivery vector are also involved directly in protein binding.205 Luminespib was conjugated to a mitochondria-penetrating peptide via a monosubstituted disulfide as shown in Figure 2.5A (compound 5). A non-cleavable analog (compound 6) was also generated as
Figure 2.4 – Localization of reporter conjugates in living cells. Peptide fluorescence is shown in the green channel and mitochondria labeled with the mitochondria-specific dye Mitotracker Deep Red is shown in the red channel. Insets are outlined by the dashed boxes. Pearson’s coefficients for Compound 2 = 0.95, Compound 3 = 0.76, Compound 4 = 0.77. Scale bar represents 20 µm.
Figure 2.5 – Application of linker strategy for Luminespib. (A) Chemical structure of the releasable Luminespib MPP conjugate, compound 5. (B) Chemical structure of the uncleavable Luminespib conjugate, compound 6. (C) Chemical structure of parent drug, Luminespib. (D) Mechanism of disulfide cleavage and linker self-immolation. (E) Structure of the peptide only control of compound 5.
shown in Figure 2.5 B. Cleavage of the disulfide linker in compound 5 by glutathione in the mitochondrial matrix was designed to trigger the release and regeneration of Luminespib through self-immolation of the thiol-carbonate (Figure 2.5 D). The regeneration of Luminespib after linker cleavage was shown to occur rapidly (Figure 2.6), and mitochondrial localization of a fluorescently-labeled analog was confirmed (Figure 2.7).

Leukemia cells treated with compound 5 exhibited a time-dependent increase in cell toxicity over 48 hours (Figure 2.8 A) which was distinct from the growth inhibition induced by Luminespib alone (Figure 2.9). In contrast, the uncleavable compound 6 exhibited low levels of toxicity that remained static over time (Figure 2.8 B). At two hours, only a small amount of Luminespib would have been generated from compound 5, and the similarity between the toxicity between the two peptides suggests that the effects observed are from the nonspecific toxicity of the peptides themselves, rather than an effect from the released Luminespib. Conversely, the time-dependent toxicity observed only with compound 5, and not the uncleavable compound 6, suggests that the difference in effects between the peptides is due to the cleavage and regeneration of Luminespib.

2.2.3 Mitochondrial HSP90 Inhibition Induces Apoptotic Cell Death

In order establish that the mechanism of cytotoxicity of mitochondrially-targeted Luminespib (5) was linked to mitochondrial effects, we monitored the mode of cell death, effects on mitochondrial mass, and mitochondrial depolarization. As controls, the parent compound Luminespib (Figure 2.5 C, compound 7), and the empty disulfide vector (Figure 2.5 E) were also tested. The cells were treated with 2.5 µM of each compound. Compound 5 produced significant populations of early and late apoptotic cells after 24 hours as visualized by Annexin V staining, as opposed to the parent compound and the peptide controls which exhibited no increase when tested (Figure 2.10). In addition, co-treatment of the parent compound with either the uncleavable compound 6 or the empty vector did not induce apoptosis, indicating that the effects observed with mitochondrially targeted Luminespib were not due to a nonspecific
Figure 2.6 – Reductive cleavage and regeneration of Compound 5

(A) A 50 µM stock solution of Compound 5 in PBS was run through RP-HPLC on a C18 column with a 5-95% MeCN/H₂O 1.5%/min gradient with 0.1% TFA. Retention time is labeled. (B) A 50 µM stock solution of Compound 5 in PBS treated with 25 mM TCEP and immediately run through HPLC. The peaks were identified by positive ionization ESI mass spectrometry. Expected m/z for Luminespib = 466.23, found m/z = 466.23. Expected m/z for Compound 5 Cleaved = 1032.67, found m/z = 1032.67. (C) A 50 µM stock solution of Compound 5 in PBS treated with 10 mM glutathione, incubated for 1 hour at room temperature and run through HPLC. The peaks were identified by positive ionization ESI mass spectrometry. Expected m/z for Luminespib = 466.23, found m/z = 466.23. Expected m/z for Compound 5 Cleaved = 1032.67, found m/z = 1032.67. (D) Structure of Compound 5 Cleaved.
Figure 2.7 – Mitochondrial localization of fluorescently labeled compound 5. (A) Chemical structure of the fluorescently labelled analogue of compound 5. (B) Localization of a fluorescently labeled compound 5, with peptide fluorescence is shown in the green channel and mitochondria labeled with Mitotracker Deep Red in the red channel. Insets are outlined by the dashed boxes. The Pearson’s coefficient for this compound was 0.92. Scale bar represents 20 µm.
Figure 2.8 – Toxicity of Luminespib conjugates to K562 leukemia cells. (A) Toxicity of compound 5 at different time points in K562 cells. (B) Toxicity of compound 6 at different time points in K562 cells.
Figure 2.9 – Growth inhibition induced by Luminespib in K562 cells over time.
Apoptosis visualized via Annexin V staining of K562 cells treated for 24 hours with 2.5 µM of the compounds indicated. Cell populations were gated with Annexin V+/Sytox red- cells as early apoptotic, and Annexin V+/Sytox red+ cells as late apoptotic. Necrotic cells, defined as Annexin V-/Sytox red+ cells were excluded as levels were negligible [<1%].
a synergistic effect between the peptide and cytosolic HSP90 inhibition by Luminespib. The mitochondrial mass of cells treated with the mitochondrially-targeted Luminespib (5) exclusively exhibited an increase in mitochondrial mass at 24 hours (Figure 2.11). These results suggest a cleavage specific induction of mitochondrial swelling, an indicator of mitochondrial toxicity and mitochondrial dependent apoptosis. Cells treated with mitochondrially-targeted Luminespib (5) also exhibited mitochondrial depolarization, suggesting compromised mitochondrial integrity (Figure 2.12). In both experiments, no induction of mitochondrial dysfunction was observed in any of the control compounds or any compound at 2 hours, suggesting the effects induced by mitochondrially-targeted Luminespib (5) derive from free Luminespib generated in the mitochondrial matrix and not from the vector itself.
Figure 2.11 – Changes in mitochondrial mass over 24 hours following treatment with Luminespib and MPP derivatives. Mitochondrial mass as measured by incubation with Mitotracker Green FM staining of K562 cells treated with 2.5 µM of the indicated compounds for 2 or 24 hours. Cells with Sytox red staining were gated against and excluded.
Figure 2.12 – Mitochondrial membrane polarization of cells treated with Luminespib and derivative compounds over 24 hours. Mitochondrial membrane depolarization of K562 cells as measured by TMRM staining treated with 2.5 µM of the indicated compounds for 2 or 24 hours.
2.3 Discussion and Conclusions

Our work describes a method for the release and regeneration of small molecule cargoes in mitochondria using a disulfide-based linker system. Using a mitochondrially targeted probe, we show that the breakdown of disulfides in mitochondria can be directly related to their \textit{in vitro} stability. Disulfide stability can be modulated both by the introduction of steric hindrance around the disulfide and by modifying the electron density of the bond. In our experiments, we found that unmodified disulfides appeared to exhibit low levels of compound release (<10%) prior to mitochondrial localization, although because of the innate time required for experimental processing a portion of that effect could very well represent cleavage in mitochondria during workup. This indicates that simple disulfides could likely be used for compounds where minor compound release in the cytoplasm is tolerable. The differences between the stability of the unsubstituted and the methyl substituted disulfides also indicate that the stability of these disulfides can be fine tuned for specific functions, either by increasing stability as conducted in this experiment or by decreasing stability through the use of electronically deficient thiols such as thiophenol\textsuperscript{207}.

Interestingly, a significant difference in the observed rate of disulfide cleavage was observed from \textit{in vitro} experiments compared to the \textit{in cellulo} results. Even at concentrations of glutathione which are on the lower bounds of mitochondrial concentrations, the disulfides were cleaved at a rate \~50 times faster \textit{in vitro} compared to in mitochondria. It is likely that the mitochondrial matrix environment is significantly different than what can be replicated by PBS buffer-based experiments \textit{in vitro}. The mitochondrial matrix is highly concentrated in complex molecules and has significantly higher viscosity than PBS buffer\textsuperscript{208}, both of which could slow down reaction kinetics in mitochondria. In addition, all direct mitochondrial targeting vectors are designed to hydrophobically interact with biological membranes in order to cross them. It is possible that in the membrane rich environment of the matrix compound 5 spends a significant amount of time associated with the inner leaflet of the mitochondrial inner membrane, which could sterically limit the cleavage of the disulfide by glutathione. Unfortunately, little data exists to compare the intracellular cleavage kinetics of disulfides. While many experiments have used cleavable disulfide for cytosolic compound delivery, few
investigate the kinetics of cleavage of individual disulfide bonds. However, it is noted that the diffusion is also limited by in the cytoplasm\textsuperscript{209, 210}, therefore it is likely that the cleavage of disulfides would also be hindered to some degree. It is likely that in vitro cleavage experiments represent an ideal system in which disulfide reduction can occur unhindered and highlights the necessity of in cellulo investigation of cleavage experiments for matrix targeted disulfides and possibly cytosolic disulfides as well.

Our experiments also show that cargoes released in mitochondria are done so in an active form, as indicated by the recovery of Luminespib activity following treatment with compound 5. The recovered activity of Luminespib indicates that the regeneration of the compound is successful as the conjugation site of the MPP-linker complex on Luminespib on one of the resorcinol hydroxyl actively inhibits the interactions between the Asp93 residue on HSP90 and a water molecule found in the crystal structure\textsuperscript{211}. In addition, the resorcinol group faces towards the center of the HSP90 binding pocket, which explains the lack of activity observed with compound 6. In addition, these inhibitory effects should have persisted if the remaining carbonate-thiol residual had not self-immolated after disulfide cleavage, indicating that this process does occur within cells. In the case of mitochondrial actions of Luminespib, the binding mode of the compound to TRAP-1, the mitochondrial HSP90 analog must also be taken into account. While no direct crystallographic data exists for Luminespib binding to TRAP-1, molecular docking experiments indicate that the binding mode is similar to that of HSP90 and that the same structural determinants would be in effect\textsuperscript{204}.

The linker system presented in this work was designed to be generally applicable to as wide a variety of cargo and delivery systems as possible. In terms of other mitotropic vectors, the small size and non-ionic character of the linker mean that vectors which are mono-cationic or more sensitive to the chemical properties of the cargo should largely be compatible. A possible exception may exist for vector system which contains accessible thiols within its structure as this could negatively affect linker stability. In terms of cargo variety, the linker chemistry can accommodate both hydroxyl and amine based chemical groups. In these experiments, the self-immolation of the residual linker was found to be rapid upon disulfide cleavage in carbonate-based systems. This effect should also translate to carbamate based systems, although the
reaction kinetics may be slower due to the increased stability of carbamate bonds generally\textsuperscript{212}.

An additional consideration for cargo release in mitochondria is the degree to which a cargo remains in the mitochondrial matrix following release. For uncharged species undergoing simple diffusion the rate at which the compound effluxes from mitochondria should be similar to the rate of influx. Therefore, compounds which are mitochondrial membrane impermeable should be retained in the matrix well following linker release. For charged species, the membrane potential may play a role in the retention of the compound in a similar manner to MPPs themselves. Cationic cargoes should be retained well in mitochondria as defined by the Nernst equation\textsuperscript{102}, particularly those which are hydrophilic and have the added benefit of a high energy barrier for membrane permeation. In contrast, the efflux of anionic cargoes from mitochondria would likely be enhanced by mitochondrial membrane potential, therefore lipophilic anions may be retained poorly in mitochondria following release. However, the precise relationship between compound charge, lipophilicity, and mitochondrial retention has as yet not been studied directly. Considering recent strategies developed for mitochondrial cargo release presented in this paper and by other mitotropic\textsuperscript{112, 161} and nanoparticle\textsuperscript{119} based technologies, further studies into compound retention by mitochondria may aid in choosing appropriate compounds for highly specific and effective mitochondrial targeting.

In conclusion, we have outlined a method for chemical cargo release from mitochondrial-targeting vectors in a flexible and enzyme-independent fashion. This strategy can be used to localize compounds to mitochondria which have critical functional groups that otherwise make them incompatible with targeting vectors. We have also shown that the chemical cleavage of disulfide linkers in mitochondria differs significantly than what could be expected from \textit{in vitro} data and outline a reporter system that can be used to determine linker stability in mitochondria.
2.4 Materials and Methods

**General Cell Culture**

HeLa cells were cultured in Minimum Essential Medium Alpha (MEM alpha, Gibco, Gaithersburg MD) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad CA) at 37°C with 5% CO₂. K562 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Gaithersburg MD) at 37°C supplemented with 10% fetal bovine serum with 5% CO₂.

**General Peptide Synthesis**

Solid phase peptide synthesis was performed on Rink amide MBHA resin (Novabiochem, UK) using a Prelude Protein Technologies peptide synthesizer as described previously [1]. Fx = L-cyclohexylalanine, r = Nω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyle)D-arginine, K(Mtt) = N-ε-4-methyltrityl-L-lysine.

**Synthesis of S-trityl-2-mercaptopropionic acid**

10 mmol 2-mercaptopropionic acid (Sigma-Aldrich, St. Louis MO) was dissolved in 5 mL dichloromethane (DCM) with trityl-chloride (1.1 eq, Sigma-Aldrich). The reaction was mixed for 72 hours, dried, and purified using RP-HPLC on a C18 column with an Acetonitrile/H₂O gradient with 0.1% TFA. The compound was identified by DART mass spectrometry, expected m/z = 347.11, found m/z = 347.1.

**Synthesis of Compound 1**

25 µmol of NH₂-Fx-r-Fx-r-Fx-r on resin was reacted with N-α-Fmoc-N-ε-4-methyltrityl-L-lysine (4 eq, ChemPep Inc.), O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyl-uronium hexafluorophosphate (HBTU, 4 eq, Protein Technologies, Tucson AZ), and N,N-diisopropylethylamine (DIPEA, 8 eq, Sigma-Aldrich) in 1 mL N,N- dimethyl formamide (DMF) for 2 hours at room temperature. The resin was washed twice with DMF, methanol (MeOH), and DCM and deprotected using trifluoroacetic acid:triisopropylsilane:DCM (3:3:94, 2 x 15 minutes). The beads were washed then
reacted with BHQ-2 carboxylic acid (2 eq, BioSearch Technologies, Petaluma CA), PyBOP (2 eq, ChemPep Inc.), and DIPEA (4 eq) in 1 mL DMF overnight. The peptide was washed and deprotected twice with 1 mL 20% piperidine in DMF (Protein Technologies) for 20 minutes. The peptide was washed and reacted with 5-Carboxytetramethylrhodamine (2 eq, Anaspec, Freemont, CA), HBTU (2 eq), and DIPEA (4 eq) in 0.5 mL DMF for 2 hours. The peptide was cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The peptide was purified by HPLC, then lyophilized. The peptide was identified by ESI mass spectrometry, expected m/z = 1973.09, found m/z = 1973.10.

**Synthesis of Compound 2**

25 µmol of NH$_2$-Fx-r-Fx-r-Fx-r on resin was reacted with N-α-Fmoc-S-trityl-L-cysteine (4 eq, ChemPep Inc., Wellington FL), HBTU (4 eq, Protein Technologies, Tucson AZ), and DIPEA (8 eq) in 1 mL N,N- dimethyl formamide (DMF) for 2 hours at room temperature. The resin was washed twice with DMF, methanol (MeOH), and DCM and deprotected using trifluoroacetic acid:triisopropylsilane:DCM (3:3:94, 2 x 15 minutes). The beads were then equilibrated in acetonitrile:water (5:1) for 5 minutes, and cysteamine (20 eq, Sigma-Aldrich) in 1 mL acetonitrile:water (5:1) was added under mixing followed by iodine (10 eq, Sigma-Aldrich). The reaction was stirred vigorously for 30 minutes, followed washing (2 x DMF/MeOH/DCM). The beads were then reacted with BHQ-2 carboxylic acid (2 eq), PyBOP (2 eq) and DIPEA (4 eq) in 1 mL DMF overnight. The peptide was washed (2 x DMF/MeOH/DCM) and deprotected twice with 1mL 20% piperidine in DMF for 20 minutes. The peptide was cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The peptide was purified using RP-HPLC on a C18 column with a MeCN/H$_2$O gradient with 0.1% TFA. The peptide was lyophilized and reacted with 5-Carboxytetramethylrhodamine (2 eq), HBTU (2 eq), and DIPEA (4 eq) in 0.5 mL DMF for 2 hours. The peptide was reprecipitated in ether at -20°C for 1 hour and then purified using RP-HPLC. The peptide was identified by ESI mass spectrometry, expected m/z = 2024.03, found m/z = 2024.03.
Synthesis of Compound 3

50 µmol of NH₂-Fx-r-Fx-r-Fx-r on resin was reacted with Fmoc-Nβ-Boc-L-2,3-diaminopropionic acid (4 eq, ChemPep Inc.), HBTU (4 eq), and DIPEA (8 eq) in 1 mL N,N-dimethyl formamide (DMF) for 2 hours at room temperature. The peptide was washed (2 x DMF/MeOH/DCM), cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The precipitate was purified by RP-HPLC, lyophilized, and reacted with S-trityl-2-mercaptopropionic acid (4 eq), PyBOP (4 eq), and DIPEA (8 eq) in 0.5 mL DMF. The peptide was reprecipitated in ether and then dried under vacuum for 1 hour. The peptide was deprotected using 0.5 mL trifluoroacetic acid:triisopropylsilane:DCM (5:3:92, 15 minutes), precipitated in ether and purified by HPLC. The peptide was dried under vacuum and dissolved in 0.5 mL acetonitrile:water (5:1). Cysteamine (20 eq) was added to the reaction mixture followed by iodine (10 eq) and the reaction was stirred for 30 minutes. The reaction mixture was precipitated in ether and purified by HPLC. The peptide was lyophilized and reacted with BHQ-2 carboxylic acid (2 eq), PyBOP (2 eq), and DIPEA (4 eq) overnight in 0.5 mL DMF. The peptide was precipitated in ether, dried, and deprotected in 1mL 20% piperidine in DMF for 20 minutes. The peptide was purified by HPLC, lyophilized, and reacted with 5-Carboxytetramethylrhodamine (2 eq), HBTU (2 eq), and DIPEA (4 eq) in 0.5 mL DMF for 2 hours. The peptide was precipitated in ether and purified by HPLC. The peptide was identified by ESI mass spectrometry, expected m/z = 2094.07, found m/z = 2094.07.

Synthesis of Compound 4

25 µmol of NH₂-Fx-r-Fx-r-Fx-r on resin was reacted with Fmoc-S-trityl-L-penicillamine (4 eq, ChemPep Inc.), HBTU (4 eq), and DIPEA (8 eq) in 1 mL N,N-dimethyl formamide (DMF) for 2 hours at room temperature. The peptide was then reacted identically as Compound 2. The peptide was identified by ESI mass spectrometry, expected m/z = 2051.06, found m/z = 2051.06.
**Fluorescence Reporter Peptide Quantification**

Fluorescence reporter peptides were quantified via absorbance spectrophotometry using a SpectraMax M5 spectrophotometer (Molecular Devices, Sunnyvale CA). The BHQ-2 absorbance at 600 nm was used to quantify the peptides, with an extinction coefficient of 31700 M$^{-1}$cm$^{-1}$ in 50/50 PBS:MeOH.

**Synthesis of Compound 5**

25 µmol of NH$_2$-Fx-r-Fx-r-Fx-r on resin was reacted with S-trityl-2-mercaptopropionic acid (4 eq), PyBOP (4 eq), and DIPEA (8 eq) in 1 mL DMF. The peptide was washed (2 x DMF/MeOH/DCM), cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The precipitate was purified by RP-HPLC, dried under vacuum and dissolved in 0.5 mL acetonitrile:water (5:1). 2-mercaptoethanol (20 eq, Sigma-Aldrich) was added to the reaction mixture followed by iodine (10 eq) and the reaction was stirred for 30 minutes. The peptide was purified by HPLC and lyophilized. 5-(2,4-Dihydroxy-5-isopropylphenyl)-N-ethyl-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (Luminespib, 3 eq, Adooq Bioscience, Irvine CA) was reacted with N,N’-Disuccinimidyl carbonate (3 eq, Sigma-Aldrich) and 4-(Dimethylamino)pyridine (12 eq, Sigma-Aldrich) in 0.4 mL DMF for 1 hour. The peptide was dissolved in 0.1 mL DMF and added to the reaction mixture and the solution was left stirring overnight. The peptide was precipitated in ether and purified by HPLC. Two isomers were identified during HPLC purification, likely due to attachment to either of the two resorcinol hydroxyls. The earlier eluting isomer was purified and tested due to its higher relative abundance. The solution was frozen in dry ice as the compound eluted from the column and lyophilized. The peptide was identified by ESI mass spectrometry, expected m/z = 1599.88, found m/z = 1599.88. The peptide was quantified via absorbance spectrophotometry using a SpectraMax M5 spectrophotometer. The absorbance profile of Compound 5 was found to be shifted as compared to Luminespib itself, therefore the peptide was quantified by cleavage in 25 mM TCEP in PBS pH 7.4 for 10 minutes, then measuring free Luminespib absorbance at 305 nm with an extinction coefficient of 8520 M$^{-1}$cm$^{-1}$. TCEP was not found to affect the extinction coefficient of Luminespib.
Synthesis of Compound 6

25 µmol of NH₂-Fx-r-Fx-r-Fx-r on resin was reacted with 3-[2-(2-Bromoethoxy)ethoxy]propanoic acid (4 eq, BroadPharm, San Diego CA), HBTU (4 eq), and DIPEA (8 eq) in 1 mL DMF. The peptide was washed (2 x DMF/MeOH/DCM), cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The precipitate was purified by RP-HPLC, lyophilized, and dissolved in 1 mL DMF. Luminespib (2 eq), and solid potassium carbonate (10 eq, Sigma-Aldrich) was added to the reaction mixture. The suspension was stirred overnight, filtered, then precipitated in ether and purified by HPLC. Two isomers were identified during HPLC purification, likely due to attachment to either of the two resorcinol hydroxyls. The earlier eluting isomer was purified and tested due to its higher relative abundance. The peptide was identified by ESI mass spectrometry, expected m/z = 1551.97, found m/z = 1551.97. The absorbance profile of Compound 6 was not found to be shifted as compared to Luminespib itself, therefore the peptide was quantified by measuring Luminespib absorbance at 305 nm with an extinction coefficient of 8520 M⁻¹cm⁻¹.

Synthesis of Fluorescently Labelled Compound 5

25 µmol of NH₂-Fx-r-Fx-r-Fx-r-K(Mtt) on resin was reacted with S-trityl-2-mercaptopropionic acid (4 eq), PyBOP (4 eq), and DIPEA (8 eq) in 1 mL DMF. The peptide was washed (2 x DMF/MeOH/DCM) and deprotected with trifluoroacetic acid:triisopropylsilane:DCM (5:3:92, 2 x 15 minutes). The peptide washed and equilibrated in acetonitrile:water (5:1). Cysteamine (20 eq) was dissolved in 1 mL acetonitrile:water (5:1) and added to the reaction mixture followed by iodine (10 eq). The reaction was stirred for 30 minutes. The peptide was washed (2 x DMF:MeOH:DCM) and reacted with 5-Carboxytetramethylrhodamine (2 eq), HBTU (2 eq), and DIPEA (4 eq) in 0.5 mL DMF for 2 hours. The peptide was washed, cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. The precipitate was purified by HPLC and lyophilized. 5-(2,4-Dihydroxy-5-isopropylphenyl)-N-ethyl-4-(4-(morpholinomethyl)phenyl)isoxazole-3-carboxamide (Luminespib, 3 eq, Adooq
Bioscience, Irvine CA) was reacted with N,N′-Disuccinimidyl carbonate (3 eq, Sigma-Aldrich) and 4-(Dimethylamino)pyridine (12 eq, Sigma-Aldrich) in 0.4 mL DMF for 1 hour. The peptide was dissolved in 0.1 mL DMF and added to the reaction mixture and the solution was left stirring overnight. The peptide was precipitated in ether and purified by HPLC. The earlier eluting isomer was purified and tested due to its higher relative abundance. The solution was frozen in dry ice as the compound eluted from the column and lyophilized. The peptide was identified by ESI mass spectrometry, expected m/z = 2140.12, found m/z = 2140.12. The peptide was quantified using the 5-Carboxytetramethylrhodamine absorbance at 547 nm with an extinction coefficient of 92000 M⁻¹cm⁻¹.

**Synthesis of Peptide Only Control**

The peptide was synthesized identically to Compound 5 until 2-mercaptoethanol addition, at which point the peptide was purified by HPLC and lyophilized. The peptide was identified by ESI mass spectrometry, expected m/z = 1108.67, found m/z = 1108.67. The peptide was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham MA).

**Microscopy**

HeLa cells were seeded in 8 well µ–slides (iBidi, Germany) at a density of 25000 cells per well one day prior to experiments in MEM alpha with 10% FBS. On treatment day, cells were washed once in MEM alpha with no FBS, and incubated with 6 µM compound 2-4 for 30 minutes, with the exception of the fluorescently labeled compound 5 which was incubated at 10 µM. For experiments with mitotracker, 5 nM Mitotracker Deep Red FM (Thermo Fisher Scientific) was added to 25 nM 10 minutes into the incubation with peptide. Cells were then washed twice in MEM alpha with no FBS and imaged using an inverted Zeiss Observer.Z1 microscope. For fluorescence time course experiments, cells were incubated in MEM alpha with 10% FBS for the indicated time at 37°C with 5% CO₂, then imaged.
**In vitro Fluorescent Reporter Peptide Cleavage**

200 uL of a 5 uM solution of the fluorescent reporter peptide in PBS with 1% DMSO was plated out into a Greiner CELLSTAR® 96 well black opaque plate (Sigma-Aldrich). Fresh 1 M DTT in PBS was added to 0.5 mM or fresh 200 mM glutathione in PBS pH 7 was added to 2 mM for each compound and the fluorescence was read with excitation at 540 nm and emission at 579 nm using a SpectraMax M5 spectrophotometer.

**In Cellulo Fluorescent Reporter Peptide Cleavage**

K562 cells were washed once with IMDM without FBS. 3 mL of 1x10⁶ K562 cells/mL was brought to 1.0 uM of fluorescent reporter peptide in IMDM + 0.5% DMSO and incubated for 30 minutes. The cells were washed three times in IMDM + 10% FBS. The cells were resuspended and plated on 6 well plates. The plates were processed at 0, 4, 8, 24 and 48 hours. Samples were spun down, aspirated, washed once in PBS and lysed in 450 uL of guanidine lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate, 1 mM EDTA, pH 6.0). 200 uL of each sample was added to two wells a 96 well black opaque plate. 10 uL of 1 M TCEP was added to one well of each sample, mixed and incubated for 10 minutes at room temperature. The fluorescence of each sample was read at 540 nm excitation and 579 emission.

**Analysis of Viability**

K562 cells were spun down at 500 g for 3 minutes and washed once with IMDM without FBS. The Luminespib conjugates were plated at 2x treatment concentration in IMDM without FBS, 50 uL per well in a conical bottom 96 well plate. 50 uL 6x10⁵ cells/mL K562s were plated on top of the treatment solution for the 2-hour time point, 3x10⁵ cells/ml for the 24-hour time point, and 1.5x10⁵ cells/ml for the 48-hour time point. The cells were incubated for 2 hours at 25°C with 5% CO₂, then spun down at 500 g for 3 minutes and washed 3 x with IMDM with 10% FBS. Cells were then either processed immediately for the 2-hour samples or incubated at 37°C with 5% CO₂. Cell viability was analyzed using CCK-8 viability dye (Dojindo, Rockville, MD) at an absorbance of 450 nm.
For the Luminespib viability experiments, K562 cells were spun down at 500 g for 3 minutes and washed once with IMDM with 10% FBS. Luminespib was plated at 2x treatment concentration in IMDM with 10% FBS, 50 uL per well in a flat bottom 96 well plate. 50 uL 6x10^5 cells/mL K562s were plated on top of the treatment solution for the 2-hour time point, 3x10^5 cells/ml for the 24-hour time point, and 1.5x10^5 cells/ml for the 48-hour time point. Cells were incubated at 37°C with 5% CO₂. Cell viability was analyzed using CCK-8 viability dye without washing.

**Annexin V Assay**

K562 cells were washed in IMDM no FBS and diluted to 2x10^5 cells/mL, 0.5 mL for each sample. 0.5 mL treatment solution was added at a 2x concentration in IMDM no FBS and cells were treated for 2 hours at 25°C with 5% CO₂. Cells were washed 3 times and resuspended in 1 mL IMDM with 10% FBS. Cells were plated on a 12-well plate and incubated for an additional 22 hours. Cells were analyzed using Annexin V, Alexa Fluor® 488 conjugate (Thermo Fisher). Briefly, cells were washed in PBS at 4°C and resuspended in 100 uL Annexin V binding buffer with 5 nM SYTOX® Red Dead Cell Stain at 4°C. 5 uL of annexin V conjugate was added and cells were incubated for 15 minutes at room temperature. Cells were diluted in binding buffer to 250 uL and read on a BD FACSCanto flow cytometer on ice.

**Mitochondrial Mass Assay**

K562 cells were treated identically as for the Annexin V experiments. For 24 hour samples, cells were plated on a 12-well plate and incubated for an additional 22 hours. For the 2 hour samples, cells were incubated in media for 30 minutes and processed immediately. Cells were then washed in PBS at 4°C and resuspended in 300 uL of 25 nM Mitotracker Green FM (Thermo Fisher Scientific) with 5 nM SYTOX® Red. Cells were incubated at 37°C for 30 minutes read on a BD FACSCanto flow cytometer on ice.

**Membrane Polarity Assay**

K562 cells were treated identically as for the Annexin V experiments. For 24 hour samples, cells were plated on a 12-well plate and incubated for an additional 22 hours.
For the 2 hour samples, cells were incubated in media for 30 minutes and processed immediately. Cells were then washed in PBS at 4°C and resuspended in 300 uL of 100 nM Tetramethylrhodamine (TMRM, Thermo Fisher Scientific) with 5 nM SYTOX® Red. Cells were incubated at 37°C for 30 minutes read on a BD FACSCanto flow cytometer on ice.
Chapter 3

Tuning the intracellular bacterial targeting of peptidic vectors.

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*Data contributions:*

Cellular microscopy images of intracellular Listeria in Figures were contributed by Mark P. Pereira.
### 3.1 Introduction

Many deadly pathogens live and divide within human cells, an approach that provides protection against the host immune response and antimicrobial treatment. When applied against intracellular pathogens, antibiotics show variable efficacy due to factors such as low cellular accumulation and deactivation within the cell, making the development of treatments difficult and further narrowing the pipeline of effective antibiotics\(^{213-215}\). *Listeria monocytogenes* is an example of an intracellular pathogen that is very dangerous and often deadly\(^{216}\).

The search for drugs that can specifically target intracellular pathogens is particularly complex, and not amenable to the in vitro methods that produce leads for extracellular pathogens\(^{217-219}\). Efforts in targeting intracellular pathogens have previously focused on the use of nanoparticle delivery vectors such as liposomes and polymer-based nanoparticles to enhance therapeutic potential and overcome cellular barriers\(^{220-225}\). The use of liposomes as a method of antimicrobial delivery has been studied extensively\(^{220}\) and has been shown to enhance uptake of membrane impermeable antibiotics and increase intracellular bacterial clearance\(^{221}\). However; instability in biological fluids, low entrapment efficiencies, and difficulties in ensuring consistent formulation highlights the need for additional work in this area\(^{222}\). The use of polymer-based nanoparticles as a delivery vector has also been studied as a successful method of targeting intracellular pathogens. These methods have been found to provide prolonged, steady release times and increased therapeutic efficiency\(^{223, 224}\). However, similar to their liposomal counterparts, polymeric nanoparticles display poor entrapment of water soluble cargos, requiring considerable effort being put towards entrapment optimization for each new cargo\(^{225}\). Receptor-based methods have also been explored to deliver therapeutics to intracellular bacteria\(^{226}\).

Recent work in our laboratory has focused on the use of peptide-based vectors for intracellular targeting of molecules, particularly to mitochondria of mammalian cells\(^{149, 150}\). Given the evolutionary link between bacteria and mitochondria, we hypothesized that similar peptides could be used to target intracellular bacteria. Moreover, previous studies indicated that conjugation of a methotrexate (Mtx) to a
peptide specific for mitochondria of human cells can be used to increase the therapeutic window of this molecule when used as an antimicrobial. Mtx, a bacteriostatic dihydrofolate reductase (DHFR) inhibitor, exhibits appreciable toxicity towards human cells, but this activity can be suppressed if the drug is kept away from cytosolic DHFR and directed to mitochondria\textsuperscript{181}. Here, we explore whether a Mtx-peptide conjugate engineered to specifically target intracellular bacteria might be active against \textit{Listeria monocytogenes}. We report on engineered Mtx-peptide conjugates as antimicrobial delivery vectors for the intracellular clearance of \textit{L. monocytogenes} from mammalian cells (Figure 3.1 A). This is the first report of peptidic delivery vectors that can target intracellular pathogens and clear them from mammalian cells through the action of an antibacterial drug.

3.2 Results

3.2.1 Localization of MPP Conjugates to Intracellular \textit{L. monocytogenes}

To evaluate whether a Mtx-peptide conjugate would localize to \textit{L. monocytogenes} within human cells, a sequence containing six amino acids and featuring alternating hydrophobic (cyclohexylalanine) and cationic residues (\textit{D}-arginine) was tested (Figure 3.1 B). The hydrophobic character of the peptide allows for efficient penetration of lipophilic biological membranes while the cationic residues promote penetration of energized membranes and localization within the mitochondrial matrix or bacterial cytosol. In order to test the specific cellular and bacterial localization of the peptide delivery vector, a dye labeled peptide was developed by conjugation of the base peptide with the fluorescent compound TAMRA. Fluorescently labeled peptides show specific localization to mitochondria and, in infected cells, to intracellular \textit{L. monocytogenes} (Figure 3.2). \textit{L. monocytogenes} in infected HeLa cells show characteristic actin polymerization activity\textsuperscript{227}, indicating bacterial viability (Figure 3.3).

3.2.2 Antimicrobial Activity of Mitochondrially Targeted Mtx

The Mtx-peptide conjugate was tested for toxicity against cultured \textit{L. monocytogenes} and exhibited potent growth inhibition with a MIC\textsubscript{50} of 3.7 ± 0.2 µM. This corresponds
Figure 3.1 – Schematic representation of peptide-based Mtx targeting of intracellular *L. monocytogenes*. A) The bacteria induce the formation of a vacuole in non-phagocytic cells\textsuperscript{216}. Lysis of the vacuoles release of *L. monocytogenes* into the cytoplasm. Conjugation of Mtx to a peptide allows for specific delivery into *L. monocytogenes* while sequestering excess Mtx into mitochondria at higher concentrations. B) Structure of initial Mtx-peptide conjugate tested against *L. monocytogenes*.
Figure 3.2 – Localization of MPPs into intracellular *L. monocytogenes*.  

A) Fluorescently labeled peptides localize specifically to mitochondria and *L. monocytogenes* in infected HeLa cells (shown in green). *L. monocytogenes* labeled with commercially available CellTrace™ Far Red show strong colocalization with peptide fluorescence (shown in red). Addition of an *L. monocytogenes* specific antibody indicates the position of extracellular bacteria (shown in blue). The lack of staining indicates an intracellular bacterium. The boxed region has been expanded to clearly demonstrate the colocalization between the peptide staining and the *L. monocytogenes* bacterium.  

B) Expanded insets of *L. monocytogenes* in HeLa cells.
Figure 3.3 – Polymerization of actin monomers by intracellular *L. monocytogenes* indicates motility and viability of *L. monocytogenes* inside infected HeLa cells. Alexa Fluor 350 phalloidin labeled actin is shown in blue. *Listeria* labeled with commercially available CellTrace™ Far Red DDAO-SE are shown in red.
to a slight increase in inhibition when compared to unconjugated Mtx, which exhibited a \( \text{MIC}_{50} \) of \( 8.8 \pm 0.9 \mu M \) (Figure 3.4 A). When tested against cells infected with \( L. \) \textit{monocytogenes}, the Mtx-peptide maintained growth inhibitory activity against intracellular \( L. \) \textit{monocytogenes} with a \( \text{MIC}_{50} \) of \( 13.1 \pm 1.4 \mu M \) (Figure 3.4 B). Unconjugated Mtx also caused growth inhibition of intracellular \( L. \) \textit{monocytogenes}, with a \( \text{MIC}_{50} \) at \( 19.1 \pm 4.2 \mu M \). However, the drug also exhibited considerable toxicity against HeLa cells with an \( \text{LD}_{50} \) of \( 14.8 \pm 0.1 \text{nM} \) (Figure 3.4 C), indicating that most of the host cells were dead before the effects of the antimicrobial could be realized against the pathogen (Figure 3.5). Overall, these results indicate that the peptide conjugate is effective against an intracellular pathogen, and unlike the parent drug, has little toxicity towards human cells at concentrations required for clearance of \( L. \) \textit{monocytogenes}.

### 3.2.3 Chemical Properties of MPP and Their Relation to Bacterial Targeting

Having successfully applied a Mtx-peptide conjugate to target intracellular \( L. \) \textit{monocytogenes}, we conducted a study designed to elucidate the optimal properties of an intracellular antimicrobial delivery vector. Previous studies indicated that charge and hydrophobicity are the main determinants of peptide localization into mitochondria\textsuperscript{149}. Therefore, a panel of peptides was designed in order to determine the effect of charge and hydrophobicity on peptide partitioning between bacteria and mitochondria (Figure 3.6). These two parameters have also been suggested to promote entry of peptides into bacteria\textsuperscript{228, 229}. Using the original peptide tested (1) as a starting point, a series of peptides with increased positive charge (and similar hydrophobicity) (5-7) or decreasing hydrophobicity (and consistent positive charge) (2-4) were synthesized. Peptide hydrophobicity was modified through substitution of cyclohexylalanine with less hydrophobic residues and was characterized by measuring retention time on a C18 reverse phase column (Table 3.1). In order to study the subcellular localization of the peptides, fluorescently labeled derivatives were generated by N-terminal conjugation to the fluorescent dye carboxytetramethylrhodamine (TAMRA). Fluorescence microscopy of cells treated with equimolar concentrations of TAMRA-peptides revealed that all of the peptides localized specifically to mitochondria in the absence of internalized bacteria (Figure 3.7). Treatment of cells with TAMRA alone or non-MPP peptide conjugates
Figure 3.4 – Activity of methotrexate and Mtx-peptide conjugate 1 against *L. monocytogenes* versus HeLa cells. A) *In vitro* antimicrobial activity of Mtx-peptide conjugate 1 and Mtx alone against *L. monocytogenes*. B) *In cellulo* antimicrobial activity of 1 against *L. monocytogenes* in HeLa cells. C) *In cellulo* antimicrobial activity of Mtx alone against *L. monocytogenes* in HeLa cells.
**Figure 3.5 – Effect of methotrexate and Mtx-peptide 1 on HeLa cell morphology.**

Differential interference contrast microscopy of *L. monocytogenes* infected HeLa cells treated with 20 µM Mtx and 1. *L. monocytogenes* infected HeLa cells treated with Mtx *L. monocytogenes* show signs of significant cellular toxicity. The Mtx-peptide conjugate, however, exhibits no signs of altered morphology.
Figure 3.6 – Impact of MPP chemical properties on relative uptake between bacteria and mitochondria. Partitioning of fluorescently labeled peptides is affected by modification of peptide chemical properties. HeLa cells were treated with 5 µM of each labeled peptide, with the exception of 3 and 4 which were treated at 20 µM. *L. monocytogenes* labeled with commercially available CellTrace™ Far Red show similar position to peptide fluorescence.
Table 3.1 – Chemical properties of unconjugated peptides

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<th>Z$^b$</th>
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<th>% Ac Elution$^d$</th>
<th>Relative Uptake$^e$</th>
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Figure 3.7 – Mitochondrial localization of TAMRA labeled peptides in uninfected HeLa cells. TAMRA fluorescence is shown in green, while respective differential interference contrast images are shown below. HeLas were treated for 30 minutes at the concentrations indicated; 1: 10 µM treatment, 2: 20 µM treatment, 3: 70 µM treatment, 4: 90 µM treatment, 5: 6 µM treatment, 6: 8 µM treatment, 7: 8 µM treatment
show no mitochondrial staining at relevant concentrations (Figure 3.8). Interestingly, the localization patterns changed in the presence of intracellular bacteria, with increased cationic character resulting in increased partitioning in bacteria (Figure 3.6; 1, 5-7). Similarly to peptide 1, peptide 5 showed little difference in partitioning between mitochondria and Listeria, however peptide 7 showed a clear increase in bacterial staining as compared to mitochondrial staining. In addition, decreasing hydrophobicity was also found to increase bacterial partitioning, however, higher concentrations were necessary to observe fluorescence for the low hydrophobicity peptides (Figure 3.6, 2-4). While increasing bacterial partitioning was observed with each peptide series, peptides 4 and 7 showed the most significant effect.

3.2.4 Optimized MPP Properties for Increased Bacterial Targeting

To determine the effect of the peptide sequence changes on intracellular concentrations of peptide, flow cytometry was used to evaluate uptake. Uptake was observed to decrease upon reduction in hydrophobicity with the least hydrophobic peptide 4 having considerably less uptake than 1. Conversely, increase in charge resulted in an initial increase in uptake between 1 and 5, followed by a reduction in uptake as the charge was increased as shown by 6-7.

Interestingly, peptide 7 was found to have a similar level of overall uptake to 1 (Table 3.1), indicating that the increase in bacterial partitioning was due to an increase in the relative uptake of 7 into the bacterium, rather than an increase in overall uptake (Figure 3.9). Therefore, peptide 7 was chosen as the ideal candidate to assess whether improvements in the delivery of an antibacterial were realized. A Mtx-peptide conjugate was generated with peptide 7 and compared to the original compound made with 1. This compound was found to potently inhibit the growth of cultured L. monocytogenes, exhibiting a MIC$_{50}$ of 0.68 ± 0.06 µM, corresponding to a greater than 5-fold increase in antimicrobial ability as compared to 1 (Figure 3.10 A). When tested against intracellular L. monocytogenes, Mtx-peptide conjugate 7 was found to be more potent relative to 1, with a MIC$_{50}$ of 4.8 ± 0.2 µM (Figure 3.10 B). Conjugate 7 also showed little toxicity towards human cells at the concentration required for clearance. The observed increase in the efficacy of conjugate 7 both in mammalian cells and in
Figure 3.8 – Localization of TAMRA, TAMRA-Lysine, and TAMRA-(Yr)$_3$ in HeLa cells. TAMRA fluorescence is shown in green, while respective differential interference contrast images are shown below. HeLAs were treated for 30 minutes at the concentrations indicated; TAMRA: 20µM, TAMRA-Lysine: 20µM, TAMRA(Yr)$_3$: 80 µM.
Figure 3.9 – Quantification of MPP uptake in uninfected HeLa cells. Measurements made with flow cytometry for HeLa cells treated with 5 µM of each labeled peptide.
Figure 3.10 – Antimicrobial activity of Mtx-peptide 1 compared to Mtx-peptide 7

A) *In vitro* antimicrobial activity of Mtx-peptide 7 against *L. monocytogenes*. Data for Mtx-peptide 1 from Figure 3.4 A is shown as a dotted line for comparison. Mtx-peptide 7 shows potent inhibition of *L. monocytogenes* growth.  

B) Mtx-peptide 7 inhibition of *L. monocytogenes* growth is maintained *in cellulo*. Data for Mtx-peptide 1 from Figure 3.4 B is shown as a dotted line for comparison. HeLa cell toxicity for Mtx-peptide 7 is shown as a dashed line for comparison. Similarly to *in vitro* *L. monocytogenes* treatment, Mtx-peptide 7 shows increased inhibition of *L. monocytogenes* growth compared Mtx-peptide 1.
culture indicates that molecular charge is an important determinant of the activity of a peptide vector targeting an intracellular pathogen. Increased partitioning of the potentiometric dye JC-1 towards *L. monocytogenes* suggests a stronger membrane potential compared to mitochondria, possibly accounting for increased uptake of peptide 7 (Figure 3.11). In addition, the addition of the uncoupling reagent CCCP eliminated JC-1 aggregate formation in both bacteria and mitochondria, suggesting that the uptake of JC-1 and other lipophilic cations in bacteria is potential dependent (Figure 3.12).

### 3.2.5 Inhibition of Bacterial Growth by Mtx-MPPs is Due to DHFR Inhibition

The activity of the Mtx-peptide conjugates was linked to the activity of the appended drug by testing the effects of media supplemented with high levels of nucleotides (Figure 3.13). Under these conditions, the pathway methotrexate inhibits is not essential. Indeed, significant decreases in toxicity for both 1 and 7 are observed under these conditions, indicating that it is the effects of the appended drug that underlie the activity. This observation differentiates this approach from work leveraging the effects of antimicrobial peptides that disrupt bacterial membranes.
Figure 3.11 – Relative membrane potential of mitochondria compared to L. monocytogenes in infected HeLa cells. Partitioning of the potentiometric dye JC-1 in HeLa cells infected with L. monocytogenes shows a preference for bacteria. JC-1 accumulates within areas of high potential and forms J-aggregates upon reaching a critical concentration, resulting in a shift in emission wavelength. Monomeric JC-1 fluorescence is shown in green. Fluorescence from J-aggregates indicative of membrane potential is shown in red. L. monocytogenes labeled with commercially available CellTrace™ Far Red show similar position to J-aggregate fluorescence (shown in gray).
Figure 3.12 – Bacterial depolarization eliminates uptake of potential dependent dyes in *L. monocytogenes* infected HeLa cells. Depolarization of *L. monocytogenes* and mitochondrial membrane potential by 20 μM FCCP eliminates J-aggregate formation. Monomeric JC-1 fluorescence is shown in green. Fluorescence from J-aggregates indicative of membrane potential is shown in red. *L. monocytogenes* labeled with CellTrace™ Far Red are shown in gray.
**Figure 3.13 – Activity of Mtx-peptides against L. monocytogenes in enriched media.** A) Efficacy of Mtx-peptide 1 is reduced upon treatment in enriched media. Data for Mtx-peptide 1 from Figure 3.4 A is shown as a dotted line for comparison. B) Efficacy of Mtx-peptide 1 is reduced upon treatment in enriched media. Data for Mtx-peptide 7 from Figure 3.4 B is shown as a dotted line for comparison.
3.3 Discussion and Conclusions

The similar physicochemical properties of mitochondria and bacteria can either present a pharmacological advantage or disadvantage depending on the design of the prospective drug. Our studies indicate that MPPs exhibit a high degree of cross targeting between these two species and that this can be used to great effect in increasing the pharmacological properties of appended antimicrobials. The relative targeting of bacteria as compared to mitochondria by MPPs was shown to depend on the hydrophobicity and cationic character of the peptide, with higher charge and lower hydrophobicity disposing the MPP towards bacteria.

Our results also show that the standard peptide 1 showed decreased relative targeting of bacteria compared to the high charge peptide 7, despite the two peptides having similar overall mitochondrial uptake. Therefore, this effect is truly due to increased bacterial targeting specifically rather than a decrease in mitochondrial targeting, as also indicated by the antibacterial activity profile of methotrexate conjugated to the two peptides. The relation between low hydrophobicity and high cationic character in relative bacterial targeting may be indicative of differences between the membrane structure of the two species. The results would seem to indicate that bacterial membrane may be less exclusive as compared to mitochondria. While the hydrophobicity of peptide 1 and peptide 7 was designed to be very similar, the increased cationic character of peptide 7 alone would make its inclusion into a hydrophobic membrane less energetically favorable.

In mitochondria, it is likely that the increased energetic penalty of higher cationic charge balances out the increased impact of the electrochemical gradient in promoting its uptake into mitochondria. However, if the bacterial membrane was more accommodating towards cationic molecules it may explain why peptide 7 showed a marked increase in uptake. It is also possible that the membrane potential differences noted by the increased uptake of JC-1 dye by bacteria may explain this difference, as it is possible that the increased potential difference could better drive the uptake of less membrane favorable peptides relative to mitochondria. However, while JC-1 dye uptake is known to be potential dependent, it is difficult to directly correlate the increase
in dye uptake by bacteria as compared to mitochondria to membrane potential as other physical differences between these species may have played a role. Direct electrochemical studies on bacterial membrane potential have indicated significant variance depending on cell cycle and bacterial metabolic status, however for actively replicating bacteria membrane potential has been found to be as high as 220 mV during division\textsuperscript{230}, while HeLa cell mitochondria exhibit a potential around 120 mV\textsuperscript{231}. As *Listeria* actively replicates in the cell cytoplasm, it is likely that their membrane potential would be at its peak during intracellular infection, possibly driving the uptake of peptides with lower penetration.

Another factor relates to the impact of the peptidoglycan layer on the uptake of MPPs by gram-positive bacteria such as *Listeria*. The peptidoglycan layer of Listeria is composed primarily of carbohydrates and ionic amino acids, making the overall composition highly hydrophilic\textsuperscript{232, 233}. Therefore, the increased uptake of low hydrophobicity and highly ionic peptides could be due to their increased ability to traverse the peptidoglycan layer, increasing the local concentration of the peptide around the bacterial membrane. Other anionic bacterial membrane components such as teichoic acids may also play a role in the selectivity towards higher cationic character in MPPs, as has been observed previously\textsuperscript{234}. Further studies into the nature of the physical interactions between MPPs and the bacterial membrane may better elucidate the relative impact of the features discussed on uptake.

The ability of MPPs to target intracellular cytosolic bacteria was clearly shown using *Listeria* infection as a model system. However, an increase in the MIC\textsubscript{50} was observed with both peptides while targeting intracellular Listeria as compared to in media treatments. Some decrease in targeting affinity is expected for any potential based targeting agent, as the mammalian cell cytosol contains a net negative charge compare to the extracellular environment, as evidenced by the potential difference across the cytoplasmic membrane. Therefore, as the relative bacterial membrane potential is decreased in the cytosol, MPP targeting would likely decrease. It is also possible that growth of *Listeria* in the cytosol is decreased compared to ideal *in vitro* growth conditions, which would likely lead to a commensurate decrease in membrane potential and MPP targeting. Additionally, the appended antimicrobial cargo
methotrexate induced antimicrobial activity by inhibiting bacterial DHFR, which in bacteria is required for the synthesis of purines, thymidine, methionine, and other amino acids depending on bacterial species. As such, our in vitro toxicity experiments utilized nucleotide free media in order to allow for DHFR inhibition to have an effect on bacterial growth. It is likely that intracellular bacteria are able to scavenge nucleotides from the cytosolic environment, which may explain the decrease in peptide activity.

Despite the decrease in toxicity, compound 7 was able to reduce intracellular bacteria load by around 80 percent prior to HeLa cell toxicity, indicating that MPPs can effectively target cytosolic bacteria with antimicrobial cargos. Compound 7 was also more effective than methotrexate alone in inhibiting the intracellular growth of Listeria, indicating that the MPP can also enhance the delivery of cargoes to intracellular bacteria, even compared to antimicrobials which are actively transported into the cytoplasm such as methotrexate. Therefore, other intracellular pathogens which replicate in the cytoplasm such as Shigella flexneri, Burkholderia pseudomallei, Francisella tularensis and Rickettsia may also be amenable to targeting by MPPs, however, additional research will be necessary to confirm these effects.

In addition to cytosolic growth, many intracellular pathogens grow in membrane encapsulated compartments such as Salmonella enterica, Mycobacterium tuberculosis, Chlamydia trachomatis. The addition of a second membrane structure makes targeting these bacteria even more difficult than their cytosolic counterparts. In addition, most of the intracellular vesicles are acidified compared to the cytoplasm, which would actively prevent the uptake of cationic molecules. Therefore, it is unlikely that MPP targeting alone would be generally applicable to these bacterial species. Recent studies using MPPs in targeting models of Mycobacterium intracellular infection have revealed that strategies which first localize MPPs to the endosome and release them can effectively target these pathogens. Strategies for targeting these bacteria will likely be species specific and vary depending on the properties of their membrane compartments and cellular biology, however, our work shows that MPPs can clearly contribute to the specificity and activity of antimicrobial cargos.
In summary, we have demonstrated that engineered peptides can provide vectors for efficient delivery of antimicrobial compounds against intracellular Listeria monocytogenes. In addition, we have shown that peptides can be chemically modified for greater bacterial specificity while maintaining mitochondria as a drug reservoir, a feature critical for the reduction of cargo toxicity. This work also highlights the importance of strong cationic character in intracellular bacterial targeting with peptide-based vectors. The results are the first showing that peptides can facilitate targeting intracellular bacteria and provide evidence that this method can be used to increase antimicrobial efficacy against intracellular pathogens.

3.4 Materials and Methods

**General Cell Culture and Bacterial Growth Conditions**

HeLa cells were cultured in MEM alpha (Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. *L. monocytogenes* was grown in Bacto™ Brain Heart Infusion media (BD, Franklin Lakes NJ) at 37°C.

**Peptide Synthesis and Characterization**

Solid phase synthesis was performed on Rink amide or Rink amide MBHA resin (Novabiochem, UK) using a Prelude Protein Technologies peptide synthesizer as described previously[1]. Peptides were synthesized on a 50 µmol scale. Methotrexate conjugated peptides were synthesized as described previously[2]. Fluorescently labeled peptides were synthesized by N-terminal coupling of 5(6)-Carboxytetramethylrhodamine (Anaspec, Freemont, CA) to 16.7 µmol of peptide on resin with O-(benzotriazol-1-yl)-N,N,N’,N’-tetramethyl-uronium hexafluorophosphate (HBTU, 4 eq, Protein Technologies, Tucson) and N,N-diisopropylethylamine (DIPEA, 8 eq, Sigma-Aldrich, St. Louis), in 1 ml N,N- dimethyl formamide (DMF) for 2 hours at room temperature. Peptides were cleaved from the resin using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5) and precipitated in ether at -20°C for 1 hour. Peptides were purified using RP-HPLC on a C18 column with an H₂O/MeCN gradient with 0.1% TFA to >95% purity. Peptide product identities were confirmed by electrospray ionization mass spectroscopy. Methotrexate conjugated peptides were
quantified at 302 nm in Dulbecco’s phosphate buffered saline (D-PBS, Invitrogen, Carlsbad CA) using the methotrexate extinction coefficient of 22700 M\(^{-1}\)cm\(^{-1}\). Carboxytetramethylrhodamine conjugated peptides were quantified at 553 nm in D-PBS using the extinction coefficient of 40000 M\(^{-1}\)cm\(^{-1}\). Unlabeled peptides were quantified using a BCA assay (Pierce, Rockford IL). Peptide sequences: NH\(_2\)-Fx-r-Fx-r-Fx-r-CONH\(_2\); Expected m/z = 944.68, Found = 944.7. NH\(_2\)-Fx-r-Fx-r-Fx-r-NH\(_2\); Expected m/z = 938.63, Found = 938.6. NH\(_2\)-F-r-Fx-r-Fx-r-CONH\(_2\); Expected m/z = 932.58, Found = 932.6. NH\(_2\)-F-r-Fx-r-Fx-r-CONH\(_2\); Expected m/z = 926.54, Found = 926.6. NH\(_2\)-F-r-Fx-r-Fx-r-CONH\(_2\); Expected m/z = 1247.85, Found = 1247.9. NH\(_2\)-F-r-Fx-r-Fx-r-Fx-r-CONH\(_2\); Expected m/z = 1551.02, Found = 1551.0. NH\(_2\)-F-r-A-r-Fx-r-F-r-A-r-CONH\(_2\); Expected m/z = 1626.09, Found = 1626.1. TAMRA-Peptide sequences: TAMRA-Fx-r-Fx-r-Fx-r-CONH\(_2\); Expected m/z = 1356.81, Found = 1356.8. TAMRA-Fx-r-F-x-r-Fx-r-CONH\(_2\); Expected m/z = 1350.77, Found = 1350.8. TAMRA-Fx-r-F-x-r-CONH\(_2\); Expected m/z = 1344.72, Found = 1344.7. TAMRA-Fx-r-F-x-r-CONH\(_2\); Expected m/z = 1338.68, Found = 1338.7. TAMRA-Fx-r-F-x-r-CONH\(_2\); Expected m/z = 1659.99, Found = 1660.0. TAMRA-Fx-r-F-x-r-CONH\(_2\); Expected m/z = 1963.16, Found = 1963.2. TAMRA-Fx-r-A-r-Fx-r-F-x-r-A-r-CONH\(_2\); Expected m/z = 2038.23, Found = 2038.3. TAMRA-Y-x-Y-r-Y-r-CONH\(_2\); Expected m/z = 1386.66, Found = 1386.7. Mtx-Peptide sequences: Mtx-Fx-r-F-x-r-CONH\(_2\); Expected m/z = 1380.83, Found = 1380.8. Mtx- Fx-r-A-r-Fx-r-A-r-CONH\(_2\); Expected m/z = 2062.25, Found = 2062.3. Fx = L-cyclohexylalanine, r = D-arginine, F = L-phenylalanine, A = L-alanine, Mtx = methotrexate, TAMRA = 5(6)-Carboxytetramethylrhodamine

**Microscopy**

Cells were seeded in 8 well µ–slides (iBidi, Germany) at a density of 12500 or 25000 cells per well one day prior to experiments in MEM alpha supplemented with 10% fetal bovine serum. Cells were washed once prior to peptide incubation for 30 minutes in MEM alpha. Cells were then washed twice in MEM alpha and imaged using an inverted Zeiss Observer.Z1 microscope. For intracellular *L. monocytogenes* localization imaging of the fluorescently labeled modified peptides, the OD\(_{600}\) of an overnight culture of *L. monocytogenes* was measured followed by centrifugation of a 1 mL sample at...
7,500 × g for 3 minutes. The bacterial pellet was washed once with D-PBS followed by incubation in CellTrace™ Far Red DDAO-SE (Invitrogen, Carlsbad CA, 10 µM) in D-PBS at a final OD\textsubscript{600} of 1.0 for 15 minutes. *L. monocytogenes* was washed twice in D-PBS following incubation. HeLa cells were infected with a multiplicity of infection (MOI) of 50 or 100 for two and a half hours in MEM alpha at 37°C. Infected HeLa cells were washed three times with MEM alpha followed by peptide incubation for 30 minutes or JC-1 incubation for 20 minutes. JC-1 treatment stocks were spun down prior to treatment at 13000×g for 1 minute to remove sediments. For cells treated with Carbonyl cyanide 4- (trifluoromethoxy)phenylhydrazone (FCCP, Sigma-Aldrich, St. Louis) and JC-1, cells were incubated with 20 µM FCCP for 20 minutes prior to JC-1 treatment and all subsequent media used contained 20 µM FCCP. Cells were then washed twice in MEM alpha and imaged. Post infection staining of *L. monocytogenes* was accomplished through fixation of HeLa cells with 4% formaldehyde/1% glutaraldehyde in D-PBS for 5 minutes at room temperature. Cells were blocked and stained with Rabbit pAb to *Listeria monocytogenes* (Abcam, Toronto, ON) and imaged using Alexa Fluor 350 labeled Goat anti-rabbit IgG. Actin staining was accomplished post fixation (4% formaldehyde/1% glutaraldehyde) and permeabilization (0.1% Triton in D-PBS) using Alexa Fluor 350 phalloidin (Invitrogen, Carlsbad, CA).

**Flow Cytometry**

Cells were seeded in 12 well flat bottom tissue culture plates (Corning, Kennebunk, ME) at a density of 100000 cells per well one day prior to experiments in MEM alpha supplemented with 10% fetal bovine serum. Cells were washed once with MEM alpha prior to peptide incubation for 30 minutes in MEM alpha. Cells were then washed twice and 500 µL of 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA) was added to each well followed by incubation at 37°C for 3 minutes. 1 mL of MEM alpha was added to each well and samples were then centrifuged at 700×g for 5 minutes at 4°C. The samples were decanted and washed once with 4°C D-PBS. Cells were then resuspended in D-PBS with 5 nM SYTOX Red Dead Cell Stain (Invitrogen, Carlsbad, CA) and incubated for 5 minutes at 4°C. Samples were analyzed by on a BD FACSCanto flow cytometer (BD Biosciences) with excitation at 488 nm and emission wavelengths of 564 to 606 collected.
Analysis of Toxicity

HeLa cell toxicity was analyzed as described previously[1]. HeLas were seeded at 1250 cells per well in 96-well flat bottom tissue culture plates (Starstedt, NC) one day prior to treatment. Cells were washed once with MEM alpha (-nucleosides) prior to treatment with peptide compound for 72 hours in MEM alpha (-nucleosides) supplemented with 10% dialyzed FBS. Cells were then washed with MEM alpha (-nucleosides) followed by determination of cell viability using CCK-8 viability dye (Dojindo, Rockville, MD) at an absorbance of 450 nm.

Minimum inhibitory concentration (MIC) determinations for methotrexate and methotrexate-conjugated peptides against Listeria in vitro were determined as described below. Overnight Listeria cultures were subcultured at 1:60 and grown to an OD$_{600}$ of 0.2 – 0.5 to ensure logarithmic growth. Cells were diluted 1:10000 into fresh media with test compounds. MEM alpha (-nucleosides, Invitrogen, Carlsbad, CA) was used as a defined media for bacterial growth. Bacto™ Brain Heart Infusion media (BD, Franklin Lakes NJ) was used as an enriched media for bacterial growth. Cells were incubated for 24 hours at 37°C. Growth was measured using absorbance at 600 nm.

Minimum inhibitory concentration (MIC) determinations for methotrexate and methotrexate-conjugated peptides against in cellulo Listeria were determined as described below. HeLa cells were seeded on a 24 well plate at 50000 cells per well 1 day before infection with Listeria. Overnight Listeria cultures were subcultured at 1:60 and grown to an OD$_{600}$ of 0.2 – 0.5 to ensure logarithmic growth. HeLas were washed twice with MEM alpha (-nucleosides) and incubated in MEM alpha (-nucleosides) for one hour. 500 µL of log phase L. monocytogenes was pelleted at 7,500 × g for 3 minutes, washed once with D-PBS and then resuspended in 500 µL D-PBS. HeLa cells were washed once with MEM alpha (-nucleosides) and infected with L. monocytogenes at a multiplicity of infection of 5 for 1 hour at 37°C. Infected HeLas were washed twice with MEM alpha (-nucleosides) and treated with test compounds for 17 hours. Infected HeLas were then washed twice with 500 µL and once with 900 µL MEM alpha (minus nucleotides). HeLas were then lysed for 15 minutes with 200 µL 0.1% Triton X-100 in D-PBS while shaking. 1800uL of D-PBS was then added to each well followed by serial
dilution to 1 in 1000 in D-PBS. 20 µL of the 1 in 1000 dilution was spotted on fresh BHI plates and incubated at 37°C overnight. Viable intracellular bacteria were determined by counting individual colonies. The cellular viability of infected HeLa cells after treatment with either Mtx or Mtx-(Fxr)$_3$ (each at 20 µM) was assessed using differential interference contrast microscopy. HeLa cells were plated in 8 well µ–slides (iBidi, Germany) at a density of 12500 or 25000 cells per well one day prior to experiments in MEM alpha supplemented with 10% fetal bovine serum. Cells were infected with L. monocytogenes and treated with compound as described above. Cellular morphology was observed using differential interference contrast microscopy.
Development of an *In Cellulo* Probe of Mitochondrial RNA Structure
4.1 Introduction

Though most often recognized as a messaging intermediate between the genetic code and the protein production machinery, RNA plays a number of other key roles in cellular function, serving as structural components of enzymes, gene expression silencers, and direct catalysts of cellular reactions. This diversity of function is due in part to the ability of RNA to form defined structural motifs through base pairing interactions between the nucleic bases called secondary structure. Therefore, determination of the secondary structure of these molecules is important in order to understand the mechanism by which these molecules function and how this is affected by environmental and mutational changes. While a number of computational and chemical tools exist for the probing of RNA structure in the cytoplasm and the nucleus, significantly fewer strategies exist for understanding RNA structure in mitochondria. Mitochondria have a specific and unique set of highly structured tRNA and rRNAs, however, the study of the structure-function relationship of these molecules have been largely restricted to computational and in vitro studies which may not fully recapitulate their native environment. Given the significant impact that diseases of these mitochondrial RNAs account for a significant portion of mitochondrial disease, we sought to develop novel tools for facile probing of these molecules in their native environment.

In order to determine the in cellulo structure of these biologically critical molecules, we chose to develop a chemical probe of RNA secondary structure which can target and penetrate the difficult to access mitochondrial matrix. We hypothesized that combining 2-methyl-3-furoic acid imidazolide (FAI), a reagent used previously for cytosolic in cellulo SHAPE probing, with our MPP vector would allow for efficient delivery to the mitochondrial matrix and structural probing of the RNAs within. While we found that modifications of MPPs can make the platform amenable for RNA SHAPE probing, difficulties in delivering adequate concentrations of the compound to mitochondria may necessitate additional research into more RNA specific SHAPE probes going forward.
4.2 Results

4.2.1 Synthesis of a Mitochondrially Targeted SHAPE Probe

In order to design an *in cellulo* mitochondrial SHAPE probe, we first developed a direct conjugate between our MPP platform and a previously validated cytosolic *in cellulo* SHAPE reagent, 2-methyl-3-furoic acid imidazolide (FAI). FAI undergoes nucleophilic attack by the ribose 2'-hydroxyl at the imidazole bond, liberating the imidazole and modifying the RNA backbone with a furanyl ester. In order to conjugate FAI to the peptide, we synthesized a derivative containing a second carboxylic acid in a meta position as compared to the reactive carboxylic acid (Figure 4.1 A). The derivative was conjugated to the MPP and activated with 1,1'-carbonyldiimidazole to yield the final product, SHAPE-MPP 1 (Figure 4.1 B). A second peptide was also synthesized with a fluorescently labeled MPP in order to test for mitochondrial localization (Figure 4.2 A). This compound was found to localize with high specificity to mitochondria, indicating that the SHAPE group did not interfere with mitochondrial localization. (Figure 4.2 B)

4.2.2 MPPs Enhance SHAPE Activity but Inhibit Analysis

In order to investigate the impact of MPP conjugation to the activity of the SHAPE group, the compound was tested by probing the structure of cytosolic 5S rRNA in purified whole cell RNA samples. 5S rRNA was selected as the test species due to its high relative abundance in total RNA samples and the fact that its structure had been previously probed using the parent reagent FAI\(^{189}\). The modification pattern of 5s rRNA determined by FAI (Figure 4.3, 4.4) was used as a positive control of SHAPE modification. Treatment of the RNA samples with SHAPE-MPP 1 revealed that the solubility limit of the conjugate was lower than the recommended concentration of FAI used for *in vitro* probing. However, purified RNA treated with the SHAPE-MPP conjugate was found to precipitate during subsequent purification with TRIzol, a phenol-chloroform extraction-based reagent. Normally, RNA remains in the aqueous layer, suggesting that an interaction between SHAPE-MPP 1 and the RNA which was resistant to the chaotropic properties of the TRIzol reagent was occurring. This suggested that even at low concentrations the conjugate was able to covalently modify RNA and that the MPP contributed towards the affinity of the conjugate towards RNA,
**Figure 4.1 – Chemical structure of SHAPE-MPP conjugates.** A) Chemical structure of the parent reagent FAI. B) Chemical structure of SHAPE-MPP 1. C) Chemical structure of the disulfide linked SHAPE-MPP 2. D) Chemical structure of the photocleavable SHAPE-MPP 3.
Figure 4.2 – Mitochondrial localization of the fluorescently labeled derivative of a SHAPE-MPP. A) HeLa cells were treated for 30 minutes with 10 µM fluorescent SHAPE-MPP. Peptide fluorescence is shown in green, bright field images are shown in gray. B) Structure of the fluorescent SHAPE-MPP derivative.
Figure 4.3 – Secondary structure of mammalian 5S rRNA. Non-base paired residues are shown in red, loop regions are labeled, and non-canonical base pairing interactions are denoted with a circle between bases. Structural data obtained from previous research with this reagent\textsuperscript{189}.
Figure 4.4 – RNA SHAPE probing pattern of human 5S rRNA by FAI reagent.
Major loop regions are labeled by the gradient lines.
possibly due to ionic interactions between the cationic peptide and the RNA phosphate backbone. The precipitate was found to be sparingly soluble in buffer after purification which allowed for testing of the effectiveness of RNA modification. When compared to a control treated with FAI itself, SHAPE-MPP 1 was found to exhibit a modification pattern which was somewhat similar to FAI itself (Figure 4.5). However, the conjugate also exhibited rapid signal drop-off, which led to almost no labeling of residues near the 5’ end of the RNA. Despite this, a substantial signal for the full-length 5S rRNA was observed during primer extension (data not shown), thereby ruling out the possibility of over-labeling as that would have prevented completion of the reverse transcriptase reaction. We hypothesized that the inclusion of the positively charged MPP onto the RNA may have caused bunching of the RNA which could impact primer extension. Therefore, removal of the MPP from the point of modification was found to be required for successful analysis of RNA modification sites.

4.2.3 Disulfides for Peptide Removal Following RNA Modification

A disulfide-based linker system was included in the structure of a new SHAPE-MPP in order to allow for peptide removal from RNA similar to what was discussed in Chapter 2. However, since the release of the SHAPE group during treatment was not required in this case, nor the regeneration of the original SHAPE compound, a simple disubstituted disulfide was used as the linker agent, resulting in SHAPE-MPP 2 (Figure 4.1 C). Whole cell RNA samples were treated with this compound, purified, then incubated with TCEP as a reducing agent. The samples were then re-purified and used as a substrate for primer extension. Removal of the peptide using the disulfide linker significantly increased the signal coverage of SHAPE-MPP 2 (Figure 4.6). A comparison of the modification signal of SHAPE-MPP 2 and the FAI reagent indicated good agreement between the modified residues of these two reagents as well as known structural elements of 5s rRNA. However, there was a noted decrease in the signal to noise ratio of SHAPE-MPP 2 treated samples as compared to those treated with the parent compound. This indicated that some aspect of the treatment was affecting primer extension efficiency. It is possible this could have been due to residual TCEP used during disulfide cleavage, or due to some property of the residual thiol itself such
Figure 4.5 – RNA SHAPE probing pattern of human 5S rRNA by SHAPE-MPP 1 as compared to FAI reagent.
Figure 4.6 – RNA SHAPE probing pattern of human 5S rRNA by SHAPE-MPP 2 as compared to FAI reagent.
as inter/intrastrand crosslinking or by interaction with some aspect of the reverse transcription reaction.

4.2.4 Photocleavage for Reagent-less Peptide Removal

Due to the possibility of the low signal observed for SHAPE-MPP 2 complicating experiments in the future we tried an alternative photocleavable linker system which would release the carboxylic acid as the residual group, resulting in SHAPE-MPP 3 (Figure 4.1 D). We hypothesized that the addition of a hydrophilic group with a negative ionic charge would better integrate into the natural properties of RNA and be less likely to interact with itself or components of the reverse transcription reaction. The linker exhibited complete release of the active group upon irradiation under a 365 nm UV lamp (Figure 4.7). In addition, the photocleavable reagent exhibited stronger signal to background for whole cell 5S rRNA treatment as compared to the SHAPE-MPP (Figure 4.8). The photocleavable compound was also applied to mt-tRNA^{Lys} in purified mitochondrial RNA. FAI was shown to modify mt-tRNA^{Lys} in purified mitochondrial RNA and generate a modification pattern which closely matched the expected secondary structure features of the RNA (Figure 4.9, 4.10), and SHAPE-MPP 3 was able to generate a similar signal which also fit the expected secondary structure of the tRNA (Figure 4.11).

4.2.5 In Cellulo Mitochondrial RNA Modification May Require Alternative SHAPE Reagents

Using the photocleavable SHAPE-MPP 3 we attempted to conduct SHAPE probing within live mitochondria. Cells maintained viability following treatment with SHAPE-MPP 3 up to 10 µM, which was taken as the maximum treatment concentration (Figure 4.12). Cells were treated with 1-10 µM SHAPE-MPP 3 following which mitochondria were separated and purified from treated cells and extracted of RNA. The RNA extracts were then exposed to UV irradiation, re-purified and probed for mt-tRNA^{Lys} modification. No modification was observed for FAI or SHAPE-MPP 3 treated cells and modification of the treatment parameters did not increase the signal to the point where observable modification could be detected over the DMSO controls. An alternative strategy was also attempted where a biotin label was integrated into the
Figure 4.7 – Photocleavage of SHAPE-MPP 3 following UV exposure. A) HPLC trace of a sample of purified SHAPE-MPP 3. B) HPLC trace of a sample of purified SHAPE-MPP 3 following 1 hour of 365 nm UV light exposure. Structure of the two major fragments. The structure of Fragment A was validated based off of HPLC retention time compared to a purified standard. Fragment B was validated based off mass spectrometry, expected m/z = 1223.75, found m/z = 1223.8.
Figure 4.8 – RNA SHAPE probing pattern of human 5S rRNA by SHAPE-MPP 3 as compared to FAI reagent.
Figure 4.9 – Secondary structure of human mitochondrial tRNA$^{\text{Lys}}$. Non-base paired residues are shown in red, loop regions are labeled, and non-canonical base pairing interactions are denoted with a circle between bases. Structural data obtained from previous research\textsuperscript{241}. 
Figure 4.10 – RNA SHAPE probing pattern of human tRNA$^{\text{Lys}}$ by FAI reagent. Major loop regions are labeled by the gradient lines.
Figure 4.11 – RNA SHAPE probing pattern of human tRNA\textsubscript{Lys} by SHAPE-MPP 3 as compared to FAI reagent.
Figure 4.12 – Viability of K562 cells treated for 2 hours with SHAPE-MPP 3.
photocleavable SHAPE-MPP to allow for affinity-based purification of modified RNAs using streptavidin beads (Figure 4.1 D). Some RNA was extracted from mitochondrial RNA samples using this method, however, no discernible modification pattern was observed upon reverse transcription indicating that the RNA isolated corresponded to non-specific binding. Interestingly, not even substitution of the targeting vector with a triphenylphosphonium cation was able to generate a measurable signal, suggesting that the problem was a quality of the FAI based reagent (Data not shown).

4.3 Discussion

Our results indicate that significant barriers exist towards organelle specific SHAPE labeling, perhaps more so than the typical delivery of bioactive molecules to the mitochondrial matrix. Our observations that removal of the MPP is required to detect modification indicates that there are innate difficulties in targeting mitochondrial matrix RNAs by SHAPE reagents. In vitro treatment of RNAs by SHAPE-MPP 1 resulted in accurate probing of a short span of RNA residues at the 3’ end of the RNA of interest. With typical SHAPE reagents, this is indicative of excessive compound in the treatment solution. Treatment with high SHAPE reagent concentrations can result in RNAs with multiple modified residues, and under these conditions, the reverse polymerase extension reaction reaches a modified residue soon after initiating the reaction. This results in strong labeling of the 3’ residues while the signal from residues closer to the 5’ end is depleted, similarly to what we observed with SHAPE-MPP 1. However, in this case, if the RNA has been modified by the SHAPE reagent to the point individual RNAs regularly contain multiple modifications, the chances that an RNA will have zero modified residues and therefore result in a full-length signal are near zero.

In our SHAPE-MPP 1 treated samples, we still observed a relatively strong full-length extension signal, indicating that over-modification was not the problem and that the MPP directly inhibited the polymerase extension reaction in a non-specific manner. It is possible that the cationic charge of the MPP could cause it to interact nonspecifically with the RNA phosphate backbone which could negatively affect the ability of the reverse transcriptase to process or even bind to the RNA in the first place. Regardless, as most small molecule mitotropic vectors contain cationic charge the
requirement for vector release will likely be necessary for any future mitochondrial RNA probing endeavors.

Interestingly, we found that conjugation of FAI to the peptide decreased the concentration required to produce a comparable modification pattern to the parent compound, at least in an *in vitro* setting. Treatment with SHAPE-MPPs required concentrations more than two orders of magnitude lower than for FAI to observe similar labeling. It is possible that the modifications done to FAI in order to conjugate the compound to the MPP could have increased the activity of the reagent. However, the reactive half-life of FAI itself was previously measured to be around 40 minutes\textsuperscript{189}, therefore almost all the FAI would have reacted during the two-hour treatments used for the *in vitro* modification tests. Therefore, it seems unlikely that the discrepancy would have been to a difference in the overall reactivity of the two compounds as both compounds would have been fully consumed. It seems more likely that the ionic interactions between the MPP and negatively charged RNA could have enhanced the affinity of the molecule towards RNA as a whole, making intermolecular interactions between the FAI group and the RNA 2’ hydroxyl more frequent. In this case, conjugation of SHAPE reagents to cationic vectors could actually increase the activity of the active reagent itself, although tests with different SHAPE reagents and vectors would be required to confirm this phenomenon.

Despite successfully probing RNA in an *in vitro* environment, the SHAPE-MPPs developed in this chapter failed to modify mitochondrial RNA *in cellulo*. This did not change with modification of the peptide or by changing the identity of the mitotropic vector. Fluorescent labeling of the SHAPE-MPP conjugates did reveal a small amount of non-mitochondrial punctate staining, which could be caused by the premature reaction of the compound with other nucleophilic cell components. However, the majority of the compound was found to be localized to mitochondria, and therefore some aspect of the SHAPE group must be incompatible with *in cellulo* probing. It is possible that the SHAPE-MPP does not reach adequate concentration for probing in mitochondria, however, mitochondrial targeting vectors are known to concentrate in the mitochondrial matrix from two to three orders of magnitude above the extracellular concentration. In this case, the concentration of SHAPE-MPP in the matrix would be
comparable to the treatment concentrations used during the *in vitro* experiments. It is also possible that the beneficial effects of the MPP on the reactivity of the conjugates observed *in vitro* does not occur in the mitochondrial matrix. If this phenomenon is due to the ionic interaction between RNA and the MPP vector, then the matrix environment may prevent some aspect of this interaction, decreasing the effectiveness of RNA labeling.

Additionally, it is likely that the cytosol and mitochondrial matrix contain nucleophilic species other than RNA which could also potentially react with the SHAPE reagent. As the SHAPE reagent used in this experiment is based on a carbonylimidazole bond, it is likely that the SHAPE reagent reacts with a number of molecules such as amines, thiols, and phenolic species. Carbonylimidazoles have been used as intermediates to the formation of esters, amides, thiols, and many other nucleophiles. They are highly reactive and have little selectivity for the identity of the nucleophile. Therefore, it is likely that FAI and other CDI based SHAPE reagents modify other molecules during *in cellulo* experiments. Previous experiments with FAI in modifying cytosolic RNAs used high concentrations of reagent, typically around 100 mM, for labelling. In this case, the sheer amount of reagent was adequate to label the RNA in addition to the proteins and other small molecules which would have also consumed the reagent. However, in the case of mitochondrial delivery, the reactivity of the reagent towards alternative species may have meant that the reagent was already consumed by the time it reached the mitochondrial matrix or have been insufficient to label the RNA as well as the high concentration of other molecular species in the matrix environment. In addition, all mitotropic reagents are limited by the degree to which reagent can be concentrated in the matrix, localization of too much cationic charge to mitochondria would result in depolarization and induction of cell toxicity or apoptosis. Therefore, it is likely necessary to develop SHAPE reagents which are more selective for hydroxyl modification in order to modify subcellular RNA pools.
4.4 Conclusions

In this chapter, we present the initial step required to develop an in cellulo RNA SHAPE probe of the mitochondrial matrix. We show that the removal of the MPP following RNA purification is absolutely necessary for downstream processing and highlight two cleavable linker strategies which are amenable for this purpose. We also show that the probing of RNA structure in live mitochondria will require SHAPE probes which are more efficient and selective for the RNA of interest. Future efforts into the development of more stable or selective SHAPE probes, combined with the strategies discussed in this work, may allow for facile and accurate structural probing of these complex, dynamic, and medically important RNA species.

4.5 Materials and Methods

General Cell Culture

HeLa cells were cultured in Minimum Essential Medium Alpha (MEM alpha, Gibco, Gaithersburg MD) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad CA) at 37°C with 5% CO₂. K562 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Gaithersburg MD) at 37°C supplemented with 10% fetal bovine serum with 5% CO₂.

General Peptide Synthesis

Solid phase peptide synthesis was performed on Rink amide MBHA resin (Novabiochem, UK) using a Prelude Protein Technologies peptide synthesizer as described previously [1]. Fx = L-cyclohexylalanine, r = Nω-(2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl)-D-arginine, K(Biotin) = N-ε-Biotinyl-L-lysine.

Synthesis of 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid

1 mmol of glyceraldehyde (70.8 µL, Sigma-Aldrich, St. Louis MO) was dissolved in 300 µL DMF (Sigma-Aldrich) and 1.3 equivalents tert-butyl acetoacetate (205.6 mg, Sigma-Aldrich) was added to the reaction. The reaction was heated at 90°C for 8 hours under a nitrogen atmosphere. The compound was dried under rotary evaporation. The
resulting residue was dissolved in 2 mL of acetonitrile (Caledon Laboratories Ltd, Georgetown ON) and 0.05 equivalents copper (I) chloride (5.0 mg) was added to the mixture. 5 equivalents of tert-butyl hydroperoxide (905 µL of 5.5 M tert-butyl hydroperoxide in decane over 4 Å molecular sieves, Sigma-Aldrich) was added dropwise to the solution under stirring. The solution was left stirring overnight. The reaction was then dried under rotary evaporation and dissolved in 5 mL of 0.02M NaOH (Sigma-Aldrich). The solution was put into a separatory funnel and extracted three times with 20 mL diethyl ether (Sigma-Aldrich). The organic layer was discarded and the aqueous layer was acidified to a pH of 1.0 with hydrochloric acid (Sigma-Aldrich). The solution was extracted three times with 20 mL diethyl ether, and the aqueous layer was discarded and organic layers were combined. The compounds were dried over excess magnesium sulfate, collected, and dried under rotary evaporation. The compound was purified using RP-HPLC on a C18 column with an acetonitrile/H$_2$O gradient with 0.1% TFA. The compound was identified by positive ionization DART mass spectrometry, expected m/z = 226.08. The compound was found to exhibit two major peaks in DART MS, a [+H] peak with an m/z = 227.09, and a [+NH$_4$] peak with an m/z = 244.12. The identity of these peaks was further identified by Accurate Mass DART MS with a 0.005 mass unit tolerance. The [+H] peak had an expected m/z = 227.092, found m/z = 227.092, and the [+NH$_4$] peak had an expected m/z = 244.119, found m/z = 244.118. The compound was found to have a molar extinction coefficient of 12000 M$^{-1}$cm$^{-1}$ in methanol at 250 nm.

**Synthesis of SHAPE-MPP 1**

50 µmol of NH2-Fx-r-Fx-r-Fx-r on resin was reacted with 6-(Fmoc-amino)hexanoic acid (4 eq, Sigma-Aldrich), O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium hexafluorophosphatate (4 eq, ChemPep, Wellington FL), and DIPEA (8 eq, Sigma Aldrich) in 1 mL N,N- dimethyl formamide (DMF) for 2 hours at room temperature. The peptide was washed (2 x DMF/MeOH/DCM) and deprotected twice with 1mL 20% piperidine in DMF for 20 minutes. The beads were washed and reacted with 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid (2 eq), benzotriazol-1-yl-oxytrypyrrolidinophosphonium hexafluorophosphatate (2 eq, ChemPep), and DIPEA (4 eq) in 1 mL DMF for 2 hours. The beads were then washed, dried, and cleaved from resin
over 2 hours using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5). The compound was precipitated in ether at -20°C for 1 hour, washed with ether, then dried and purified by RP-HPLC. The purified compound was lyophilized and dissolved in 20 µL DMSO (Sigma-Aldrich). The solution was quantified by UV/VIS spectroscopy using the extinction coefficient for 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid in methanol. 1.5 equivalents of 1,1′-carbonyldiimidazole (Sigma-Aldrich) was dissolved in 10 µL DMSO and added to the peptide solution. The compound was allowed to react for 90 minutes. The final compound was identified by ESI mass spectrometry, expected m/z = 1259.8, found m/z = 1259.7.

**Synthesis of SHAPE-MPP 2**

50 µmol of NH2-Fx-r-Fx-r-Fx-r on resin was reacted with N-Acetyl-D-penicillamine (4 eq, Sigma-Aldrich), PyBOP (4 eq), and DIPEA (8 eq) in 1 mL DMF for 2 hours at room temperature. The beads were washed (2 x DMF/MeOH/DCM) and the coupling was repeated to increase the yield. The beads were then washed and equilibrated in acetonitrile:water (5:1) for 5 minutes, and cysteamine (20 eq, Sigma-Aldrich) in 1 mL acetonitrile:water (5:1) was added under mixing followed by iodine (10 eq, Sigma-Aldrich). The reaction was stirred vigorously for 30 minutes, followed washing (2 x DMF/MeOH/DCM). The beads were washed and reacted with 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid (2 eq), PyBOP (2 eq), and DIPEA (4 eq) in 1 mL DMF for 2 hours. The beads were then washed, dried, and cleaved from resin over 2 hours using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5). The compound was precipitated in ether at -20°C for 1 hour, washed with ether, then dried and purified by RP-HPLC. The purified compound was lyophilized and dissolved in 20 µL DMSO (Sigma-Aldrich). The solution was quantified by UV/VIS spectroscopy using the extinction coefficient for 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid in methanol. 1.5 equivalents of 1,1′-carbonyldiimidazole (Sigma-Aldrich) was dissolved in 10 µL DMSO and added to the peptide solution. The compound was allowed to react for 90 minutes. The final compound was identified by ESI mass spectrometry, expected m/z = 1394.78, found m/z = 1394.8.
Synthesis of SHAPE-MPP 3

50 µmol of NH2-Fx-r-Fx-r-Fx-r on resin was reacted with 4-[4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxyl]butyric acid (4 eq, EMD Millipore, Billerica MA), PyBOP (4 eq), and DIPEA (8 eq) in 1 mL DMF for 2.5 hours at room temperature protected from light. The beads were washed (2 x DMF/MeOH/DCM). The beads were equilibrated in DCM, then suspended in 1 mL DCM containing 1,1'-carbonyldiimidazole (2 eq), DMAP (0.2 eq), and 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid (2 eq) at 4°C. The reaction was mixed for 1 hour at 4°C, then at room temperature overnight. The beads were then washed, dried, and cleaved from resin over 2 hours using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5). The compound was precipitated in ether at -20°C for 1 hour, washed with ether, then dried and purified by RP-HPLC. The purified compound was lyophilized and dissolved in 20 µL DMSO (Sigma-Aldrich). The solution was quantified by UV/VIS spectroscopy using the extinction coefficient for 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid in methanol. 1.5 equivalents of 1,1'-carbonyldiimidazole (Sigma-Aldrich) was dissolved in 10 µL DMSO and added to the peptide solution. The compound was allowed to react for 90 minutes. The final compound was identified by ESI mass spectrometry, expected m/z = 1427.80, found m/z = 1427.8

Synthesis of Fluorescently Labeled SHAPE-MPP

50 µmol of NH2-Fx-r-Fx-r-Fx-r on resin was reacted with N-Fmoc-S-trityl-D-penicillamine (4 eq, ChemPep), PyBOP (4 eq), and DIPEA (8 eq) in 1 mL DMF for 2 hours at room temperature. The beads were washed (2 x DMF/MeOH/DCM), and deprotected with trifluoroacetic acid:triisopropylsilane:DCM (3:3:94, 2 x 15 minutes). The beads were then washed and equilibrated in acetonitrile:water (5:1) for 5 minutes, and cysteamine (20 eq, Sigma-Aldrich) in 1 mL acetonitrile:water (5:1) was added under mixing followed by iodine (10 eq, Sigma-Aldrich). The reaction was stirred vigorously for 30 minutes, followed washing (2 x DMF/MeOH/DCM). The beads were washed and reacted with 4-(tert-butoxycarbonyl)-5-methylfuran-2-carboxylic acid (2 eq), PyBOP (2 eq), and DIPEA (4 eq) in 1 mL DMF for 2 hours. The beads were then washed and deprotected twice with 1 mL 20% piperidine in DMF (Protein Technologies) for 20
minutes. The beads were washed and reacted with 5-Carboxytetramethylrhodamine (2 eq, Anaspec, Freemont, CA), HBTU (2 eq), and DIPEA (4 eq) in 0.5 mL DMF for 2 hours. The beads were then washed, dried, and cleaved from resin over 2 hours using trifluoroacetic acid:triisopropylsilane:water (95:2.5:2.5). The compound was precipitated in ether at -20°C for 1 hour, washed with ether, then dried and purified by RP-HPLC. The purified compound was lyophilized and dissolved in 20 µL DMSO (Sigma-Aldrich). The solution was quantified by UV/VIS spectroscopy using the extinction coefficient for TAMRA of 92000 M⁻¹cm⁻¹ 547 nm in methanol. 1.5 equivalents of 1,1'-carbonyldiimidazole (Sigma-Aldrich) was dissolved in 10 µL DMSO and added to the peptide solution. The compound was allowed to react for 90 minutes. The final compound was identified by ESI mass spectrometry, expected m/z = 1765.9, found m/z = 1765.9.

**Synthesis of Biotin Labeled SHAPE-MPP 3**

50 µmol of NH2-Fx-r-Fx-r-Fx-r-K(Biotin) was reacted identically as in the synthesis of SHAPE-MPP 3. The final compound was identified by ESI mass spectrometry, expected m/z = 1732.0, found m/z = 1731.9

**In Vitro RNA SHAPE Treatment**

24 µL of 1 mg/mL total cell or mitochondrial RNA was added to each centrifuge tube. The tube was heated at 95°C for 2.5 minutes on a heat block and cooled in ice for 1 minute. 12 µL of ice cold 3x SHAPE buffer (333 mM HEPES, pH 8.0, 20 mM MgCl2 and 333 mM NaCl) was added to each tube. The solution was incubated at 37°C for 10 minutes. FAI was added to 100 mM or SHAPE-MPP was added to 1 mM along with 4 µL DMSO. RNA was incubated for 2 hours at 37°C. 1 mL TRizol reagent (ThermoFisher) was added to each sample, and samples were incubated at room temperature for 5 minutes. 200 µL chloroform was added to each sample, mixed for 1 minute, incubated for 3 minutes. The samples were centrifuged at 12000 g at 4°C for 15 minutes. For samples treated with the photocleavable and disulfide linked SHAPE-MPPs, the aqueous phase and interphase were collected separately, while for other samples only the aqueous phase was collected. 500 µL of isopropanol was added to the aqueous phase samples and the solutions were incubated at room temperature for 10
minutes. The samples were spun down at 12000g for 10 minutes at 4°C, and the RNA washed with 1 mL cold 75% ethanol in water. The ethanol was removed, and the samples were dried under atmosphere for 3 minutes then dissolved in 50 µL RNase-free water. For the interphase samples, the interphase was dissolved in 100 µL back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris base) and treated with either 25 µL of 1 M TCEP pH 7.0 for the disulfide SHAPE-MPP or 365 nm light for 1 hour for the photocleavable SHAPE-MPP.

First strand extension

3 µg of RNA per tube was added to a 0.2 mL PCR strip tube. 2 pmol of 5’-fluorescein labeled primer against the target RNA of interest was added to the tube, and total volume was brought up to 13 µL with water. Samples were heated to 95°C for 2 minutes, followed by cooling at 4°C for 1 minute on a thermocycler. At 4°C, 1 uL of 10 mM dNTP mix, 1 µL of 100 mM DTT, and 4 µL of Superscript III first strand buffer (ThermoFisher) were added to the tubes. The solution was heated to 52°C and held for 1 minute in a thermocycler. 1 µL of 200 units/µL Superscript III reverse transcriptase (ThermoFisher) was added to the tube, and the reaction was held at 52°C for 30 minutes on the thermocycler. The polymerase was then heat inactivated at 70°C for 15 minutes. The samples were cooled to 37°C, and 1 µL of 5 U/µL RNase H (Sigma Aldrich) was added and incubated for 20 minutes. Samples were stored at 4°C and sent for gel electrophoresis. Electrophoresis was completed at the Centre for Applied Genomics, Genetic Analysis Facility, 686 Bay Street, Room 139800.

Analysis of Viability

K562 cells were spun down at 500 g for 3 minutes and washed once with IMDM without FBS. The SHAPE-MPPs were plated at 2x treatment concentration in IMDM without FBS, 50 uL per well in a conical bottom 96 well plate. 50 uL 6x10^5 cells/mL K562s were plated on top of the treatment solution. The cells were incubated for 2 hours at 25°C with 5% CO₂. Cell viability was analyzed using CCK-8 viability dye (Dojindo, Rockville, MD) at an absorbance of 450 nm.
**In cellulo SHAPE Treatments**

K562 cells were spun down, washed once with IMDM (no FBS), and resuspended in 10 mL of IMDM. The cells were split into two 5 mL samples and treated with SHAPE-MPP for one sample and an equivalent amount of DMSO for the other. The tubes were incubated at 37°C for 2 hours, spun down, and washed in PBS. The samples were then suspended in 3 mL of isolation buffer (10 mM Tris adjusted to pH 7.4 with MOPS, 1 mM EGTA adjusted to pH 7.4 with Tris, 200 mM sucrose). The cells were lysed with 100 passes on in a 5 mL Teflon/glass homogenizer, and the lysate spun down for 10 minutes at 500g at 4°C. The supernatants were collected and spun down at 7000g for 10 minutes at 4°C. The isolated mitochondria were washed once with isolation buffer and lysed in 200 uL Guanidine lysis (4 M guanidine isothiocyanate, 25 mM sodium acetate, 1 mM EDTA, pH 6.0) buffer. The sample was then exposed to 365 nm UV light for 1 hour and purified using TRIzol. For samples which were treated with biotin tagged SHAPE-MPP, mitochondria were lysed in 0.5% Triton, 200 mM KCl, 1 mM PMSF and Halt protease inhibitor cocktail (ThermoFisher) and affinity purified using streptavidin magnetic beads prior to UV cleavage (New England Biolabs, Ipswich, MA). The beads were washed 3x in wash buffer (0.1% Triton, 5 mM EDTA, 30 mM Tris pH 7.4) and once with water. The lysate was added to the beads and incubated for 1 hour at 4°C. The beads were washed 3x in lysis buffer and once with water. The beads were extracted with 100 uL guanidine lysis buffer and heated at 95°C. The beads were magnetically removed and the solutions were cleaved with UV and purified with TRIzol.

**Primer Sequences Used in Experiments**

Cytosolic 5S rRNA

5’-AAAGCCTACAGCACCCGGAT-3’

tRNA Lys

5’-TGGTCACTGTAAGAGGTTG-3’
Chapter 5

Summary and Future Directions
5.1 Summary

Mitochondrial targeting of biochemically active compounds is a strategy which is gaining interest due to the persistent efforts of a number of research groups uncovering examples of how organelle specific targeting can help generate new and effective compounds. However, the previous work within the field of mitochondrial vectors focused on a small number of highly specific pharmacological applications. For example, large bodies of work have been done on DNA damaging agents and alkylators for chemotherapy or antioxidants for metabolic and neurodegenerative diseases, and many other classes of chemical compounds have been overlooked due to difficulty in adapting them for use with mitotropic vectors. The strength of mitochondria penetrating peptides in comparison to other mitotropic vectors is their malleability, as their chemical properties can be modified to suit the purposes of a variety of cargoes and delivery conditions. The studies presented in this work highlight methods to utilize the flexible chemical nature of MPPs in order to expand the repertoire of compounds and strategies which can be applied to this delivery system. In addition, we highlight how MPPs can provide compounds with properties which make them more suited for purposes alternative to their typical uses.

We provided a method for release and regeneration of small molecule compounds in the mitochondrial matrix following localization. A significant limiting factor in the use of mitotropic vectors is the fact that cargoes must be attached covalently. This fact may account for the apparent bias against small molecule ligands in the compounds selected as cargoes in previous literature (Table 1.2), as they are more sensitive to structural perturbation. We found that our linker system could be used to regenerate free Luminespib following mitochondrial localization, a compound we confirmed to be deactivated by conjugation. This allowed for the development of a novel potential chemotherapeutic compound which would have been impossible with standard MPP coupling chemistry. We also showed that the release kinetics of the linker could be controlled for faster or slower release, a property which could be used with compounds which require rapid concentration of active molecules or a slow, consistent release profile respectively.
We also show that MPPs exhibit highly effective cross targeting towards bacteria both in vitro and in an intracellular infection model. Interestingly, the relative targeting of MPPs towards bacteria was positively correlated with positive charge and lower hydrophobicity, even in peptides which exhibit similar mitochondrial uptake. These considerations can be used in future experiments in subcellular targeting of antimicrobials, as we show that mitotropic vectors can be used to effectively target intracellular bacteria which are normally protected by the cellular membrane. Additionally, our results suggest that intracellular bacteria appear to readily uptake lipophilic cations such as MPPs and mitochondrial dyes at higher concentrations than mitochondria themselves, a property which appears to be tied to an increased relative membrane potential. Taken together, these results show that potential exists for subcellular targeting vectors to provide the same benefits to compound activity and specificity in the field of antimicrobials as they have already done for mitochondria-centric compounds.

Finally, in Chapter 4 we presented our efforts towards the development of a novel class of in cellulo mitochondrial RNA structural probe. We were able to modify a previously existing RNA SHAPE probe for MPP conjugation without affecting its activity. Our results indicate that MPPs are able to localize the majority reactive SHAPE reagents into mitochondria, although some evidence of side reactions with other cellular components was noted. We also found that MPPs are an active inhibitor of reverse transcription and therefore must be removed prior to first strand extension analysis of modified RNAs. We highlight two methods of releasing MPPs from modified RNA, one by use of reducing agents to cleave a disulfide bond between the vector and the cargo, and another by a photocleavable linker. We found that both strategies were able to release the MPP from RNA and recapitulate the activity or the parent compound, with the photocleavable linker being more convenient and effective. However, in the end, the compounds developed in this project were unable to modify mitochondrial RNA to detectable levels in cellulo. Despite these setbacks, we have laid the groundwork for the design requirements for mitochondrial SHAPE probing, and future developments in the design of SHAPE probes may be able to utilize this research for future efforts.
In conclusion, this body of work shows that MPPs can be applied to a number of chemical and pharmacological problems both inside and outside of the realm of mitochondrial targeting. The flexible nature of MPPs can allow them to overcome application barriers provided careful consideration and design. We hope that the research presented in this work will be used to inform future efforts into understanding the full potential of MPPs and subcellular targeting vectors as a whole.

5.2 Future Directions

5.2.1 Expanding the Understanding of Mitochondrial Cargo Release

Although we have presented a method for small molecule release from MPPs, a number of questions about how compounds behave in mitochondria must still be answered to fully understand the scope of this strategy. One of the most pressing matters which should be investigated is the degree to which compounds are retained in mitochondria following release, and how the chemical properties of the compound affect these properties. Because the primary factor for mitochondrial retention has been lost following cleavage, certain cargo molecules may be able to efflux from mitochondria following release. While the innate impermeability of the inner mitochondrial membrane may aid in compound retention, particularly those which are hydrophilic and ionic, it is possible that molecules which are hydrophobic enough to penetrate the membrane may diffuse out of mitochondria.

One possible method to study these effects is to utilize tracer molecules to observe the concentration of molecules retained in mitochondria after release. Fluorescent dyes may be useful for this purpose, as many different classes are available and cover a wide range of chemical properties. Measuring the relative mitochondrial fluorescence over time of an MPP using the releasable linker compared to a control would indicate what percentage of the compound is being lost over time. Understanding the factors behind mitochondrial compound retention could help inform which types of compound are and are not amenable to this system.

Another avenue to investigate would be more fine control of the release kinetics of the linker. In the work described in Chapter 2, we found that simple methyl
substitution of the vector thiol provided around a fourfold range of linker half-life, with
the most stable linker having a half-life of around 24 hours. A more stable linker would
be possible with increased methyl substitution, and we found that tetrasubstitution
around the disulfide rendered the linker indistinguishable from an uncleavable control
(data not shown). However, decreased linker stability should also be possible using
substitutions which decrease the electron density of the disulfide bond. The inclusion of
electronegative substitutions around the peptide thiol or switching the alkyl chain with a
thiophenol may allow for a significantly decreased linker half-life without affecting the
self-immolation of the compound side thiol. The design of linkers which are quicker to
cleave may be essential for compounds which require a more rapid buildup of free
compound to elicit a biological effect. It would also be interesting to observe the degree
to which fast-cleaving linkers affect the mitochondrial specificity of the appended cargo.
Even the fastest cleaving linker tested in Chapter 2 required hours before significant
cleavage was observed, therefore it is unlikely that significant cleavage within the
cytoplasm occurred during the half an hour incubation period required to observe
mitochondrial fluorescence. The time any individual molecule would spend in the
cytoplasm would likely be a fraction of that time, however, it would be interesting to
observe the fastest cleavage time possible with this linker system without observing
significant cytoplasmic cleavage.

Finally, the release of small molecules from MPPs in mitochondria was a concept
designed around the fact that many small molecule ligands are highly sensitive to
structural perturbation. Therefore, this linker system opens up MPPs for use with a
number of protein inhibitors and activators. Given the previous success we have had in
using MPPs to develop probes of mitochondrial biology\textsuperscript{195}, the ability to also send
protein specific activity modulators could open a new chapter in mitochondrial probing.
In addition, this system could also be used with numerous pharmacological targets in
mitochondria to elicit a beneficial effect. For example, mitochondrial uncoupling with
protonophores such as dinitrophenol was a concept briefly investigated as a method to
treat obesity in the mid-19\textsuperscript{th} century, however, it has since fallen out of favor in the
medical community due to the risk of overdose resulting in mitochondrial toxicity and
death\textsuperscript{245}. However, the use of uncouplers with mitotropic vectors is a concept which
has garnered interest due to the possibility that a vector would limit the toxicity of the compound as uptake would decrease as membrane polarity is reduced. Therefore, the self-limiting uptake of the compound would maintain concentrations at a stable level. This compound was previously investigated using triphenylphosphine as a vector, however, it was found that the strong mitochondrial localization of the conjugate prevented the uncoupling activity of dinitrophenol itself\textsuperscript{246}, as unhindered passage through the membrane is required for activity. The releasable linker presented in this work reveals a potential solution to this problem and illustrates how a releasable linker could solve problems previously endemic to mitochondrial vectors.

5.2.2 Expanding the bacterial spectrum of MPP vectors

Our results show that MPPs can significantly enhance the potency of antimicrobials both by increasing their bacterial uptake and by decreasing off target effects by sequestration in mitochondria. Previous studies have shown that MPP can target a number of bacterial species\textsuperscript{181}, and many of these species are of high importance from a human health perspective. However, these species are primarily gram-positive pathogens, and there is still work to be done in investigating the ability of MPPs to target gram-negative pathogens which have historically exhibited more innate resistance to antimicrobials. Preliminary data indicate that some MPPs are able to target certain gram-negative species (data not shown), however, we have not yet discovered an MPP with true generalizable uptake into all bacterial species.

To develop an effective peptide against gram-negatives, the basis of bacterial resistance to MPP uptake must be determined. Gram-negative bacteria typically produce a number of membrane efflux pumps which may facilitate resistance to MPP uptake\textsuperscript{247}. Investigations into efflux knock out bacterial lines may help confirm this possibility, and systematic variation of MPP chemical properties or conversion of MPP residues into more artificial analogs may help combat this problem. It is also possible that differences in gram-negative cell wall structure such as changes in the peptidoglycan composition or bacterial capsule may affect MPP penetration. In this case, it may be prudent to model changes to MPP composition to reflect the chemical properties of compounds which are effective in gaining access to gram-negative
bacteria while attempting to retain mitochondrial targeting properties. Hopefully, the chemical flexibility of a peptide-based system will allow MPPs to overcome these targeting problems and expand the effectiveness of antimicrobial compounds against pathogenic bacterial species.

Our experiments also show that MPPs can effectively target intracellular pathogens which grow in the cytoplasm and may, therefore, be effective against species such as *Shigella*, *Burkholderia*, *Francisella*, and *Rickettsia*. While the effectiveness of MPP conjugated antimicrobials against these species should be tested, a more difficult and pressing matter is the targeting of intracellular bacteria which grow in membrane bound bodies. These species include a number of high priority targets such as *Mycobacterium*, *Chlamydia*, and *Salmonella*. Due to the fact that these membranes are often mildly acidified compared to the cytosol, it is unlikely that MPPs will effectively target these compartments on their own. Work completed by our lab show that targeting MPPs to endosomal compartments can help increase targeting towards bacterial compartments and enhance overall targeting of entrapped bacteria.

However, as the structural features and mechanics of formation of each species compartment are different, the design of targeting strategies may have to be species specific. However, the innate difficulties in targeting these bacteria with traditional antibiotics are significant, and the fact that MPPs appear to be effective in providing a solution for targeting these species may warrant further study.

Finally, the potential of MPPs to target other classes of human pathogens such as fungal and parasitic species may be an interesting field of study. MPPs may be effective at targeting mitochondria of eukaryotic pathogenic species, and it is possible that the chemical properties of MPPs may be adjusted to provide a targeting bias towards these pathogens as what was observed with the results in bacteria. Developing therapeutics against *Plasmodium* species is an obvious target for MPP based therapeutics, either by targeting these parasites in infected cells or in circulation to prevent disease progression. Targeting mitochondria of eukaryotic pathogens may also provide an opportunity to pursue novel molecular targets which have not been previously investigated.
5.2.3 Continuing the Development of a Mitochondrial SHAPE Probe

Future direction for the development of a mitochondrial SHAPE probe should revolve around increasing the delivery of active SHAPE reagent to mitochondria. As discussed in Chapter 4, the presence of a portion of non-mitochondrial fluorescence in the labeled SHAPE peptide indicates that the compound may be reacting with other cellular components. The promiscuity of the carbonylimidazole active group of the SHAPE reagent makes it prone to attack by a number of nucleophilic groups. A method of increasing the chemoselectivity of SHAPE reagents towards RNA, either by directly modulating the selectivity of the reagent towards the 2’ hydroxyl or by shielding the SHAPE reaction until proximal to the RNA, may be necessary in order to achieve the level of labeling necessary for detection.

Previous studies into SHAPE probing of cytosolic RNA utilized high treatment concentrations to obtain adequate labeling efficiency, and could therefore largely ignore off target reactions. Because of this, little data exists measuring the relative nucleophilicity of the activated 2’ hydroxyl as compared to other common cellular nucleophiles. If the nucleophilicity of the activated hydroxyl is sufficiently stronger than those of primary amines and thiols common in the cell, it would be possible to increase the selectivity of the SHAPE reagent by simply decreasing the effectiveness of the leaving group. Fortunately, a method to study this already exists in the form of hyperactivated RNA residues in certain RNA species observed in previous studies. The reaction kinetics of SHAPE reagents with these constitutively activated residues could be compared to those of off target cellular components in order to test for compounds which have a higher selectivity for RNA molecules.

It would also be possible to enrich samples of modified mitochondrial RNA using affinity-based purification. In our studies, we attempted to utilize a biotin labeled SHAPE-MPP in order to purify modified RNA using a streptavidin matrix. We found that this strategy was insufficient to purify modified RNAs, however, it is possible that this effect was due to the interference of endogenously biotinylated mitochondrial components. Retrying this strategy with a non-endogenous tag molecule may yield better success in a purification strategy. For example, FITC labeled SHAPE reagents
paired with a high-affinity anti-FITC antibody may be more effective than biotin in allowing for specific purification of modified RNAs.

Alternatively, delivery vectors which can introduce higher overall concentrations of SHAPE reagent into the mitochondrial matrix may help achieve adequate labeling efficiency. Nanoparticle based delivery vectors such as liposomes may aid in this purpose and they may be able to deliver highly concentrated solutions of SHAPE reagent directly to the mitochondrial matrix. In addition to increasing the total amount of reagent delivered to mitochondria, the nanoparticle may also help shield the reagent from the cellular environment prior to delivery. Unfortunately, the reactivity of the SHAPE reagent combined with its instability in ionic solutions may make particle loading a challenge. However, if an adequate material can be found, nanoparticle-based delivery of SHAPE reagents may be worth studying.

5.2.4 Final Statements

In summary, the majority of the work presented in this thesis focuses on charting new avenues of research which are possible with MPPs. As such, it is my hope that this work can aid in future experiments which push the envelope of what is possible with mitochondrial research and the chemical manipulation of mitochondrial biology. Additionally, the conceptual similarities between MPPs and other mitotropic vectors mean that many of the concepts presented in this work can apply to the work of other scientific groups as well. Hopefully, the research highlighted in this thesis can help inform and inspire others who are interested in studying this multifaceted organelle and those interested in the betterment of society as a whole.
References


