Mitochondria-Penetrating Peptides: Characterization and Cargo Delivery

By

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Abstract

A class of mitochondria-penetrating peptides (MPPs) was studied in an effort to optimize their applications in the delivery of bioactive cargo to this therapeutically important organelle. The sequence requirements for mitochondrial entry were monitored, and it was discovered that while an alternating cationic/hydrophobic residue motif is not required, the inclusion of a stretch of adjacent cationic amino acids can impede access to the organelle. In addition, a variety of C-terminal cargos were tested to determine if there are limitations to the lipophilicity, charge, or polarity of compounds that can be transported to mitochondria by MPPs. Furthermore, these systematic studies aided the design and synthesis of a copper-binding MPP for the delivery of copper ions to mitochondria for the potential rescue of disorders associated with copper-deficiency. The results reported demonstrate that MPPs are versatile transporters that may have a wide range of biological applications.
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Abbreviations

Fmoc: 9-fluorenylmethylloxycarbonyl

HBTU: O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyl-uronium hexafluorophosphate;

NMM: 4-methylmorpholine

DMF: N,N-dimethyl formamide

to: 2-[[1-(5-carboxypentyl)-4(1H)-quinolinylidene]methyl]-3-methylbenzothiazolium bromide

rox: 5-(and-6)-carboxy-x-rhodamine

fl: 6-carboxy-fluorescein

DIPEA: N,N-diisopropylethylamine

TFA: trifluoroacetic acid

TIS: triisopropylsilane

PBS: phosphate buffered saline

EDTA: ethylenediaminetetraacetic acid

DIC: differential interference contrast

Fx: cyclohexylalanine

Fn: pyridylalanine

k = (D)-lysine

r = (D)-arginine
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1. Chapter 1: Introduction

This chapter represents published work:

Introduction

Mitochondria are not purely energy-producing compartments; they possess critical roles in a myriad of vital cellular processes. Many of these processes are relevant to disease, and thus, targeting this organelle with synthetic, drug-carrying vectors may have therapeutic benefits. For example, the discovery of a link between mitochondrial DNA (mtDNA) mutations and human myopathies over 20 years ago\textsuperscript{1, 2} indicates that the delivery of nucleic acids is a highly worthwhile objective. Another rationale for targeting the mitochondrion arises from its ability to propagate reactive oxygen species (ROS) and oxidative stress signaling. Energy production through oxidative phosphorylation, a process involving the oxidation of substrates with molecular oxygen coupled to ATP synthesis, requires a continuous flow of electrons. As such, mitochondria are the major sites of ROS, which are produced as byproducts from the electron transport chain (Figure 1). Mitochondrial ROS have been closely linked to various diseases, including cancer, diabetes, Alzheimer’s, and other neurodegenerative disorders; this renders the mitochondrion an important target for the delivery of radical scavengers.\textsuperscript{3} In addition, mitochondria have an indispensable role in the execution of the intrinsic apoptotic pathway. As resistance to apoptosis contributes to tumor cell survival, it is of interest to trigger apoptosis by delivering relevant biomolecules to mitochondria to override these alterations.\textsuperscript{4-6} The importance of mitochondrial processes directly links all mitochondrial components to cell survival and normal function; thus, it is not surprising that deficiencies in intramitochondrial complexes lead to mitochondrial dysfunction and abnormal cell development. For instance, deficiencies in metal ions that play a role in mitochondrial complexes (\textit{i.e.}, copper, zinc, iron, manganese) cause a series of human disorders that are linked to mitochondrial dysfunction.\textsuperscript{7-10}

Despite the numerous therapeutic applications that could be realized by targeting mitochondria, there are very few examples of mitoactive drugs. This is in part a reflection of the difficulty in delivering drugs into this organelle. For example,
**Figure 1. Schematic of a mitochondrion with subcompartments and components.**

The mitochondrion is composed of four compartments: the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane, and the matrix. The matrix houses mitochondrial DNA (mtDNA), approximately 17 kbp sized plasmids. The electron transport chain (ETC), found on the inner membrane, is comprised of several protein complexes that maintain an electrochemical gradient needed for ATP synthesis, giving rise to the -180 mV membrane potential. The ETC is a major source of reactive oxygen species (ROS), which can induce apoptosis if left to accumulate. The protein import machinery, composed of the TOM (translocase of the outer membrane) and TIM (translocase of the inner membrane), recognizes the mitochondrial targeting sequence (MTS) of proteins by receptors on the TOM, which subsequently import the unfolded protein to its designated compartment. The MTS is then removed within the matrix by matrix metalloproteinases, and the protein refolds.

drug molecules must initially cross the cellular membrane before ultimately targeting this intracellular compartment, which possesses a convoluted and complex structure that prohibits access to this organelle. The mitochondrion is comprised of four distinct compartments: the outer mitochondrial membrane (OMM), the inner membrane space (IMS), the inner mitochondrial membrane (IMM), and the mitochondrial matrix (Figure 1). Unassisted entry into the IMS is only possible for moieties that can freely diffuse through the 2 nm aqueous pores in the OMM.\textsuperscript{11} Gaining access to the matrix is very difficult as the IMM is highly convoluted, densely packed with proteins, and acts as a rigid barrier to the passive diffusion of all molecules. The protein-to-lipid ratio for the IMM is 3:1, and 1:1 for the plasma membrane\textsuperscript{12} (Figure 2). Furthermore, anionic molecules face an additional hindrance in accessing the mitochondrial matrix due to the strong negative membrane potential (-180 mV) maintained by the IMM for the function of the electron transport chain.

In order to efficiently deliver biologically relevant cargo molecules to this organelle, a variety of methods for achieving mitochondrial accumulation in living cells have been developed. Several of these methods have been reviewed in detail.\textsuperscript{13-19} This section will summarize the types of transporters available, describe their physicochemical properties, and highlight their strengths and weaknesses as applied to the mitochondrial delivery of bioactive cargos (Figure 3).

1.1. Delocalized Lipophilic Cations as Mitochondrial Localizers and Transporters

One way to selectively target mitochondria is to take advantage of the substantial electrochemical potential maintained across the IMM, a distinguishing characteristic of this organelle. While all cations are attracted to the negative potential across this membrane, one class of molecules, referred to as delocalized lipophilic cations (DLCs), is particularly effective at crossing the hydrophobic membranes and, hence, preferentially accumulates into mitochondria of living cells.\textsuperscript{20} DLCs are small
Figure 2. Membrane barriers that must be penetrated for mitochondrial targeting.

Cell penetrating peptides, such as Tat (shown) must cross the hydrophobic cell membrane to reach their target, while mitochondria-specific compounds such as mitochondria penetrating peptides (MPPs) must cross additional barriers (two mitochondrial membranes) to reach their target. The inner mitochondrial membrane acts as a rigid barrier to the passive transport of all molecules due to its highly packed structure whereas the cell membrane is less packed and more permeable (insets).

molecules that have a positive charge, but, unlike inorganic cations (e.g., Na\(^+\), Ca\(^{2+}\)), possess an electronic structure that achieves delocalization of this charge through resonance stabilization. The transport of these charged, lipophilic molecules is energetically favorable because the charge is spread over a large molecular area, and thus forms a greater ionic radius.\(^{21}\) This effectively lowers the enthalpy associated with desolvating charged species and placing them into a lipid environment (specifically referred to as the Born energy).\(^{21}\) Because of this, DLCs readily accumulate within the cell and then preferentially localize to the mitochondrial matrix, driven by membrane potentials as described by the Nernst equation:

\[
\Delta \psi = \frac{2.303RT}{F} \log \left( \frac{[\text{cation}]_{\text{in}}}{[\text{cation}]_{\text{out}}} \right) \quad (\text{Equation 1})
\]

This relationship predicts that a plasma membrane potential of -60 mV and mitochondrial membrane potential of -180 mV will result in DLCs accumulating ten-fold in the cytoplasm and 10,000-fold within the mitochondria.\(^{22}\)

DLCs are widely used for visualizing and probing mitochondrial morphology and physiology. As early as the 1890s, DLCs such as fuschin, Janus green, and crystal violet were used by histologists to selectively stain mitochondria, but the origin of the specificity observed with these agents was not understood at the time.\(^{23}\) Over 60 years later, studies of the dibenzylammonium cation in isolated mitochondria\(^{24}\) and the fluorescent dye Rhodamine 123 in cultured cells prompted the suggestion that DLCs actively accumulate in mitochondria in a potential-dependent manner.\(^{25}\) Today, DLCs like Mitotracker, tetraphenylphosphonium (TPP) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazolcarbocyanine iodide (JC-1) are commonly used as mitochondria-specific dyes for staining and studying mitochondrial physiology.
DLCs have been used to transport exogenous cargo such as antioxidants and nucleic acid mimics into the mitochondria; however, this class of molecules has some limitations as mitochondrial delivery vectors. Toxicity is a particular concern for some DLCs, as they have been shown to compromise mitochondrial function at high concentrations. For example, they can depolarize the membrane potential due to charge accumulation\textsuperscript{26} or interfere with various complexes of the electron transport chain.\textsuperscript{27, 28} In fact, DLCs such as AA1 and F16, have been used as cytotoxic agents to target tumor cells, which have been shown to exhibit increased membrane potentials relative to nontransformed cells.\textsuperscript{29, 30} Therefore, delivery applications realized with this type of transporter must include careful monitoring of cellular toxicity in vitro and cardiotoxicity in vivo. Finally, while DLC-mediated delivery is a promising tool for small molecule transport, it is not as effective with large polar constructs, presumably because the properties of larger molecules overwhelm the influence of the DLCs in dictating whether a conjugate may pass through the mitochondrial membranes.\textsuperscript{31}

1.2. Mitochondrial Targeting Sequences for Organellar Delivery

Another strategy used to selectively target mitochondria involves harnessing the mitochondrial protein import machinery, which is naturally utilized by cells for the delivery of nuclear encoded mitochondrial proteins. These proteins are directed to the mitochondrion post-translationally through cleavable N-terminal peptide sequences. Mitochondrial targeting sequences (MTSs) are commonly 20–40 amino acids in length with structural motifs recognized by the mitochondrial import machinery.\textsuperscript{32} Initially, the MTSs of different nuclear-encoded mitochondrial proteins were expected to be conserved; however, it was discovered that a consensus sequence does not exist. Instead, these peptides display large numbers of both positively charged and hydrophobic residues that stretch over the full length of the sequence.\textsuperscript{33} While some MTSs are amphiphilic and have primarily $\alpha$-helical structures in membrane-like environments, this secondary structure may not be a
Figure 3. Commonly used delivery strategies to target mitochondria.

The three most common ways to access mitochondria are via a delocalized lipophilic cation (DLC), a mitochondria targeting sequence (MTS), or by vesicle-based methods. Each strategy is associated with its advantages and disadvantages.

required feature for import. Following synthesis, MTS-targeted proteins enter mitochondria using the protein import machinery. Once a MTS is recognized by receptors on the outer membrane, the attached protein is transported into the IMS by threading through the pore of the TOM (see refs. and for a full review). In concert with the ATP-dependent activity of matrix-localized hsp70 (mhsp70), the precursor is then electrophoresed through the TIM by using the electrical potential gradient across the IMM. Once inside the matrix, the MTS is cleaved in one or two proteolytic steps by mitochondrial processing peptidases, and, with the help of matrix-localized chaperones such as mhsp70, the protein refolds into its mature form.

The use of MTSs for mitochondrial delivery has been successful with a variety of cargo molecules, including proteins, nucleic acids, and endonucleases. This approach is therefore a powerful means to transport biomolecular species. It does, however, have drawbacks, as the most effective MTSs are quite long, and therefore the synthesis of fusion constructs can be challenging. Furthermore, the aqueous solubility and cellular permeability of these constructs is a limiting factor for exogenous delivery, as many of these MTSs display hydrophobic stretches of amino acids (Figure 3).

1.3. Vesicle-Based Mitochondrial Transporters
A third class of mitochondrial delivery vector that is particularly advantageous for transporting large or impermeable cargo is the vesicle-based transporter (Figure 3). For example, a novel construct called MITO-Porter displays surface-bound cationic peptides to deliver a liposome-based carrier for macromolecular delivery to the mitochondria. MITO-Porter is comprised of a liposome, which encapsulates the cargo, modified with a dense surface layer of octaarginine (R₈) peptides. The entire assembly is internalized into cells by macropinocytosis. Following endosomal escape, MITO-Porter is able to fuse with the mitochondrial outer membrane due to the highly fusogenic lipids in the liposome and the electrostatic interactions of R8
with negatively charged surface lipids of the mitochondrial outer membrane (e.g., sphingomyelin or phosphotidic acid). Evidence of successful mitochondrial delivery facilitated by this system was obtained by detecting delivered GFP by using fluorescence microscopy and western blot analysis of the mitochondrial compartments, as well as detecting gold nanoparticles by transmission electron microscopy. While this system is hindered by a low efficiency of internalization (only 10% of cells were transfected with GFP), it clearly holds promise for future delivery applications of large macromolecules.

The DQAsome, or dequalinium-based liposome vesicle, constitutes another class of liposome-based delivery vectors. Dequalinium (DQA) is a symmetrical delocalized lipophilic dication that resembles balaform electrolytes and forms vesicles upon sonication. DQA vesicles with diameters ranging between 70-700 nm can be generated, and mitochondrial delivery of encapsulated cargo was verified for this carrier by using fluorescence colocalization. The unique self-assembly of DQA into liposome-like aggregates, as well as its efficient mitochondrial localization, alleviates both synthetic challenges and cargo size limitations involved in designing a universal mitochondrial targeting strategy.

1.4. Synthetic Peptide and Amino Acid-Based Mitochondrial Transporters
The ease of peptide synthesis and the success in using this type of building block to assemble cellular transporters has inspired the development of a variety of systems that use artificial structures and sequences to gain access to mitochondria. Quite recently, a variety of amino acid and peptide-based mitochondrial transporters have been described that are designed to exploit charge-driven uptake into the organelle. While these transporters are in earlier stages of development than those described above, they show significant promise as tunable and versatile mitochondrial delivery agents.
The Kelley laboratory recently reported a novel set of peptide-based transporters that display both efficient cellular uptake and mitochondrial localization.\textsuperscript{46} Inspired by the successful delivery of various cargo mediated by cell-penetrating peptides (CPPs), a class of short peptide sequences with physicochemical properties compatible with both cellular and mitochondrial uptake was designed (Figure 2). These mitochondria penetrating peptides (MPPs) display both natural and synthetic amino acid residues that are either cationic (\textit{e.g.}, arginine, lysine) or hydrophobic (\textit{e.g.}, phenylalanine, cyclohexylalanine).\textsuperscript{46} By systematically studying sequences featuring subtle structural modifications, it was determined that these highly charged peptides could achieve mitochondrial access as long as a critical lipophilicity threshold is met. MPPs possess the necessary balance of charge and lipophilicity that provides the electrostatic driving force for uptake through energized barriers (\textit{i.e.}, cellular and mitochondrial membranes) while still maintaining ability to cross the hydrophobic membranes.\textsuperscript{46} As discussed above, DLCs are thought to gain access to the mitochondria for the same reason; here, however, in MPPs, the molecular charge is not delocalized over the entire molecule. This observation indicates that point charges can traverse the mitochondrial membranes if they are embedded in a sufficiently lipophilic vector. These peptides are currently under study as transporters for a wide variety of biological cargo molecules. Initial efforts to transport model cargo into mitochondria by using MPPs indicate that molecules with diverse physicochemical properties can be carried with these agents.\textsuperscript{47} The tunable scaffold should provide versatility and generality in mitochondrial delivery applications.

Other synthetic mitochondrial localizers recently developed feature the amino acid arginine and rely on the special properties of guanidinium moieties as delocalized cations.\textsuperscript{48-50} For example, a nonhydrolysable tetraguanidinium oligomer comprised of chiral bicyclic guanidinium subunits conjugated through thioether linkages was shown to exhibit exceptional cell permeability and was reported to
rapidly accumulate into mitochondria of live HeLa cells within five minutes.\textsuperscript{48} Another mitochondrial localizer of this class was made by using a sorbitol (d-glucitol) scaffold, chosen for its water solubility and low toxicity\textsuperscript{49} and contains eight guanidine residues tethered through two branched chains. The intracellular localization in cultured cells displayed mitochondrial sequestration, and preferentially accumulated in heart and brain tissues of mice.\textsuperscript{49} Other variants were generated featuring multiple guanidine groups attached to a lactose scaffold through alkyl chains of differing lengths.\textsuperscript{50} The anomeric hydroxyl group of the lactose scaffold serves as a functional moiety for the conjugation of different cargo molecules, while the remaining seven are tethered to guanidine groups through alkyl linkages. Interestingly, mitochondrial targeting in HeLa cells was achieved with linear hexanoic acid linkers, while similar molecules featuring octanoic acid linkers were excluded from the organelle and, instead, displayed endosomal and lysosomal sequestration. The lactose transporters were readily taken up by cells and were present in the spleen, liver, and brain of mice; such organs are associated with elevated numbers of mitochondria due to higher energy demands.

An interesting commonality amongst all of these synthetic constructs with very diverse scaffolds (peptide, bicyclic oligomers, or carbohydrate) is the inclusion of guanadinium groups. This amino acid, with its delocalized charge, may be an important contributor to the mitochondrial access that these vectors exhibit, and make the behavior of these agents conceptually quite similar to that of DLCs.

1.5. Conclusions
Mitochondria play central roles in both cell survival and death. As more details emerge that associate dysfunctions in this organelle to human diseases, the need for targeting mitochondria will be increasingly important. Moreover, the capability to selectively probe and perturb this organelle within a living cell affords a novel means to examine mitochondrial functions that are relevant to biology and medicine. Hence, the development of mitochondria-specific transporters that exhibit
efficient translocation across mitochondrial as well as cellular membranes is essential to utilize them as tools for cell biology. These motivations will continue to advance research in the area of mitochondrial delivery agents, with synthetic and bioinspired systems continuing to provide new tools for research and therapeutics.
2. Chapter 2: Materials and Methods
**Materials and Methods**

**Peptide synthesis:** Solid-phase synthesis was performed at a 35-50 μmole scale on MBHA Rink amide resin (0.6-0.7 mmol/g, 100-200 mesh) (NovaBiochem) using a Prelude automated peptide synthesizer (Protein Technologies, Inc). Couplings were performed with 4 equivalents of Fmoc-protected D or L-amino acids (Advanced ChemTech, Fmoc= 9-fluorenylmethyloxycarbonyl), HBTU (4 equiv, Protein Technologies Inc., HBTU=O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyl-uronium hexafluorophosphosphate), and NMM (8 equiv, Protein Technologies, Inc.; NMM=4-methylmorpholine) in N,N-dimethyl formamide (DMF) for 1h. Double couplings were performed for all arginine residues. The Fmoc group was removed with piperidine (20% v/v) in DMF (2x 10min).

**Synthesis and purification of peptide conjugates:** The deprotected N-termini of the completed peptides were conjugated to a carboxy-derivatized thiazole orange fluorophore (to) 2-[1-(5-carboxypentyl)-4(1H)-quinolinylidene]methyl]-3-methyl-benzothiazolium bromide) (4 equiv.) with HBTU (4 equiv.) and DIPEA (8 equiv.) for 3 hrs in SPE filter columns (Alltech). The derivatized to dye was synthesized as described previously. The fl and rox fluorophores were conjugated to the deprotected N-terminus of the peptide in a similar manner as described above. 6-carboxy-fluorescein (3 equiv., Invitrogen) was coupled to the peptide in the presence of 3 equiv. of HBTU and 6 equiv. of DIPEA. For the rox conjugation, 5-(and-6)-carboxy-x-rhodamine, succinimidyl ester (3 equiv., Invitrogen) was coupled to the N-terminus of the peptide in the presence of 6 equiv. of DIPEA. The coupling reactions were allowed to run for 3 hours before washing the resin with DMF, MeOH, and CH₂Cl₂.

The dye-peptide conjugates were detached from the resin and deprotected in a single step by adding 95/2.5/2.5 % TFA/H₂O/TIS v/v (TFA= trifluoroacetic acid; TIS= triisopropylsilane, Sigma) to the beads for 2 hr. The solvent was allowed to drip slowly through the resin bed and collected into a 50 mL conical tube. After washing the resin with ~5 mL of MeOH, cold Et₂O (40 mL) was added and the
resulting crude mixture was centrifuged to isolate the peptide. The resulting solid was dissolved in TFA/H₂O (0.1% v/v) and purified by reverse-phase HPLC with a Shimatzu preparative LC-8A series HPLC equipped with a diode array detector using preparative column (Zorbax 300SB C-18, 7 micron, 21.2 x 250 mm) at a flow rate of 10 mL/min. The mobile phases were MeCN/0.1 % TFA and H₂O/0.1 % TFA and the gradient was 0.8%/min. Purity of the resultant product was assessed by an analytical Agilent 1100 series HPLC using a linear solvent gradient from 5 to 95 % B over 60 min (solvent A = 0.1 % TFA in H₂O; solvent B = 0.1 % TFA in CH₃CN) with an analytical column (Varian C18, 5 micron, 250 x 4.6 mm) at a flow rate of 1.0 mL/min; retention times are reported in the Supporting Information. Purity of all peptides used in this study was greater than or equal to 95%. ESI mass spectroscopy was used to confirm the identity of the conjugate. A molar extinction coefficient of 63,000 M⁻¹ cm⁻¹ at 500 nm in H₂O was used to quantify the to-peptide conjugates. A molar extinction coefficient of 78,000 M⁻¹ cm⁻¹ at 577 nm in H₂O was used to quantify the rox-peptide conjugates. A molar extinction coefficient of 88,000 M⁻¹ cm⁻¹ in 0.01 M NaOH aqueous solution was used to quantify the fl-peptide conjugates at 500 nm.

**Synthesis of C-terminal biotin and trolox cargo:** The biotin and trolox moieties were coupled to an additional C-terminal lysine residue possessing an orthogonal protecting group (K(Dde); Novabiochem). To do this, to was first coupled to the N terminus of the peptide as described above. Then, while still on the resin, the Dde protecting group was selectively removed with 4% hydrazine solution (35 wt% in H₂O; Sigma) in DMF for 3 h and washed with DMF. For 2-trolox, the exposed primary amine was conjugated to the trolox (4 equiv; Sigma) with activation by HBTU (4 equiv) in the presence of DIPEA (8 equiv). For 2-biotin, the exposed primary amine was conjugated to a succinimidyl ester derivative of biotin (Biotin-Os, 4 equiv; Sigma) in the presence of DIPEA (8 equiv). The reactions were allowed
to continue for 3 h and then the peptide conjugate was cleaved from the resin, purified, and analyzed as described above.

**Cell culture:** HeLa cells (ATCC) were cultured as subconfluent monolayers on 75 cm² cell culture plates with vent caps (Sarstedt) in minimum essential medium (MEM, ATCC) supplemented with 10% (v/v) fetal bovine serum (ATCC) in a humidified incubator (70-95 %) at 37 °C with 5 % CO₂. Cells grown to subconfluence were enzymatically dissociated from the surface with a solution of 0.05 % trypsin/ 0.53 mM EDTA (ethylenediaminetetraacetic acid, Cellgro) and plated at 15-25 x10³ cells/well 1-2 days prior to the experiment in eight-well ibitreat µ-slides (Ibidi). For uptake experiments, 1 x10⁵ cells/well were plated in a 12-well plate 1 day prior to the experiment. These conditions produced a monolayer at subconfluence for the experiments.

**Localization of peptide conjugates:** Localization of to-peptide conjugates: For intracellular localization studies, the culture medium was removed, and the cells were washed in phosphate-buffered saline (PBS; Cellgro), pH 7.4. The cells were incubated with 1-5 μM conjugate in serum-free MEM (α-MEM [no phenol red]; Invitrogen) for 60 min. The viability of cells in the absence of serum was investigated by microscopy and by monitoring the exclusion of the viability dye Propidium Iodide. Cells were washed twice with serum-free MEM. After washing, serum-free MEM was added and the slides were immediately placed on ice. Images were taken with an inverted Zeiss LSM 510 confocal microscope with a C-APO 63x water immersion lens (NA = 1.2). The excitation wavelength for visualization of to conjugates was 488 nm, and emission was collected using a long-pass filter from 505 nm. Differential interference contrast (DIC) images were taken along with fluorescence channels.

**Colocalization of peptide conjugates:** For all colocalization studies, the corresponding Mitotracker (Invitrogen) was added in the last 15 min of the
incubation to achieve a final concentration of 50–100 nM. Cells were washed twice with serum-free MEM. After washing, serum-free MEM was added and the slides were placed on ice. Images were taken immediately. For 1a and 1d, an excitation wavelength for to (thiazole orange) at 488 nm was used, and emission was collected from 505 to 550 nm. The excitation wavelength for visualization of Mitotracker Red 633 nm was 543 nm, and emission was collected with a long-pass 560 nm filter. The fluorescence images were analyzed with Colocalizer Pro software program to determine Pearson’s coefficient (Rr), and values reported are average values obtained for individual representative cells (~30 cells) over multiple experiments (≥3 days). The background was subtracted from the images with a manually selected region of interest. Mitotic and unhealthy cells, as assessed by DIC, were excluded from analysis. Because large differences in signal intensity between the two fluorescence channels can introduce artifacts, only cells with comparable signals from both channels were used for calculations.

For N-terminal dye conjugates, the cells were incubated with 2 µM of 2, 20 µM of 3 conjugates, or 2 µM of 4 in serum-free MEM for 60 min. Mitotracker Red 633 nm was used for colocalization with 2 and 3 while Mitotracker Green was used with 4 to minimize spectral overlap between the fluorophore pairs. Images were taken as described above, with an excitation wavelength for visualization of 2 and 3 at 488 nm, which was passed through a HFT 488 and NFT 490. Emission was collected from with a band pass filter at 505-550 nm. For visualization of Mitotracker Red 633 nm, a HeNe laser 633 nm was passed over a HFT 488/543/633, and emission was collected with a long pass 560 nm filter. The excitation wavelength used for 4 was 543 nm which was passed over an HFT 488/543 and emission was collected with a long-pass 560 nm filter, while the Mitotracker Green was excited with 488 nm and visualized using a bandpass 505-550 nm filter.
For all colocalization studies, DIC images were taken along with both fluorescence channels, and no bleed-through was observed with these parameters.

**Analysis of peptide uptake by flow cytometry:** After incubation with 3-5 μM peptide for 90 min, cells were enzymatically removed from the surface of the plate with trypsin/EDTA (500 μL/well) for 10 min at 37 °C. The trypsinization was quenched with 1 mL complete MEM per well, and an additional 1 mL PBS was added to each well. From this point on, the samples were maintained on ice or at 4 °C until analysis. The samples were transferred from the wells to sterile tubes, pelleted by centrifugation (6 min at 800 g), and resuspended in PBS (500 mL) containing Sytox Red (5 nM; Sigma). Samples were then analyzed by flow cytometry on a BD FACSCanto flow cytometer (BD Biosciences). A minimum of 10,000 cells were analyzed per sample. Those staining positive for Sytox Red were excluded from analysis. The fluorescence median of the live population was used for statistical analysis.

**Statistical analysis:** Statistical significance of cell and mitochondrial uptake data was determined by one-way ANOVA methods using the Prism software. Statistical significance was accepted when p < 0.05.

**Octanol/water partitioning for measurement of logP:** The logP values were measured by octanol partitioning by a modification of the shake-flask method and as described previously. An aliquot of to–peptide conjugate (100 μL, 50–300 μm) in Tris buffer (10 mM, pH 7.4) and 1-octanol (100 μL; Aldrich) were added to a microtube (0.5 mL). Buffer was employed in order to measure logP of the peptide conjugates in the protonated state observed at physiological pH. The tubes were vortexed for 2 min and centrifuged for 2 min; 25 μL of each layer was removed and diluted either in 100 μL 3:1 methanol/Tris or methanol/octanol for a final composition of 3:1:1 methanol/octanol/Tris. The aqueous layer was diluted an additional fourfold. Three dilutions were prepared per layer, each dilution (100 μL)
was pipetted into a 384-well plate, and the absorbance was read at 500 nm with a reference wavelength at 625 nm. The mean $A_{500}$ of three dilutions was calculated for each layer. The log ($A_{500}$ of the organic layer/$A_{500}$ of the aqueous layer) yielded logP. For compounds with very low lipophilicity (logP < -2.3), the volume of the octanol and aqueous layers was increased from 100 to 125 µL, and the absorbance of the octanol layer was directly measured without additional dilution (3:1:1 methanol/octanol/Tris). This procedure was repeated a minimum of four times per conjugate to calculate the mean logP and standard error. All absorbance measurements used were within the linear range of the instrument.

**Peptide stability in human serum:** Peptides at a final concentration of 30 µM in 100 % human serum (human male AB plasma, Sigma) were incubated at 37 °C. Aliquots of 50 µL were extracted at 0 min, 30 min, 90 min and added to 80 µL of 1 M HCl to stop the degradation process. The samples were first filtered then analyzed by RP-HPLC (flow: 1 mLmin⁻¹; gradient: 5-95 % B in 30 min; B = 0.1% TFA in acetonitrile).

**Mitochondrial isolation:** Mitochondria were isolated from mouse livers as previously described. All steps of mitochondrial isolation were performed at 4 °C. Mice of no particular breed or sex were sacrificed by cervical dislocation. The livers were collected into Isolation buffer (10 mM Tris-MOPS, 1 mM EGTA/Tris, 300 mM mannitol; pH 7.4), cut and washed in homogenization buffer, and homogenized by 3–4 strokes of a Potter homogenizer at a ratio of 15 mL of buffer/liver. The homogenate was centrifuged twice at 1300 g for 10 min to remove cell debris, red blood cells, and nuclei. Following this, the supernatant was divided into 1-2 mL aliquots and centrifuged at 10,000 g for 10 min to obtain the mitochondrial pellet. The pellet was resuspended in a total of 1 mL Isolation buffer, which was then divided into two and centrifuged at 10,000 g for 10 min. The pellets were resuspended into 1 mL Isolation buffer. The mitochondrial protein concentration was determined using the BCA kit. For all subsequent experiments and respiration
studies, the isolated organelles were diluted in Experimental buffer (125 mM KCl, 10 mM Tris/MOPS, 0.1 mM EGTA/Tris, 1 mM Pi (KH2PO4, pH 7.4). Respiration of isolated mitochondria was determined by measuring the mitochondrial respiratory control ratio using a Clark electrode. The ratio of oxygen consumption in experimental buffer in the presence of substrates (e.g., malate, glutamate) before and after the addition of ADP was calculated to ensure functional mitochondria prior to use in experiments.

**Relative uptake in isolated mitochondria:** All treatments following mitochondrial isolation were performed at room temperature in experimental buffer (see 53) in the presence of 5 mM glutamate and 2.5 mM malate. The relative uptake was measured using a BD FACSCanto flow cytometer equipped with a 488 nm Argon laser (BD Biosciences). Mitochondria were selected based on MitoTracker green staining (200 nM, 15 min, Ex 488 nm, Em 516 nm), and the debris was excluded by selectively gating this population using light-scattering properties in the SSC and FSC modes. This gate was fixed for all subsequent analyses of samples that used the same isolated mitochondria stock. The concentration of mitochondria was determined by measuring the protein content using the BCA assay (Pierce). Mitochondria (2 nM protein) were treated with peptides (1 μM) in experimental buffer (15 min; room temperature). The median of the fluorescence intensity of each sample was measured. Immediately after acquisition, heparan sulphate (HS) was added (20 μg/mL; 2 min; room temperature) in order to competitively bind membrane-bound peptides that are electrostatically interacting with the anionic lipids of the mitochondrial membrane. The HS treated samples were then reanalyzed and the median of the fluorescence intensity was recorded. The percent of peptide displaced from the membrane by HS, as displayed in Figure 5 (inset), was generated by the following calculation:

\[
\frac{[\text{Florescence Signal without HS} - \text{Florescence Signal with HS}]}{[\text{Florescence Signal without HS}]}\]
These values were normalized to the HS bound to Tat, since this peptide has been shown to be excluded from the organelle by several methods (see references\textsuperscript{[13]} and \textsuperscript{[14]}). Figure 5 represents data from seven independent experiments from three different isolations.

**Circular Dichroism (CD).** CD measurements were made using a Jasco J-720 spectropolarimeter. All samples were prepared using 10 mM Sodium Phosphate buffer at pH 7.4 with 30 mM sodium dodecyl sulfate (SDS) and 25 μM peptide concentrations. CD spectra were recorded from 250 nm to 190 nm in cells of 0.005 cm path length at room temperature. All spectra were scanned three times to improve signal to noise ratio, with 2-nm bandwidth, 50 nm min\(^{-1}\) scan speed, and 4-sec response time. The molecular ellipticity in deg cm\(^2\) dmol\(^{-1}\) was calculated based on the mean residue weight. D-(KLAKLAK\textsubscript{2}) was used as a positive control due to its ability to form secondary structure in the presence of SDS.\textsuperscript{54}

**Copper-binding studies– spectrophotometric measurements:** UV/vis analyses were performed on a Molecular Devices SpectraMax 5 spectrophotometer. Measurements were performed directly on the peptide sample (25 μM, 200 μL) in a quartz cuvette. Complex formation of the MPP-Cu(II) was monitored by adding small aliquots of a 0.02 M aqueous solution of copper sulfate to the peptide solution. The absorbance was measured 5-10 min after each addition. Spectra were recorded from 200 – 800 nm. In every case, the maximum absorption wavelength of the copper complex was observed at 630 nm.

**ICP-AES sample preparation:** Mitochondria were isolated from mouse liver and protein content was quantified as described above. Each sample (1 mL) contained 2 mg mitochondrial protein and experimental buffer (EB) (see above). Immediately following mitochondrial dilution, treatment was applied for 5-8 min at room temperature while inverting the sample tubes every minute. Mitochondria were then collected by centrifugation (10,000 g; 4 °C; 10 min). The pellet was gently
rinsed with 3 mL EB. Copper ions in the samples were dissolved in 1 mL concentrated HNO₃ for a minimum of 1 hour at room temperature. Samples were then diluted in DI H₂O (4 mL) before ICP-AES analysis.

**ICP analysis:** Elemental analyses for the metal ions were carried out on an Optima 7300 ICP-AES (PerkinElmer) spectrometer. Triplicate analyses of each sample were performed at a flow rate of 1.5 mLmin⁻¹.

**Fluorescence emission and quenching – Kq measurements:** Experiments were carried out using a Photon Technology International QM-1 fluorescence spectrometer. Measurements were performed in 5 mM MOPS, 0.1 mM Glycine (pH 7.4). Each sample (1 µM peptide; 500 µL) was placed in an acrylic cuvette with a path length of 1 cm. The excitation wavelength was set at 285 nm to selectively excite Tryptophan, and the emission spectrum was recorded from 300 to 450 nm. Excitation slit width varied from 2-4 nm, and emission slit width was in the range of 2-4 nm. Small aliquots of copper sulfate were added to the peptide sample in the cuvette and scanned 15 minutes after addition. Each point in the fluorescence titration curves represents the average of at least three measurements. Fluorescence titrations were performed at 20 – 25 °C. All curves were corrected for background fluorescence and dilution was taken into consideration.
3. Chapter 3 - Mitochondria Penetrating Peptides: Characterization and Model Cargo Transport

This chapter represents published work:


Data pertaining to the dye-panel (indicated in the Figure caption) has been collected by K.M. Stewart and has been included in this chapter to provide a complete picture of the project undertaken.
3.1. Introduction

Inefficient translocation of the cell membrane can limit the efficacy of drug molecules.\textsuperscript{55} However, the cellular uptake of bioactive molecules can be increased by synthetically modifying physicochemical parameters or through the use of delivery vectors. One class of delivery vectors, cell-penetrating peptides (CPPs), has been used with significant success in preclinical studies and is effective with a wide range of impermeable cargo molecules,\textsuperscript{56} including proteins,\textsuperscript{57} nanocrystals,\textsuperscript{58, 59} nucleic acids,\textsuperscript{60} and small-molecule drugs.\textsuperscript{61} Given the utility of CPPs, significant effort has been directed towards optimizing their properties and understanding their mechanism of action.

While CPPs are able to translocate the plasma membrane, most are not able to cross intracellular membranes; this limits organellar targeting of bioactive cargos. Mitochondria, for instance, are organelles implicated in many human diseases, such as diabetes,\textsuperscript{62} Parkinson’s disease,\textsuperscript{63} and cancer,\textsuperscript{64} and it is therefore critical to develop strategies to deliver therapeutics to this intracellular compartment. Indeed, efforts have been made to use CPPs to deliver cargo to mitochondria, but available sequences could not penetrate the organelle.\textsuperscript{31, 65} Instead, other transporters, such as lipophilic cations,\textsuperscript{66} sorbitol-based transporters,\textsuperscript{49} and oligoguanidinium vectors\textsuperscript{48} have been explored as molecular mitochondrial transporters; however, of the targeting agents tested, limitations in cargos delivery were reported.

Recently, the Kelley lab successfully engineered a set of CPPs that exhibit efficient cellular uptake as well as selective mitochondrial localization. These mitochondria-penetrating peptides (MPPs)\textsuperscript{46} feature alternating basic and hydrophobic amino acids and were systematically engineered to elucidate the exact features needed for mitochondrial localization. These peptides appear to enter cells via a direct mode of uptake and bypass endocytic uptake, thereby avoiding endosomal/lysosomal sequestration that would prohibit their ability to accumulate into mitochondria. The study of a family of sequences with varying charges and
lipophilicities revealed that peptides must meet a minimal charge-dependent lipophilicity threshold for mitochondrial localization. Indeed, a critical balance of charge and lipophilicity was shown to be required for the uptake across mitochondrial membranes.

Obtaining a broader understanding of the essential characteristics of MPPs is critical for their development as delivery vectors. Open questions remain about these peptides, including: 1) is an alternating hydrophobic-cationic motif necessary for mitochondrial localization and cargo transport; and 2) must cargos possess a particular level of polarity, charge, or hydrophobicity for effective transport? The answers to these questions would guide the application of these peptides in biology and medicine. While early studies provided a direction for MPP design, a more detailed understanding of their physiochemical properties is necessary to better optimize them as molecular delivery agents for diverse chemical cargos.

Herein, we describe a series of studies that characterize the MPP scaffold and how its exact structure correlates with mitochondrial localization. We also tested the effect of various model cargo molecules on the intracellular accumulation of MPPs. By addressing the effects of sequence, charge distribution, lipophilicity and cargo on the localization of MPPs, we gained information on the properties that permit access into the mitochondria and provided evidence that MPPs are suitable mitochondrial delivery vectors.

3.2. Results and Discussion
Most cellular delivery vectors only traverse the plasma membrane. MPPs, in contrast, need to additionally navigate through the cellular milieu in order to target mitochondria, and thus need to infiltrate membranes with distinct chemical properties relative to the plasma membrane. While CPPs can successfully deliver numerous agents intracellularly, it is anticipated that more limitations will exist for mitochondrial delivery with MPPs than for CPP-mediated delivery. For example,
since the organelle is associated with a strong negative membrane potential and a
double membrane — one being a rigid hydrophobic barrier — some cargo could be
ultimately prevented from entering. Thus, it is important to provide evidence of
successful MPP-mediated small-molecule delivery with cargos ranging in
physicochemical properties including charge, size, and hydrophobicity.
Furthermore, determining the peptide features that are critical for mitochondrial
entry can be used to engineer sequences for more difficult delivery applications.

3.2.1. Effect of sequence and charge distribution on MPP localization
MPPs previously found to penetrate mitochondria feature a motif with alternating
cationic and hydrophobic residues. CPPs (e.g., the Tat peptide from the HIV
transactivator protein with the sequence YGRKKRRQRRRP) that are not able to
access mitochondria,\textsuperscript{31, 65} however, are typically composed of stretches of cationic
residues. This observation raises the question of whether exclusion of highly basic
peptides results in part from the energetic penalty that would result if large patches
of cations need to cross a highly hydrophobic membrane. This hypothesis is testable
by altering the charge distribution within MPPs to produce stretches of positive
charge while keeping the overall sequence composition and charge the same.
Alterations in localization as a function of charge density would point to this effect
as an important one in facilitating mitochondrial access. To address how charge
distribution affects the intracellular accumulation of MPPs, an octamer with four
charged residues previously shown to primarily localize to mitochondria (1a) was
scrambled to generate three peptide analogues (1b–d) with an increasing number
of adjacent charges (Figure 4A). The peptides were labeled with thiazole orange (to)
for imaging and were assessed for mitochondrial localization in unfixed HeLa cells
by using confocal fluorescence microscopy (Figure 4B). Interestingly, while the
peptides with one, two, and three adjacent charges (1a, 1b, 1c, respectively)
ehibited mitochondrial localization, a sequence with four adjacent cationic
residues (1d) instead exhibited primarily nuclear and cytoplasmic localization. This
(continued on the following page)
Figure 4. Effect of sequence on MPP localization.

A. Peptide sequences and charge distribution. B. Images revealing intracellular localization obtained by confocal fluorescence microscopy in live HeLa cells. HeLa cells were incubated with 1-5 μM of each peptide for 45 min at 37°C. Single cells are shown for clarity. C. Colocalization images of 1a and 1d with Mitotracker and the corresponding Pearson’s Coefficient (Rr) values. D. Retention times of 1a and 1d analyzed using reversed phase HPLC monitored at 500 nm. E. Relative uptake of 1a and 1d as measured using flow cytometry. Scale bars represent 10 mm.

was further confirmed quantitatively in studies in which MitoTracker — a mitochondria-selective dye — was colocalized with the peptides, and a Pearson’s coefficient (Rr) was calculated to quantitate the degree of mitochondrial localization. Rr values of +0.4 (-0.1) for 1a and 0.0 (-0.1) for 1d were obtained (Figure 4C). These values confirm that the proximity of the four charges without intervening hydrophobic residues is not compatible with passage through the mitochondrial membranes. Furthermore, the uptake of these peptides into isolated mitochondria was also monitored (Figure 5), further confirming the trend obtained from whole cells.

Another possible explanation for the different intracellular localization observed for the parent peptide 1a, which has an alternating hydrophobic and cationic structure, compared to peptide 1d, which has clustered charges and hydrophobic residues, could stem from a change in physiochemical properties that resulted from the sequence scrambling. For example, if the overall lipophilicity of peptide 1d was reduced, this could explain its exclusion from mitochondria. To address this possibility, the logP values and the HPLC retention times of peptides 1a and 1d were measured, and, interestingly, the opposite trend was observed. Peptide 1d appears more lipophilic than 1a, with a longer retention time on reversed-phase HPLC (Figure 4D) and a logP value of -1.8 (±0.1) for 1d versus -2.4 (±0.1) for 1a. It appears that the effect of clustered polar residues is overwhelmed by the clustering of hydrophobic residues, which then makes the compound more lipophilic overall. Thus this trend points to charge distribution as an effect that alters the amount of lipophilicity required for a compound to access mitochondria. Yet another explanation is that this could be due to changes in structure caused by the sequence modifications, but from circular dichroism data it was determined that none of the peptides displayed defined secondary structure (Figure 6), thus this is not likely.

The cellular uptake of compounds 1a and 1d was also measured to determine how charge distribution affects this aspect of MPP behavior. Prior studies
Figure 5. Relative uptake of peptides in isolated mouse liver mitochondria (main graph).

Inset: percent binding of peptides to heparin sulfate (HS) with respect to Tat (calculation: [(uptake without HS – uptake with HS)/(uptake without HS)] normalized to binding of Tat to HS). Tat and k₃ were used as negative controls. The consistent amount of HS binding for the non-mitochondrial localizers indicates that these conjugates are primarily surface-bound (organelle surface) and do not penetrate the organelle, further confirming the whole cell localization images (Figure 4; 1d displayed nuclear and cytoplasmic staining).

of MPPs indicated that sequence 1a displays efficient uptake comparable to the Tat peptide.\textsuperscript{46} Interestingly, peptide 1d exhibited a small but significant increase in uptake relative to 1a (Figure 4E). Arginine-rich peptides were previously shown to exhibit uptake that increased according to the number of these charged residues present, due to increased membrane binding and direct transport,\textsuperscript{67} and thus these observations with MPPs likely reflect the same effect. However, while clustered arginines increase transport across the plasma membrane, they decrease transport into mitochondria.

Overall, in addition to revealing the impact of charge distribution on the organellar specificity of MPPs, the results described here also rule out the dependence of mitochondrial localization on the alternating scaffold as previously shown to be necessary.\textsuperscript{44,46} Rather, the observation that peptides with two and three adjacent charges can still penetrate mitochondria suggests that the overall lipophilic and cationic content are the important determinants of this intracellular localization.

3.2.2. Investigation of MPP-mediated transport of hydrophobic, polar, and charged cargos
For CPPs, it has been previously reported that a tethered molecule can affect the overall efficiency of cellular uptake\textsuperscript{68} and mechanism of entry\textsuperscript{69} of the peptides. Furthermore, we have shown that small changes in physiochemical properties of the MPP result in variations in subcellular localization.\textsuperscript{46} Thus, it is critical to assess how cargo molecules affect the intracellular localization of conjugates to determine whether there are limitations to delivery applications that can be pursued. To address this issue, a variety of N- and C-terminal cargos were appended to MPPs. Terminal tripeptides were used to generate cargos with varying properties, and in addition, a series of dyes and bioactive small molecules were used to examine the effect of changes in charge and polarity.
Figure 6. Circular dichroism spectra of peptides 1a, 1d, and d-(KLAKLAK)$_2$.

The molar ellipticity based on the mean residue weight is plotted on the y-axis to account for the differing lengths of the peptides. The diasteromers 1a and 1d do not display secondary structure when compared to d-(KLAKLAK)$_2$, a known α-helical peptide.

**Transport of highly lipophilic model cargos**

Increasing the overall lipophilicities of mitochondrially localized small molecules has been shown to directly affect accumulation into the organelle.\(^{70-72}\) Also, MPPs possessing more lipophilic character have been shown to exhibit more specific mitochondrial localization.\(^{46}\) While this property can enhance delivery to mitochondria, high levels of lipophilicity could at some point limit cellular uptake by causing ineffective transport through the plasma membrane and sequestration into this hydrophobic environment. Moreover, lack of water solubility could impede the use of highly lipophilic conjugates.

In order to study the behavior of MPPs carrying lipophilic cargos, a peptide panel was synthesized by increasing the hydrophobicity of a parent sequence by systematically adding the unnatural, hydrophobic amino acid cyclohexylalanine (Fx) to the C terminus (Figure 7A) of a to-modified sequence (2). The logP values of three compounds featuring one, two, or three appended Fx residues (2-Fx, 2-Fx\(_2\), 2-Fx\(_3\)) were measured and found to span -0.5 to +1.4 (Figure 7B). Interestingly, the localization profiles for these peptides in live HeLa cells did not differ significantly from that of the parent sequence (Figure 8A); this suggests that peptides with an overall +4 charge that maintain lipophilicity with logP values up to +1.4 can still penetrate the plasma membrane and exhibit mitochondrial localization. It is noteworthy, however, that cellular uptake was diminished as the hydrophobic residues were added (Figure 8B); thus further increases to peptide lipophilicity would likely decrease cellular accumulation to an unacceptable level.

**Transport of model N-terminal cargos of variable polarity and charge**

A panel of molecules consisting of three different fluorophores conjugated to the N terminus of a MPP was also studied as a means to assess how cargo polarity and charge affected intracellular localization (Figure 7A). The use of fluorophores for
(continued on the following page)
Figure 7. Schematic and LogP of MPP-cargo conjugates.
(A) Schematic of parent peptide appended to small molecule fluorophores, to (2), fl (3), and rox (4) as N-terminal cargos; lipophilic cargos, Fx – Fx₃; tripeptides k₃, s₃, f₃, FN₃, y₃ as C-terminal cargo mimics, and biotin, trolox as C-terminal small molecule cargos. Amino acids in the natural (L)-configuration are designated with a capital letter, and (D)-configuration with lower-case letters. (B) Experimental logP values for C-terminal cargo conjugates and (C) for N-terminal small molecule fluorophore conjugates.

this study was advantageous as this allowed for direct visualization of intracellular distribution for a range of small molecule–MPP conjugates in live cells by using confocal fluorescence microscopy. In addition to conjugate 2, which was labeled with the cationic dye to, the MPP was also labeled with the cell impermeable, anionic dye 6-carboxy-fluorescein (fl) to generate conjugate 3, and the lipophilic carboxy-x-rhodamine (rox) to generate conjugate 4. The measured logP values for these conjugates suggest that while 2 and 3 possessed a similar degree of lipophilicity (logP equal to -1.5 and -1.4, respectively), conjugate 4 is much more lipophilic with a logP of -0.6 (Figure 7C).

Each MPP–dye conjugate was incubated with HeLa cells and the localization was compared to that of MitoTracker. As shown in the representative cells in Figure 9, all three conjugates displayed predominant mitochondrial accumulation; this suggests that the MPP is able to both promote access to the cell as well as control the subcellular distribution of the conjugate, independently of the fluorophore. Interestingly, the MPP was sufficient to deliver fl and rox, despite the fact that these fluorophores are negatively charged and zwitterionic, respectively, at physiological pH. Prior work has indicated that certain lipophilic weak acids can enter mitochondria, but in general the transport of this class of molecules into the organelle is unfavorable. The fact that the large negative membrane potential (-170 mV) of mitochondria did not impede the import of the anionic dye indicates that the MPPs are able to compensate for unfavorable electrostatics of cargo. In addition, these results suggest that MPPs could successfully deliver bioactive small molecules with similar sizes and physiochemical properties to the fluorophores in this panel; this demonstrates a potential application for these organelle-specific molecules.

**Transport of model C-terminal cargos of variable polarity and charge**

To further study the effects of cargo molecules on the intracellular localization of MPPs, different tripeptides varying in structure, size, and lipophilicity were tethered to the C terminus of the to-labeled MPP (2) as small molecule cargo mimics (Figure
Figure 8. Transport of C-terminal lipophilic cargo.

A. Intracellular localization of compounds 2 – 2-Fx₃ in unfixed HeLa cells with corresponding DIC images. Cells were incubated with 1-3 µM peptide for 35 min at 37 °C. Scale bar represents 10 mm. B. Relative cellular uptake of compounds 2 – 2-Fx₃ in viable HeLa cells (non-PI positive) after treatment with peptides at 5 µM for 60 min. Median fluorescence is displayed for each peptide (n=3) with standard deviation and statistical significance, P<0.05 (*).

7A). This serves as a convenient synthetic model system to evaluate the extent of localization shifts due to the introduction of a cargo molecule. The tripeptides used in this study were chosen to span a large range of lipophilicities, with the most hydrophobic being cyclohexylalanine (2-Fx₃), as discussed above, and the most hydrophilic being lysine (2-k₃). The proteolytic stability of all C-terminal tripeptides conjugates in the natural (L) configuration was investigated by co-incubating the peptides with human serum over a period of 90 min (see Materials and Methods for more details), and assessed by HPLC (Figure 10). All of the conjugates were stable except the one bearing three (L)-lysines. Thus, the unnatural (D)-lysine tripeptides was used for this construct. From their intracellular localization profiles (Figure 11A), it is evident that MPPs are able to promote the uptake of different small-molecule cargos as long as they meet the required degree of lipophilicity. The conjugate with three terminal lysines (2-k₃) has a logP equal to -2.8, which falls below the minimal threshold for mitochondrial access (log P -2.5, taken from ref. 46), and was therefore unable to access this organelle. The remaining five conjugates with varying logP values (Figure 7B) successfully accessed this organelle; this suggests that the MPP’s properties dominated those of the full conjugates.

For further proof-of-principle that MPPs can be used to deliver diverse cargos, two bioactive small molecules, biotin and trolox (a water soluble analogue of vitamin E), were tethered to 2 via a primary amine of an additional C-terminal lysine residue (Figure 7A). The successful targeting of these molecules provides evidence for the ability of MPPs to facilitate the translocation of additional cargos to this important organelle (Figure 11A).

3.3. Conclusions

In order for MPPs to be effective as a mitochondrial delivery vectors, it is essential to generate a concise understanding of the versatility for these peptides in the delivery of various molecules into the mitochondria of living cells. Notably, by
Figure 9. Effect of N-terminal cargo on MPP localization.

HeLa cells were incubated for 60 min with 2 μM 2, 20 μM 3, or 2 μM 4 in serum-free MEM; 50 μM Mitotracker CMXRos (2, 3) and 100 μM Mitotracker green (4) was introduced 15 min prior to imaging. For each conjugate, a suitable Mitotracker was chosen to minimize bleed-through into the peptide channel. Only single cells are shown for clarity; see supporting information for additional images. The green channel corresponds to the peptide and the red channel to that of mitotracker. Overlaid and DIC images are also shown for each cell. Scale bar represents 10 μm. Data collected by K.M. Stewart.

Figure 10. Stability of C-terminal tripeptide panel.

The peptide stability of all constructs synthesized with tripeptides in the (l)-configuration were assessed by HPLC. Of the screened peptides, only the construct with the three lysine residues displayed degradation in human serum.

**Figure 11. Transport of structurally diverse C-terminal cargo.**

A. Intracellular localization of terminal tripeptides as C-terminal cargo mimics and small molecules biotin and trolox. HeLa cells were incubated with 1-3 μM of each peptide for 60 min at 37 °C to give the intracellular localization of the MPP-cargo mimics. Scale bar represents 10 μm. B. Relative cellular uptake of peptides (3 μM peptides for 90 min) in live HeLa cells. Median fluorescence is displayed for each peptide (n=3) with standard deviations.

varying the number of adjacent charges in a step-wise manner, it was evident that an alternating hydrophobic/cationic sequence is not critical to allow for these charged peptides to access the mitochondria; however, a sequence displaying more than four adjacent positive charges exhibits altered organellar specificity. This suggests that unfavorable energetics prevent the charge cluster from crossing the hydrophobic mitochondrial membranes. Also, it was demonstrated that these organelle-specific peptides are sufficient to deliver negatively charged and zwitterionic small molecules. In addition, attaching tripeptides to the C terminus of the MPP sequence provided further evidence that the MPP-mediated delivery is contingent on the overall degree of lipophilicity of the conjugate, which must fall above the threshold (logP= -2.5). Overall, the systematic studies outlined above allowed for a more comprehensive examination of the role of charge and lipophilicity in MPP localization and verified that these lipophilic yet cationic peptides are a promising tool for mitochondrial targeting applications.
4. Chapter 4: MPP-Mediated Delivery

Applications: Mitochondrial targeting of Copper Ions
4.1. Introduction

4.1.1. Metal Ions in Mitochondria
Transition metal ions play a critical role in mitochondrial function. Zinc, iron, manganese and copper are important cofactors for metalloenzymes and metalloproteins in the organelle. Zinc is associated with metalloenzymes involved in protein import as well as mitochondrial metabolism processes including alcohol oxidation and leucine biosynthesis. Iron is found in mitochondrial FeS centres and hemoproteins while the only known role of manganese is in the manganese superoxide dismutase (MnSOD2), a scavenger of superoxide anions. Copper is required for the biological activity of two mitochondrial enzymes, cytochrome c oxidase (COX) (Complex IV of the ETC) and the Cu,Zn superoxide dismutase (Cu,ZnSOD1).

4.1.2. Copper toxicity and transport
The redox properties that make copper an ideal cofactor in living systems also render it toxic. Copper ions can participate in Fenton-like reactions that generate damaging ROS. Consequently, cells have evolved pathways to distribute these ions to different compartments and to ensure that it is only transported as a complex, inert species. In fact, copper transporters, such as metallothioneins and copper chaperones, bind copper very tightly with binding constants in the pico to femtomolar range and maintain the total cellular copper levels in the 10-100 μM range.

4.1.3. Copper transport: cellular and mitochondrial
Safe levels of intracellular copper pools are maintained by controlled uptake and distribution mechanisms. Copper enters the body from dietary sources and is excreted by the biliary system. Dietary copper is absorbed at 30-50%, and is distributed from the liver to other parts of the body in the plasma as a bound species, forming complexes with proteins such as ceruloplasmin and albumin. In
fact, copper is also found coordinated to amino acids, primarily histidine. Studies have shown that human albumin forms a ternary complex with copper-histidine, which may play a role in the transport of copper across cell membranes.80

Copper levels must be tightly regulated at all stages of its transport in the plasma as well as within cells and organellar compartments. Cuprous ions are transported into cells by Ctr1 permeases that are found across cell membranes in all tissues.81 Once in the cell, copper is distributed by two transporters: ATOX1, which shuttles Cu(I) to the Menkes and Wilson P-type ATPases (ATP7A and ATP7B, respectively) and CCS, the copper chaperone for SOD1. Evidence of cuproenzyme assembly within the mitochondria (e.g. two mitochondrially encoded COX copper-binding subunits) indicates that transport pathway(s) must exist for the delivery of copper into the organelle.82 In general, ions are able access the mitochondria by initially passing through the OMM by virtue of the TOM40 and porin channels, and are then transported across the compact IMM by translocases. Copper needed for mitochondrial proteins originates from a pool in the organelle’s matrix. This dynamic pool of copper is comprised of an unidentified low molecular weight copper complex.83 Copper homeostasis in mammalian cells is maintained by several regulatory steps which are believed to link cellular and mitochondrial copper pools.82 At elevated cellular copper levels, the number of Ctr1 permeases is reduced by endocytosis and degradation steps to prevent subsequent uptake.84 Furthermore, the localization of ATP7A and ATP7B is dependent on the intracellular copper levels; such ATPases are responsible for transporting copper to the cell periphery or cytoplasmic vesicles from the trans-Golgi network at elevated levels.85 Thus, the activation and abundance of copper-dependent proteins (e.g. Sod1) is directly influenced by intracellular copper levels.86

4.1.4. Consequences of disruption in copper homeostasis
Copper deficiency and overload have severe clinical outcomes due to dysfunction of many copper-dependent enzymes. A disruption in copper homeostasis during fetal
stages leads to perinatal mortality, severe growth retardation, and neurodegeneration.\textsuperscript{9} For example, dysfunction in the aforementioned ATP7A and ATP7B P-type ATPases will not only affect the metallation of cuproenzymes, will also affect the removal of excess copper leading to strikingly different clinical phenotypes.\textsuperscript{87} Defects in ATP7B lead to Wilson’s disease, a disorder caused by toxic levels of copper that accumulate in the liver and brain due to defective copper export to the bile.\textsuperscript{87} Patients with this disorder suffer from hepatic cirrhosis and neuronal degeneration. In addition, ATP7B transfers Cu(I) to the lumen of the trans-Golgi network to be incorporated in ceruloplasmin for transport within the body. Also, a loss of function mutation in the \textit{ATP7A} gene causes the copper deficiency disorder termed Menkes disease. Due to defective copper transport from enterocytes into the circulating pools, tissues such as the brain become deficient in copper.\textsuperscript{88} This causes progressive neurodegeneration associated with psychomotoric retardation and connective tissue abnormalities.\textsuperscript{80}

\textbf{4.1.5. Copper’s role in COX}

One of the critical roles of copper in living systems is in cellular respiration. COX is a key catabolic enzyme found on the IMM that terminates the respiratory chain by transferring electrons from cytochrome \textit{c} to molecular oxygen.\textsuperscript{89} The reduction of molecular oxygen to water requires the redox activity of the two copper centres found in COX (Figure 12). The subunits necessary to form the catalytic core of COX (COX1 – COX3) are encoded by the mitochondrial genome and are assembled by a series of IMS copper-binding proteins (\textit{i.e.} Cox17, Cox19, Sco1, Cox11).\textsuperscript{90, 91} Due to the critical role of COX in cellular respiration, COX deficiency has been reported to be the most common cause of respiratory chain defects, leading to a number of different clinical phenotypes, including Leigh Syndrome and cardiomyopathy.\textsuperscript{8}

\textbf{4.2. MPP-mediated delivery of copper to the mitochondria}

The role of copper in mitochondria, and the pathologies that appear to result from disruption of these roles in the organelle, make the delivery of this metal an
Figure 12. The mitochondrial electron transport chain (ETC).

The reduced electron donor NADH transfers 2 electrons to the first of the ETC complexes imbedded in the IMM, NADH-CoQ reductase, and achieves reduction of coenzyme Q10 (CoQ). Electrons are transferred within complex I via flavin mononucleotide and a series of Fe-S clusters. Complex III, or CoQH2-Cytochrome c reductase, oxidizes ubiquinol and reduces two cytochrome c molecules. The fourth and final complex in the ETC, Cytochrome c oxidase (COX), transfers two electrons to molecular oxygen to form water. Coupled with this process is the continuous pumping of protons from the matrix to the IMS by proton pumps. Ten protons are pumped into the IMS for each NADH molecule used in the ETC, generating an electrochemical gradient that is used by ATP synthase to synthesize ATP from ADP and inorganic phosphate (not shown). Adapted from H. Lodish et al. Molecular Cell Biology (5th ed). WH Freeman and Company: New York, NY. 2003; pp. 304-330.
important target for therapeutic applications. At specific levels, copper’s redox properties render it either lethal or essential to the cell. Copper's pro-oxidant properties can be utilized for cancer chemotherapy strategies. For example, a unique ‘copper-cage’ that releases the bound metal ion upon UV activation has been designed.\textsuperscript{92} This light-activated release mechanism enables the specific targeting of copper to the affected tissue/cells, avoiding metal toxicity in other areas. Alternatively, copper delivery can be used therapeutically to supplement deficient cells to prevent or rescue cuproenzyme inactivity. In fact, copper-(histidine)\textsubscript{2} complex treatments have been previously administered to Menkes disease patients.\textsuperscript{93} While these treatments produced promising results, long-term administration of 100-900 \textmu g copper per day may lead to severe toxicity.\textsuperscript{94} Therefore, the selective delivery of copper to specific intracellular compartments where its activity is required is beneficial as this would reduce the effective dose and reduce potential side-effects. Targeted copper delivery is particularly useful for the rescue of mitochondrial COX deficiency in the ETC. COX deficiency is mainly caused by a dysfunction in its assembly, which requires the insertion of several cofactors (\textit{i.e.} heme groups and copper centres) by accessory proteins critical for its proper catalytic function. Thus, developing a system that can achieve efficient delivery of copper to mitochondria is a worthwhile objective.

4.3. Results and discussion

4.3.1. Engineering a copper-binding MPP

MPPs have demonstrated both efficient delivery as well as specific mitochondrial localization for various cargo mimics (see Chapter 3 and \textsuperscript{97}). The MPP characterization studies listed in Chapter 3 provided a better understanding of the limitations of these novel molecules for the delivery of varying model cargos; including the addition of tripeptides. Given that certain peptide sequences are known to bind copper at physiologically relevant levels, the use of amino acids appeared to be a straightforward path to generate mitochondria-penetrating copper
delivery vehicles. In an effort to engineer a copper-binding MPP, several known copper-binding peptide sequences with the desired physiochemical properties were chosen and conjugated to the C terminus of a suitable MPP to generate a panel of peptides (Table 1). The MPP (Fxr)₄ was chosen for its overall +4 charge to compensate for the increased lipophilicity and length of the added copper binding motifs (Figure 13).

Most of the sequences listed in Table 1 are rich in histidine and proline amino acids, which offer an imidazole-rich environment for the coordination of different metal ions. Indeed, H and H/P-rich sequences (e.g. H₆ or GPHHG) have been used in metal ion-affinity chromatography for protein purification. In addition, Cys residues can also coordinate copper very well, as seen in the CPDXC (CXXXC) copper-binding motif of the Sco1 copper chaperone. Thus, one of the MPP constructs was engineered to have a CPDICPG motif at its C-terminus.

Of the peptides synthesized, the HGGGW binding motif has been the most characterized in the literature. The construct with this motif was designed based on the copper-binding sequence of the Prion protein (PrP). The amino-terminal region of the human PrP contains four non-structured octapeptide repeats of the sequence (PHGGGWGQ)_4. These repeats have high affinities for divalent metal ions.

**Table 1. Engineering a copper-binding MPP**

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Full peptide construct</th>
<th>Copper motif</th>
<th>binding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>(Fxr)₄-GGHGLHH</td>
<td>GGHGLHH</td>
<td></td>
<td>[·]</td>
</tr>
<tr>
<td>6</td>
<td>(Fxr)₄GGHGGGW</td>
<td>GGHGGGW</td>
<td></td>
<td>[⁹⁹]</td>
</tr>
<tr>
<td>7</td>
<td>(Fxr)₄GGPHHG</td>
<td>GGPHHG</td>
<td></td>
<td>[⁹⁵-⁹⁷]</td>
</tr>
<tr>
<td>8</td>
<td>(Fxr)₄HPHH</td>
<td>HPHH</td>
<td></td>
<td>[¹⁰⁰]</td>
</tr>
<tr>
<td>9</td>
<td>(Fxr)₄-CPDICPG</td>
<td>CPDICPG (CPDXC)</td>
<td></td>
<td>[¹⁰¹]</td>
</tr>
</tbody>
</table>
Cu(II) in particular, and can bind up to four Cu(II) ions.\textsuperscript{99, 101} The X-ray crystallography structure of the copper-bound HG GG W has revealed equatorial coordination by the histidine imidazole, two deprotonated glycine amides, a glycine carbonyl as well as an axial water molecule that bridges the tryptophan indole.\textsuperscript{102} The MPP based on this binding sequence was synthesized with two additional Gly residues to act as spacers between the MPP and binding sequence, in an effort to reduce interference of the MPP’s on metal binding. While screening these constructs, one must keep in mind that the proposed copper delivery peptide must transport the metal while traversing multiple hydrophobic barriers – a challenge that other chaperones do not need to overcome. Thus, the MPPs with the altered C-termini must possess a high affinity to the metal, stability of the MPP-copper complex, peptide stability as well as organelle specificity.

4.3.2. Copper-binding and intracellular localization

The engineered constructs must display three main properties: (i) to bind Cu(II), (ii) to localize selectively to mitochondria, and (iii) to release the metal once it is in the organelle. Of these, the first two properties have been addressed in this study. Two copper-binding tests were used to verify metal binding. Firstly, an increase in absorbance due to coordination to copper ions was monitored at specific wavelengths (550 nm – 650 nm, based on previously reported studies)\textsuperscript{103}. Of all of the peptides screened, only the sequence having the Prion protein binding sequence (HG GGW) showed an increase in absorbance at 630 nm with the addition of CuCl\textsubscript{2} or CuSO\textsubscript{4} (Figure 14A). Secondly, copper binding was further confirmed by mass spectrometry (MS) and compared to that of the parent (Fxr)\textsubscript{4} sequence (Figure 14B). From the MS data, \textbf{6} exhibited significant metal-bound species while (Fxr)\textsubscript{4} did not. This suggests that the parent MPP is not interfering with the metal-binding activity of its C-terminal metal-binding motif and that the MPP itself is not coordinating the copper ions (data not shown). In addition, since the tethered copper-binding amino acids may interfere with the intracellular localization of the
Figure 13. Engineering copper binding peptide.

The parent MPP (Fx)\textsubscript{4} was chosen as the delivery agent due to its efficient mitochondrial localization as well as its positive residue content that will compensate for the additional lipophilicity of the C-terminal tail added. For the list of engineered peptide constructs, see Table 1. \textit{Fx}: cyclohexylalanine; \textit{r}: D-arginine; \textit{X}: residues comprising the metal binding sequence of interest.
Figure 14. Copper-binding to the engineered constructs.

A. Absorbance measurements of 6. The increase in absorbance at 630 nm is indicative of complex formation in the presence of copper. B. Metal binding assessed by mass spectrometry. Metal binding is evident from the 1924.1 amu peak that appears when copper is added to the peptide solution. The additional peak at 1899.1 amu needs additional studies to be identified.
parent MPP, the localization of to-labelled analogues in unfixed HeLa cells was monitored using confocal fluorescence microscopy. Conjugates 7 and 8 displayed no intracellular staining, conjugate 5 was primarily found in lysosomes and 6 selectively localized to the mitochondria (Figure 15). While the microscopy results were promising, they do not provide evidence for the metal-bound MPP; the MPP-Cu(II) complex may not access the organelle due to the change in secondary structure associated with metal coordination and may even release the metal prior to accessing the organelle. Nonetheless, these screens confirmed that 6 possesses both requirements of Cu(II) binding as well as mitochondrial localization and was therefore used for all subsequent studies.

4.3.3. Characterizing (Fxr)$_4$GGHGGGW as a mitochondrial copper delivery agent

To verify that 6 is a suitable mitochondrial copper delivery agent, its long-term stability, delivery capacity, as well as binding affinity were addressed. Ensuring peptide stability, particularly of the (L)-amino acids (designated in capital letters in the peptide sequences), is important for treatments in media supplemented with FBS for lengthy cell-based assays. To address the stability of 6, a to-labelled analogue was incubated in cell media (MEMα) supplemented with 2% and 10% FBS at 37 °C for a period of 5 days. Degradation was monitored by analytical-HPLC methods (see materials and methods for details) (Figure 16). The peptide construct was > 95 % stable in these conditions, suggesting that it is suitable for future cell-based assays.

The ability of 6 to bind copper and maintain stability have been confirmed; however, whether this peptide is able to transport copper to the organelle remains to be elucidated. Monitoring copper levels in mitochondria that are treated with MPP-Cu(II) would provide a direct means to answer this question. This can be achieved with the use of radiolabeled copper (e.g. $^{64}$Cu or $^{67}$Cu) and monitoring the level of radioactivity in isolated mitochondria fractions. Alternatively, a more straightforward approach is to directly quantify the absolute copper levels in
Figure 15. Localization profiles of 5, 6, 7.

Unfixed HeLa cells were treated with peptides 5, 6, 7 (3 μM; 45 min at 37 °C) and imaged by confocal microscopy (top panel). Localization of 6 (3 μM; 45 min at 37 °C) with and without the addition of CuCl₂ (1:5 peptide:CuCl₂) (lower panel). Scale bars represent 20 μm.
Figure 16. (Fxr)$_4$GHHGGGW stability in cell media.

The HPLC trace illustrates the stability of a to-labeled analogue of 6 (absorbance at 500 nm). The peptide was incubated in MEMα plus 10% FBS (37 °C) for 5 days.
isolated mitochondria by elemental analysis techniques, such as inductively-coupled plasma atomic emission spectroscopy (ICP-AES) (Figure 17). This type of emission spectroscopy uses the ICP to excite atoms and ions to emit electromagnetic radiation at wavelengths characteristic of each element. The intensity associated with the emission is indicative of the concentration of the elements within the sample (Figure 18). Indeed, this method, with a detection limit of 0.004 mg/L for copper, provided direct measurements of mitochondrial copper content after treatments of isolated mouse liver mitochondria with the MPP-Cu(II) complex. Mitochondrial isolation was performed based on published procedures immediately following tissue harvest from mice that were sacrificed by cervical dislocation. To ensure that functional mitochondrial were obtained, respiration was measured after isolation using a Clark electrode (see Materials and Methods for details). Preliminary results of copper uptake, however, do not indicate significant differences in treatments with the copper-bound 6 relative to untreated, CuSO₄ treated, and MPP treated samples (Figure 19). The observed data may be due to several factors, including a weak binding affinity to the metal; this would cause the MPP to release the copper prior to accessing the mitochondria.

Successful delivery of cargo is directly affected by the transporter’s binding affinity. Naturally found chaperones ensure binding to their ligand to avoid its loss by competition with other species. Since copper is toxic, even at very low concentrations, cuproproteins have very strong binding affinities (pico to femtomolar) to control homeostasis. In order to maintain a metal-bound species, a transporter with a high binding affinity to copper ions would be required. It is critical to note that the MPP’s affinity for copper may limit the use of this system. If there is strong binding, free MPPs will be able to sequester copper ions from other copper proteins and cause unwanted side effects. Furthermore, tight binding will also prevent the delivery vector to release the metal ion once in the mitochondria. On the other hand, if the binding affinity is weak, cuproproteins will be able to
Figure 17. Inductively coupled plasma atomic emission spectroscopy (ICP-AES).

First, the aqueous sample is pumped into the instrument, where high velocity argon gas atomizes the sample into a fine mist. The sample contents enter the hot plasma (~ 7000 °C), ionizing the elements and causing them to excite and emit light with characteristic wavelengths. The emitted light travels through a spectrometer slit onto a grating, separating the wavelengths specific for each element which are detected by photomultiplier tubes. The electronic signal from the detectors is converted into concentrations relative to reference standards. Adapted from U.S. Geological Survey, http://minerals.cr.usgs.gov/gips/na/5process.html.
deprive the MPP from the metal or the peptide may release the copper ion prematurely due to the instability of the complex. The binding affinity of the MPP-Cu(II) complex can be determined by monitoring the quenching of tryptophan fluorescence (285 nm excitation, 355 nm emission) with the addition of the metal (CuSO$_4$). In the prion protein, tryptophan indole fluorescence is quenched by a copper-bound axial water molecule that bridges the metal to the indole moiety. Using this method, the binding constant for this construct was calculated to be in the μM range ($K_D = 1.6 \mu M \pm 0.3$) (Figure 20). Several studies on the copper-binding sequences in the prion protein have reported binding affinities in this range (nM to μM). While μM binding affinity is sufficient for this cell-surface glycoprotein, this might be too weak within the cellular environment and provides a possible explanation for the inability of the MPP to deliver copper, as observed in the ICP data. As previously described in Chapter 3, mitochondrial uptake of these cationic MPPs is believed to follow a direct, potential-driven, energy independent mechanism. Thus, threading through these hydrophobic membranes may introduce a large enough energy barrier to the 6-Cu(II) complex, forcing the peptide to release the metal first and then enter the negative compartment.

Alternatively, other factors, or a combination of factors, may be playing a role in the observed lack of copper uptake. One possibility is that the functional mitochondria used in the ICP studies are able to maintain copper homeostasis and, therefore, can export the copper ions in response to elevated levels in the matrix. While this is unlikely in the conditions used, it is possible that the isolated organelles do re-establish their physiological copper levels and prevent the increased uptake of the metal. On the other hand, the unobserved increase in copper levels does not rule out the possibility that this copper-delivery system is indeed working, however, only at very low and undetectable levels. If this is the case, this copper-binding peptide would not be useful for this approach.
Figure 18. Schematic of MPP-mediated copper ion delivery.

MPP-Cu²⁺ can be administered to whole cells or isolated mitochondria and copper levels can be monitored in isolated mitochondrial fractions using ICP-AES elemental analysis.
Figure 19. ICP-AES analysis of isolated mitochondria treated with 6-Cu(II).

Mitochondria samples (2 mg mitochondrial protein per sample) were treated with conjugate 6 (5 μM; 8 min at room temperature). Standard deviation is shown. The data do not significantly differ between the samples.
Figure 20. Measuring the affinity of 6 to Cu(II) ions.

The binding affinity of conjugate 6 to Cu(II) was measured by monitoring tryptophan fluorescence quenching with the addition of CuSO₄ aliquots. Background fluorescence and dilution were taken into consideration when analysing the data. The data points (n = ≥4) were fit by a non-linear regression analysis using the GraphPad Prism curve fitting software. From this data, a Kd value of 1.6 (± 0.3) was obtained.
4.4. Conclusion

Taken together, the data reported summarizes the design and synthesis of a copper-binding, mitochondria localizing peptide. This has been achieved by tethering a well characterized MPP and the metal-binding octapeptide sequence of the prion protein, generating (Fxr)₄GGHGGGW (6). This peptide construct is stable for at least 5 days in cell media and is able to bind Cu(II) at 1.6 μΜ (± 0.3) affinity. Preliminary data suggests that the peptide construct is unable to deliver copper to the mitochondria; however, this needs further investigation. Nonetheless, these studies provide a basis for the design of a more suitable mitochondria-specific copper-delivery agent. MPP-mediated copper delivery provides a very promising approach to the delivery of metal ions and may be therapeutically relevant for the treatment of disorders associated with copper deficiency.
5. Chapter 5: Future Directions
5.1. Further characterization of MPPs

While the systematic studies outlined in Chapter 3 provided a thorough understanding of the physiochemical properties required for selective mitochondrial localization and cellular uptake, further studies may provide additional information. For instance, the effect of lipophilicity has been addressed by increasing the number of hydrophobic residues in an MPP to increase its overall lipophilicity. This approach led to aggregation and insolubility when more than three terminal Fx residues were attached. To circumvent the aggregation problems faced with an increasing C-terminal hydrophobic tail, the Fx residues can be, instead, distributed across the MPP to increase lipophilicity. A larger panel of watersoluble MPPs is predicted to exist, which will in turn provide peptides possessing a wider window of lipophilicity than the panel described in Chapter 3 due to the incorporation of more Fx residues.

5.2. Optimizing copper ion delivery to the mitochondria

Metal-ion delivery to the mitochondria is a promising approach for several applications. The work described in Chapter 4 generated a copper-binding MPP with low micromolar affinity to the metal. With this straightforward approach, variable copper-binding sequences can be screened to design an optimal metal-delivery vector. From the studies performed, the novel copper-binding MPP (6) was unable to deliver copper to isolated mouse liver mitochondria. This can be attributed to several factors, including a weak binding to the metal. Future directions for this project require the design of a novel copper-binding MPP with a stronger metal-binding affinity as well as an ability to release the metal ion within the organelle to ensure its successful delivery. Nanomolar affinity might be achieved by introducing a proline in the metal binding sequence of 6 to aid in the coordination, as found in the octapeptide sequence of the prion protein (PHGGGWG). This as well as other alterations in the sequence (i.e., increase length of Gly linkers, introduce more imidizole moieties, etc.) may be pursued to increase binding affinity. Alternatively,
another approach to engineer a mitochondrial copper-delivery vector is by conjugating the MPP to a copper-cage complex. Through light-activation, the bound copper ions are released from the copper cage. With the picomolar affinity reported for the copper-cage–Cu(II), this complex would ensure both, a strong binding to copper ions as well as a controlled release mechanism. In addition to the studies completed in this work for conjugate 6, the progress of future copper-delivery agents will require functional assays in a live cell environment. A suitable cell-based assay to verify mitochondrial delivery and copper-release can be achieved by treating COX-deficient cells with the MPP-Cu(II) construct and monitor COX rescue over time. The turnover time required for COX assembly is within one week, a timeline compatible with the stability of such peptides. Cytotoxicity as well as divalent metal competition are assays that also need to be considered.

5.3. Use of copper ions in radiosensitization

In addition to treating deficiency disorders, copper ions can also be used in molecular imaging techniques. For instance, the nuclear characteristics of $^{64}$Cu (i.e., $t_{1/2} = 12.7$ h) make it ideal for positron emission tomography (PET) imaging as well as radiotherapy. Recent studies have designed bifunctional copper chelators for applications in radiopharmaceuticals. These complexes display functional groups that enable both, tight chelation to copper ions as well as conjugation to peptides. The conjugation of MPPs to these bifunctional copper chelators would generate mitochondria-specific radiosensitizers, ideal compounds for cancer therapy as mitochondria are directly involved in the intrinsic apoptotic pathway of the cell. Such approaches would indeed advance the growing field of cancer research and therapy.
6. Chapter 6: References


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