Chemistry & Biology
Article

Mitochondria-Penetrating Peptides

Kristin L. Horton,[1,3] Kelly M. Stewart,[1,3] Sonali B. Fonseca,[2] Qian Guo,[1] and Shana O. Kelley[1,2,*]
[1]Department of Biochemistry, Faculty of Medicine
[2]Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy
University of Toronto, Toronto, ON M5S 3M2, Canada
[3]These authors contributed equally to this work.
*Correspondence: shana.kelley@utoronto.ca
DOI 10.1016/j.chembiol.2008.03.015

SUMMARY

Mitochondria are important targets for cancer chemotherapy and other disease treatments. Gaining access to this organelle can be difficult, as the inner membrane is a barrier limiting diffusive transport. A mitochondrial molecular carrier would be a boon to the development of organelle-specific therapeutics. Here, we report a significant advance in the development of mitochondrial transporters—synthetic cell-permeable peptides that are able to enter mitochondria. Efficient uptake of these mitochondria-penetrating peptides (MPPs) is observed in a variety of cell types, and organellar specificity is attained with sequences that possess specific chemical properties. The MPPs identified are cationic, but also lipophilic; this combination of characteristics facilitates permeation of the hydrophobic mitochondrial membrane. The examination of a panel of MPPs illustrates that mitochondrial localization can be rationally controlled and finely tuned by altering lipophilicity and charge.

INTRODUCTION

The mitochondrion represents a candidate of significant interest for organelle-specific delivery of exogenous molecules (Fantin and Leder, 2006; Galluzzi et al., 2006; Murphy and Smith, 2000). The roles of mitochondria in energy production and programmed cell death make this organelle a prime target in the treatment of several disease states (Galluzzi et al., 2006; Green et al., 2004; Murphy and Smith, 2000). Furthermore, cancer therapies have targeted mitochondria to induce cell death in tumors where conventional apoptotic pathways are disabled (Galluzzi et al., 2006). A significant challenge to mitochondrial drug delivery is the impervious structure of the hydrophobic inner membrane. Theoretical and experimental studies have revealed the importance of lipophilicity and positive charge in molecules that accumulate in the mitochondria, but the relationship between chemical structure and mitochondrial entry remains ill defined (Fernandez-Carneado et al., 2005; Horobin et al., 2007; Maiti et al., 2007; Modica-Napolitano and Aprille, 2001; Rosania, 2003; Rosania et al., 2003; Zhao et al., 2004). A precise understanding of the determinants for mitochondrial entry is critical to the development of a general class of synthetic mitochondrial transporters and to the optimization of therapeutics with targets within this organelle.

While little is known about engineering molecules to penetrate the mitochondrial membrane, a major breakthrough in the identification of compounds that can penetrate the plasma membrane was made when a series of short peptide sequences were identified that exhibited efficient cellular uptake (Derossi et al., 1994; Frankel and Pabo, 1988; Green and Loewenstein, 1988). One that has received a great deal of attention is the Tat peptide, derived from the HIV transactivator protein (Green and Loewenstein, 1988). The discovery of this peptide, along with others displaying membrane-crossing activities, served as the cornerstone for a new subfield focused on the use of cell-penetrating peptides (CPPs) as molecular transporters (Gupta et al., 2005; Joliot and Prochiantz, 2004; Snyder and Dowdy, 2004). Numerous applications of CPPs as molecular delivery vehicles have been demonstrated, illustrating their utility in biology and medicine (Goun et al., 2006; Gupta et al., 2005; Joliot and Prochiantz, 2004; Lewin et al., 2000; Snyder and Dowdy, 2004; Torchilin et al., 2001). Although the precise mechanisms of entry are still under debate, some highly cationic CPPs have been shown to respond to membrane potential (Fischer et al., 2005; Futaki et al., 2007; Henriques et al., 2005; Rothbard et al., 2004; Terrone et al., 2003). This begs the question: given the potential-responsive character of many mitochondrial localizers (Davis et al., 1985; Horobin et al., 2007; Modica-Napolitano and Aprille, 2001), could CPPs be engineered with the proper chemical attributes for transport into mitochondria? Peptide-based mitochondrial agents would have many advantages over other mitochondriotropics, including biocompatibility and straightforward synthesis, that would facilitate modification with therapeutic cargos. Indeed, the prior development of peptide-based antioxidants that exhibited mitochondrial localization provides an important precedent for the feasibility of such an approach (Zhao et al., 2004).

Here, we report the first, to our knowledge, systematic development of a class of synthetic peptides that exhibit efficient cellular uptake and specific mitochondrial localization. Using design principles that impart properties facilitating efficient cellular uptake and mitochondrial entry, sequences were engineered to display levels of cellular uptake that rival the Tat peptide, but with strong mitochondrial localization, a property unattainable with this CPP. Moreover, the exact physiochemical properties leading to the organellar specificity of these mitochondria-penetrating peptides (MPPs) were identified, providing an important conceptual framework for understanding how mitochondrial localization can be achieved with synthetic compounds.

 ©2008 Elsevier Ltd All rights reserved

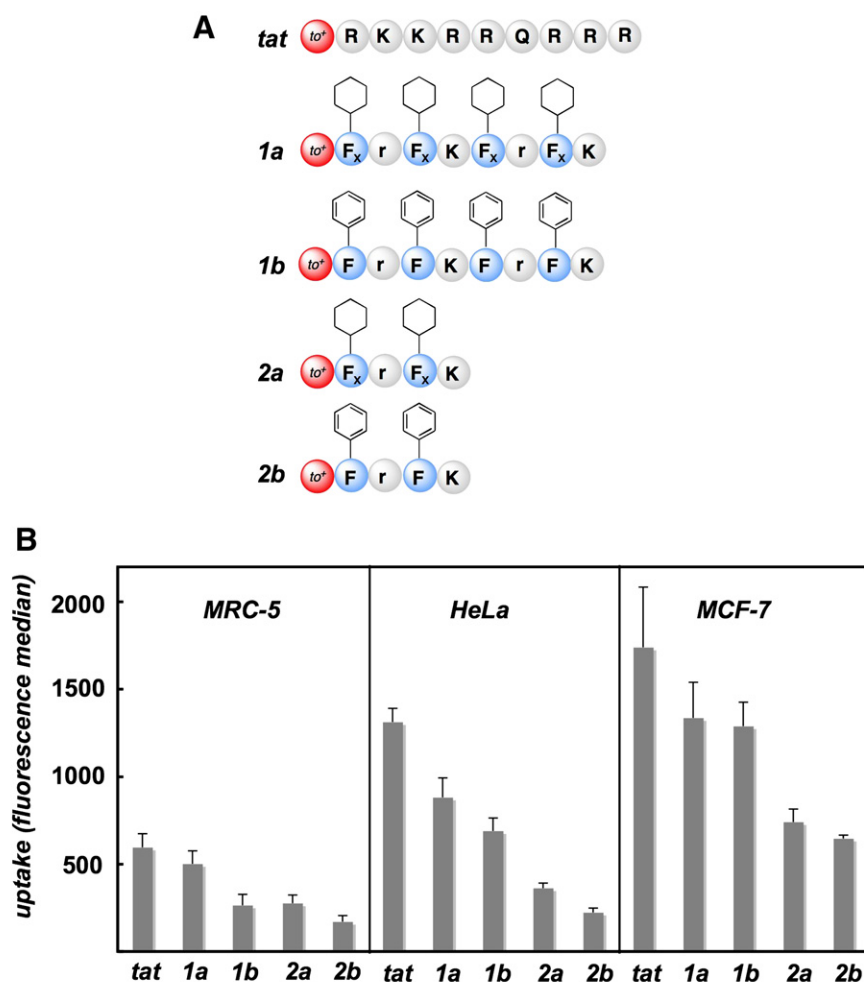

Figure 1. Uptake of MPPs

(A) Structures of peptides used for analysis. The peptides consisted of four or eight amino acids and were linked to the fluorophore, *to*, for imaging. *d*-Arginine (r) was included in the conjugates to increase cellular stability. The Tat_{49-57} peptide was also synthesized, modified with *to*, and used as a standard.

(B) Uptake of MPPs in human cell lines measured by flow cytometry. HeLa, MCF-7, or MRC-5 cells were incubated with 2.5 μM peptide for 90 min before cytometry trials. Data shown represent means and standard error of a minimum of three trials. The toxicities of the MPPs were monitored to ensure that all measurements were obtained with viable cells (see Figure S2).

RESULTS AND DISCUSSION

Design and Preparation of MPPs

The sequences of the MPPs were designed to display two properties known to be important for passage across both the plasma and mitochondrial membranes: positive charge and lipophilic character. Previous studies of arginine-based peptide oligomers indicated that high levels of cellular uptake can be achieved through the inclusion of cationic residues (Wender et al., 2000). The positive molecular charge facilitates charge-driven uptake through the plasma membrane, which exhibits a potential gradient that will electrophorese cationic species from the extracellular space into the cell (Rothbard et al., 2004; Terrone et al., 2003). Given that the mitochondrial membrane is a similarly energized interface, including cationic residues in the sequences should, in principle, allow passage through both membranes. However, the inner membrane of this organelle is much more hydrophobic than the plasma membrane, which necessitates preservation of a high degree of lipophilicity in order to allow partitioning of the peptides through the lipid bilayer. The successful delivery of a peptide antioxidant to mitochondria that contained alternating aromatic and cationic residues indicates that this motif is an effective one for mitochondrial delivery (Zhao et al., 2004). Thus, we made oligomers containing cationic and hydrophobic residues that would provide electrostatic driving force for uptake through the energized plasma and mitochondrial membranes, while preserving the lipophilic character that would facilitate passage through the latter. Lysine (K) and arginine (R) were selected to provide positive charge, and phenylalanine (F) and cyclohexylalanine (F_X) residues were used to impart lipophilicity. Peptides containing either four or eight residues were synthesized and labeled with the fluorophore thiazole orange (*to*; Figure 1A).

Analysis of MPP Cellular Uptake and Localization

The cellular uptake and localization of the sequences was evaluated in human cells by flow cytometry and confocal microscopy. The uptake of the designed peptides was analyzed in three different cell lines: HeLa (a cervical carcinoma cell line), MCF-7 (a breast carcinoma cell line), and MRC-5 (a normal lung fibroblast cell line) (Figure 1B). The Tat peptide was also modified with the same fluorophore and analyzed for comparison. In all the cell lines tested, uptake of the designed MPPs approached that of Tat, indicating that efficient transport through the plasma membrane was achieved. The uptake of the sequence $F_XrF_XKF_XrF_XK$ (**1a**) in the two cancer cell lines was particularly strong, and was comparable to that observed with Tat. These results indicate that success was achieved in generating sequences with favorable uptake, and that efficient transport of these compounds into cells is observed in a variety of cell types.

The analysis of peptide localization within HeLa cells revealed that, while uptake across the plasma membrane of Tat and the lipophilic peptides was similar, the intracellular localization was profoundly different (Figure 2). The oligomers designed to function as MPPs exhibited strong mitochondrial localization, while the Tat peptide was excluded from mitochondria and, instead, appeared within the nuclei. By colocalizing the peptides with Mitotracker CMXRos (Invitrogen) and comparing the images corresponding to the fluorescence profiles, Pearson's correlation coefficients (Rr) could be calculated reflecting mitochondrial specificity. The two peptides containing F_X residues displayed

 ©2008 Elsevier Ltd All rights reserved

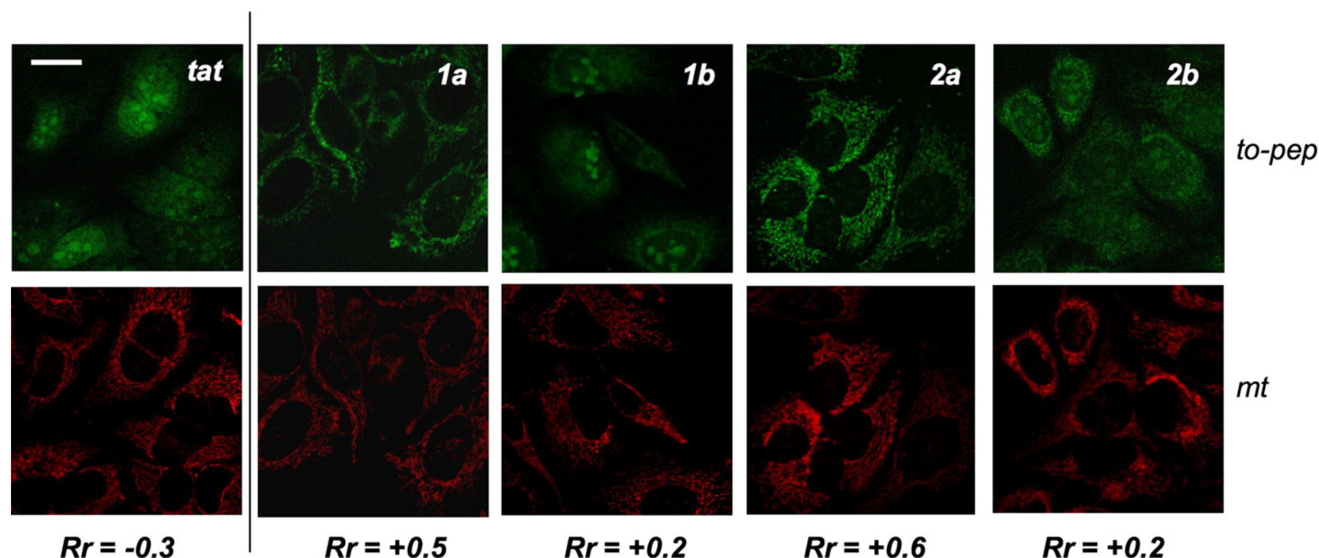

Figure 2. Localization of MPPs in Live HeLa Cells
Cells were incubated with peptide for 90 min before imaging; 25 nM Mitotracker CMXRos was introduced 15 min before images were acquired. Peptide concentrations were adjusted (1–10 μM) to achieve equivalent uptake for the panel of peptide conjugates. Images shown in green correspond to those of the peptides and those shown in red correspond to Mitotracker. Images collected for both compounds were analyzed with an algorithm for the calculation of Rr to determine the extent of mitochondrial localization for the peptides. Values reported were calculated from more than 100 cells analyzed in multiple (>3) experiments. Only live cells (as judged by the exclusion of PI) were included in the analysis. Scale bar, 10 μm.

very high levels of mitochondrial localization (Rr = +0.5 to +0.6), while those containing F residues were less mitochondrial (Rr = +0.2), and Tat displayed essentially no mitochondrial localization (Rr = −0.3). These results demonstrate that the artificial sequences we engineered were successfully targeted to mitochondria, but the different levels of mitochondrial specificity for the F_X-containing sequences relative to those containing F indicates that subtle changes to the chemical structure of the peptide had a significant effect on localization.

It is noteworthy that, in the experiments shown in Figure 1B and Figure 2, 90 min incubations were used to enhance the quality of the images obtained by confocal fluorescence microscopy. However, experiments were also performed to monitor uptake and localization of the MPPs with shorter incubation times. As described in Figure S4 in the Supplemental Data (available with this article online), uptake comparable to that of Tat was obtained and similar localization profiles were observed when shorter (e.g., 15 and 30 min) incubations were examined.

Elucidation of Molecular Requirements for Mitochondrial Localization

To explore the nature of the relationship between chemical structure and the cellular localization of MPPs, we synthesized a panel of compounds with a variety of functional groups (Figure 3 and Table 1). As we envisaged that lipophilicity would be important in modulating the localization of the conjugates, structures were selected that would exhibit significant variations in this parameter. The compounds incorporated several hydrophobic amino acids, including unnatural residues displaying diphenyl, naphthyl, or hexyl functionalities. Fluorinated F (F_F), methylated tyrosine (Y_{Me}), and tyrosine (Y) residues were also utilized to enhance chemical diversity within the panels of conjugates tested.

The calculation of correlation coefficients describing the extent of mitochondrial localization for all 14 compounds included in this study revealed that the members of the panel exhibited a wide variety of intracellular localizations (Table 1). For example, as shown in Figure 3B, within the set of compounds bearing a +3 charge, some compounds displayed exclusively mitochondrial localization, others were completely excluded from the mitochondria, and others exhibited mixed localization, appearing both in the mitochondria and nuclei of HeLa cells.

While it is not possible to determine unequivocally from this analysis the exact suborganellar location of the peptides that enter into mitochondria, it is likely that they do not remain in the mitochondrial membrane, as the sequestration of cationic peptides in this environment would be energetically unfavorable. It is therefore reasonable to expect that they would enter the matrix, but this is a difficult issue to address experimentally.

Examination of the correlation between mitochondrial localization and the lipophilicity of the +3 peptide conjugates revealed a striking trend (Figure 4). Compounds that possessed log P values higher than −1.7 exhibited strong mitochondrial localization, while those with log P values lower than −2.0 were essentially excluded from mitochondria, and instead appeared in the nuclei and cytoplasm of HeLa cells. In the range between these two values, mixed localization was observed that demonstrated a strong dependence on lipophilicity. Thus, there appears to be a critical lipophilicity threshold where mitochondrial entry is permitted, with hydrophilicity above this level decreasing compatibility with the mitochondrial membrane and precluding access. Interestingly, there is a well-defined lipophilicity threshold that is required for mitochondrial penetration. Indeed, these cationic compounds with a +3 charge must possess sufficient lipophilicity (i.e., that reflected in a log P value of approximately −2.0 or higher) to access mitochondria.

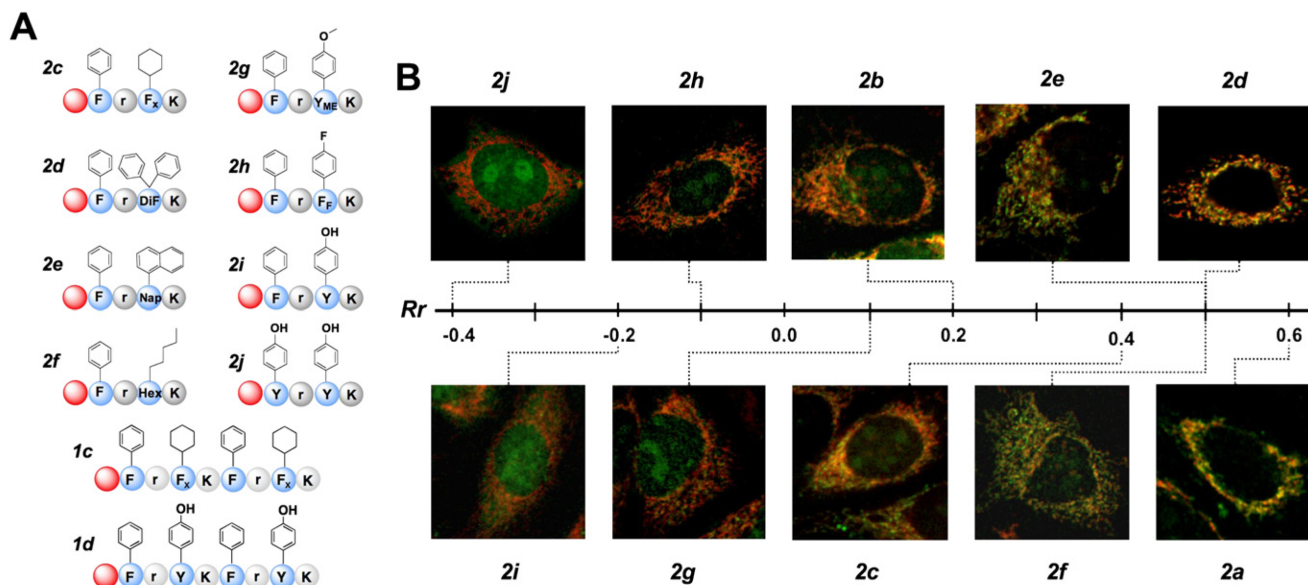

Figure 3. Evaluation of Mitochondrial Localization for Synthetic Peptide Conjugates

(A) The peptidoconjugate scaffolds tested consisted of four or eight amino acids linked to the fluorophore, *to*. The lysine and arginine residues shown schematically in gray were held constant (along with the fluorophore, *to*), and the residues in red were altered to modulate lipophilicity. The tetramer conjugates possess a charge of +3, while the octamers possess a charge of +5.

(B) Quantitation of mitochondrial localization via calculation of Rr for +3 peptide conjugates. Coefficients were calculated as described in the Experimental Procedures. Images for octameric peptides can be found in the Supplemental Data.

When the larger compounds possessing a +5 charge were examined, the same strong relationship between lipophilicity and mitochondrial localization was observed (Figure 4). However, the lipophilicity threshold for mitochondrial localization shifted relative to the +3 compounds, with more hydrophilic compounds still penetrating the organelle. Peptides with log P values of −2.5 and higher exhibited mitochondrial localization, while a log P value of −2.7 appeared to promote exclusion from mitochondria. Thus, for compounds bearing this elevated charge, the lipophilicity threshold is decreased to approximately −2.6. These results suggest that the charge-driven uptake of these conjugates into mitochondria dominates the localization, and that a greater electrochemical driving force provided by additional charge can provide enough energy to propel a more hydrophilic species through the hydrophobic mitochondrial membrane.

The approach described here, in which confocal fluorescence microscopy and flow cytometry were used to extract systematic trends describing how the chemical properties of peptides influence subcellular localization, relies on the use of an appended fluorophore, to. While this moiety could influence the behavior of these peptides, it is important to recognize that it is a constant throughout all of the experiments described, which minimizes the likelihood that the localization trends observed are related to the fluorophore. As shown, changes to the peptide sequence have a direct effect on the localization profiles of the compounds, indicating that the behavior observed truly results from the varying chemical properties of differing side chains. While *to* was the best choice as an imaging label for the experiments described because of its spectroscopic properties (e.g., its high extinction coefficient and quantum yield) and yielded high-resolution images suitable for the quantitative approach that we pursued, we also investigated the localization of peptides featuring other fluorophores to ensure that our chromophore was not influencing the localization of the MPPs. For example, the inclusion of 2-aminobenzoic acid (abz) in MPPs allowed us to assess localization in the absence of the *to* chromophore. Indeed, the localization profiles of abz-modified peptides were qualitatively comparable to those observed with *to* (see Figure S5), and confirm that the fluorophore used in the majority of our studies is not dominating the behavior observed.

Investigation of Charge-Driven Transport through the Plasma and Mitochondrial Membranes

To more directly establish that charge-driven uptake is responsible for the transport of the MPPs across the plasma and mitochondrial membranes, we tested agents that would alter membrane potentials and monitored the effects on uptake and localization. While many CPPs, including Tat, have been shown to enter cells by endocytosis (Fotin-Mleczek et al., 2005; Gump and Dowdy, 2007), some artificial sequences have been shown to traverse the plasma membrane by direct potential-driven diffusion (Henriques et al., 2005; Rothbard et al., 2004; Terrone et al., 2003). Given the results presented above, it appeared that this direct mechanism could dominate the uptake and subcellular trafficking of MPPs. Indeed, while treatment with endocytosis inhibitors did not decrease the uptake of the MPPs (data not shown), treating with gramicidin or valinomycin (two agents that alter the plasma membrane potential) (Rothbard et al., 2004; Urban et al., 1980) did have a pronounced effect on uptake (Figure 5A). The use of gramicidin to depolarize the plasma membrane decreased the uptake of peptide **1a** by 80%. Likewise, a 110% increase in uptake for **1a** was observed

 ©2008 Elsevier Ltd All rights reserved

Cell PRESS

Table 1. Lipophilicity and Mitochondrial Localization of Peptide Conjugates

Compound	Charge	Log P[a]	Rr[b]
to-F_X-r-F_X-K-F_X-r-F_X-K (**1a**)[c]	+5	−2.2	+0.5
to-F-r-F-K-F-r-F-K (**1b**)	+5	−2.5	+0.1
to-F-r-F_X-K-F-r-F_X-K (**1c**)	+5	−2.3	+0.4
to-F-r-Y-K-F-r-Y-K (**1d**)	+5	−2.7	−0.4
to-F_X-r-F_X-K (**2a**)	+3	−1.1	+0.6
to-F-r-F-K (**2b**)	+3	−1.9	+0.2
to-F-r-F_X-K (**2c**)	+3	−1.4	+0.4
to-F-r-F_2-K (**2d**)	+3	−1.6	+0.6
to-F-r-Nap-K (**2e**)	+3	−1.6	+0.5
to-F-r-Hex-K (**2f**)	+3	−1.7	+0.5
to-F-r-Y_{Me}-K (**2g**)	+3	−2.0	+0.1
to-F-r-F_F-K (**2h**)	+3	−2.0	−0.1
to-F-r-Y-K (**2i**)	+3	−2.1	−0.2
to-Y-r-Y-K (**2j**)	+3	−2.4	−0.4

Abbreviations: F, phenylalanine; F_2, diphenyl; F_X, cyclohexylalanine; Hex, hexyl; K, *l*-lysine; Nap, naphthyl; r, *d*-arginine; Rr, Pearson's correlation coefficient; *to*, thiazole orange; Y, tyrosine; Y_{Me}, methylated tyrosine.

[a] Log P values measured by the shake-flask method. See Figure 4 for standard errors of values.

[b] Rr values measured by confocal microscopy and comparison of Mitotracker with fluorescence from labeled conjugate. See Figure 4 for standard errors.

[c] The residue r was included to increase protease stability of conjugates. See the Supplemental Data for chemical structures of compounds and characterization.

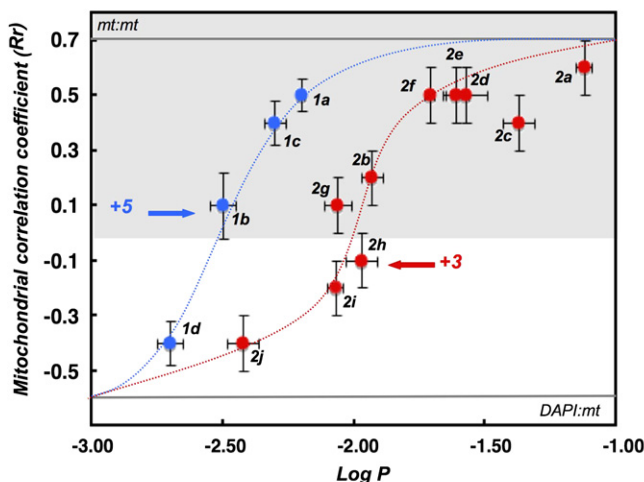

Figure 4. Correlation between Mitochondrial Localization and Lipophilicity

Rr values are plotted against experimentally determined log P values. Compounds bearing a +3 charge are plotted in red, and those with a +5 charge are plotted in blue. Solid gray lines represent limits for Rr values obtained when two mitochondrial dyes were colocalized (mt:mt) versus when one mitochondrial dye and one nuclear dye were colocalized (DAPI:mt). These control experiments indicate that the maximum positive correlation coefficient that can be obtained with the protocols employed is +0.7, and that the minimum negative correlation coefficient is −0.6. The gray-shaded area on the plot represents the values of Rr reflecting mitochondrial localization. Please note that the lines drawn through the experimental points do not represent a mathematical fit, but are instead intended to highlight the trends qualitatively. Error bars on data points represent standard error for log P and Rr determinations.

in the presence of valinomycin, indicating that an increase in membrane potential greatly enhanced transport across the lipid bilayer. The relative uptake of the compounds across the panel of cell lines (Figure 1B) also suggests a membrane potential-driven mechanism of uptake, since uptake increased with plasma membrane potential of the cell lines (MRC-5 [67 ± 9 mV] < HeLa [75 ± 7 mV] < MCF-7 [103 ± 11 mV]).

Perturbing the mitochondrial membrane potential also influenced the behavior of the MPPs. In the presence of the mitochondrial decoupler FCCP, the localization profile of compound **1a** was dramatically altered, and mitochondrial uptake was significantly decreased (Figure 5B). These results, taken together, indicate that the MPPs are successful in penetrating the plasma and mitochondrial membranes because they are able to respond to the potential gradients that exist across these interfaces. It is noteworthy that there are known examples of mitochondrially localized agents (e.g., 10-N-nonyl acridine orange [Petit et al., 1995]) that exhibit this type of organellar specificity due to association with cardiolipin. The potential dependence we observe here indicates that it is an electrochemical gradient, rather than a specific type of complexation, that is responsible for the mitochondrial localization of a subset of the peptide sequences described. However, from these experiments, we cannot rule out that cardiolipin might play a role.

Comparison of MPPs to Other Mitochondrially Localized Molecules

Prior work on the development of mitochondrial transporters has primarily focused on the use of lipophilic delocalized cations that harness the mitochondrial membrane potential to enter the organelle (Murphy and Smith, 2000; Horobin et al., 2007; Modica-Napolitano and Aprille, 2001; Davis et al., 1985). This approach has been successful when applied to deliver nonpolar species into mitochondria, but has not been successful when more polar cargos are targeted. Indeed, the conjugation of a lipophilic cation to a CPP did not facilitate its uptake into mitochondria, and this observation was taken as evidence that peptides would not permeate the inner mitochondrial membrane (Ross et al., 2004). We show here that lipophilic cationic peptides, while not possessing delocalized charge, can be engineered to enter mitochondria. Indeed, it is noteworthy that, while our work to our knowledge constitutes the first example of a systematic series of MPPs with efficient cellular uptake and highly specific organellar localization, a prior report of peptide antioxidants also indicated that small peptides could display this type of organellar specificity (Zhao et al., 2004). These compounds exhibit structural similarity to a subset of our tetrameric peptides. Clearly, this previous work, and that reported here, indicates that peptides are indeed a useful tool for mitochondrial delivery.

SIGNIFICANCE

We have engineered a class of cell-permeable peptides that efficiently enter human cells, and specifically localize to mitochondria. The studies reported are, to our knowledge, the first to systematically identify critical levels of lipophilicity

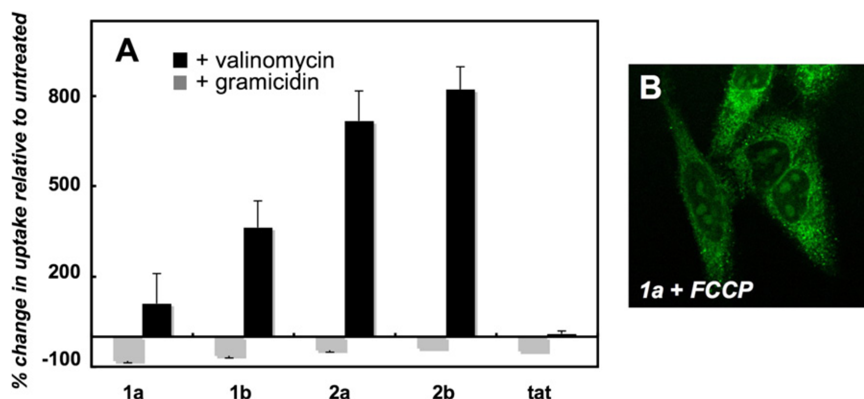

Figure 5. Effects of Membrane Potential on Uptake and Localization of MPPs

(A) Uptake dependence of MPPs and Tat on plasma membrane potential. HeLa cells were treated with valinomycin (increased membrane potential) or gramicidin (decreased membrane potential) before and during incubation with peptide conjugates. Uptake was measured by flow cytometry and is expressed as percent change relative to untreated cell uptake. Data represent the mean and standard error of a minimum of three trials.

(B) Decreased mitochondrial localization observed for *to*-F_X-r-F_X-K-F_X-r-F_X-K in the presence of FCCP. Images were acquired under the same conditions as described in Figure 1B.

that allow molecules to access mitochondria and to elucidate the effect of molecular charge on lipophilicity thresholds. The trends observed with these conjugates provide insight into the exact requirements that impart mitochondrial localization to lipophilic cations, and may provide a means to engineer the cellular trafficking of bioactive compounds. These mitochondria-penetrating peptides (MPPs), based on a peptide scaffold, possess characteristics conducive to use as a delivery vector: straightforward synthesis, facile derivitization, and biocompatibility. Thus, the MPPs described may represent a class of effective mitochondrial transporters that are well suited to traverse an organellar membrane that is difficult to penetrate.

EXPERIMENTAL PROCEDURES

Preparation of MPPs

Peptides were prepared as described previously (Carreon et al., 2007). See the Supplemental Data for protocols and characterization information.

Cell Culture

HeLa and MRC-5 cells (ATCC) were cultured as subconfluent monolayers on 25 cm^2 or 75 cm^2 cell culture plates with vent caps (Sarstedt) in minimum essential medium (MEM; ATCC) supplemented with 10% (v/v) fetal bovine serum (FBS; ATCC) in a humidified incubator (70%–95%) at 37°C with 5% CO_2. MCF-7 cells were cultured in similar conditions, expect that the MEM media were supplemented with 10% FBS and 0.01 mg/ml insulin. Cells grown to subconfluence were enzymatically dissociated from the surface with a solution of 0.05% trypsin/0.53mM EDTA (Cellgro) and plated at 15–25 $\times 10^3$ cells/well 1–2 days prior to the experiment in eight well ibiTreat μ-slides (Ibidi). For uptake experiments, 1×10^5 cells/well were plated in a 12-well plate 1 day prior to the experiment. These conditions produced a monolayer at subconfluence.

Confocal Fluorescence Microscopy

For colocalization 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 ~5 μM conjugate in serum-free MEM (α-MEM [no phenol red]; Invitrogen) for 90 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; no losses of viability were observed, as shown in Figure S6. Peptide concentrations were adjusted (1–10 μM) to achieve equivalent uptake for the panel of peptide conjugates. Mitotracker CMXRos was added to achieve a final concentration of 25–50 nM for the last 15 min of the incubation. Cells were washed three times with serum-free MEM. After washing, serum-free MEM was added and the slides were placed on ice. Images were taken with an inverted Zeiss LSM 510 confocal microscope with a water immersion lens (63×). The excitation wavelength for visualization of to conjugates was 488 nm, and emission was collected from 505 to 550 nm. The excitation wavelength for visualization of Mitotracker CMXRos was 543 nm, and emission was collected with a long-pass 560 nm filter. Differential interference contrast (DIC) images were taken along with both fluorescence channels, and no bleedthrough was observed during colocalization studies with these parameters. The fluorescence images were analyzed with the Colocalizer Pro software program to determine Rr, and values reported are average values obtained for individual representative cells (~100 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.

To assess localization in cells with altered mitochondrial membrane potential, the proton ionophore carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP) was used to decrease mitochondrial membrane potential. Cells were pretreated with 10 μM FCCP in serum-free MEM for 15 min at in a humidified incubator at 37°C with 5% CO_2. This solution was removed and the cells were incubated with 2 μM *to*-F_X-r-F_X-K-F_X-r-F_X-K in serum-free MEM in the presence of 10 μM FCCP for 30 min. After incubation, the solution was removed and the cells were washed with MEM and placed on ice until imaging.

Flow Cytometry

After incubation with 5 uM peptide for 90 min, cells were enzymatically removed from the surface of the plate with trypsin/EDTA (250 μl/well) for 10 min at 37°C. The trypsinization was quenched with 500 μl 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 (8 min at 2100 × *g*), and resuspended in 500 μl PBS containing 1 μg/ml propidium iodide (PI; 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 PI were excluded from analysis. The fluorescence median of the live population was used for statistical analysis.

In order to test the role of membrane potential on cellular uptake, the above procedure was repeated with solutions of 50 nM valinomycin (Sigma) or 1 μM gramicidin (Sigma) in serum-free MEM for pretreatment and coincubation. The cells were removed from the surface and analyzed in the same fashion. Those staining positive for PI were excluded from analysis. Treated and untreated cells were >80% viable.

Measurement of Log P

Log P values were measured via octanol partitioning by a modification of the shake-flask method and as previously described (Rothbard et al., 2004). An aliquot of 100 μl of 300 μM to-peptide conjugate in Tris buffer (10 mM, pH 7.4) and 100 μl 1-octanol (Aldrich) were added to a 0.5 ml microtube. Buffer was employed in order to measure log P of the peptide conjugates in the

 ©2008 Elsevier Ltd All rights reserved

protonation state observed at physiogloical pH. The tubes were vortexed for 1 min and centrifuged; 25 μl of each layer was removed and diluted in 100 ml 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 4-fold. Three dilutions were prepared per layer, 100 μl of each dilution was pipetted into a 384 well plate, and the absorbance 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 log P. For compounds with very low lipophilicity (log $P < -2.3$), the volume of the octanol and aqueous layers was increased from 100 to 125 μl, and the absorbance of the octanol layer was measured without any additional dilutions. This procedure was repeated a minimum of three times per conjugate to calculate the mean log P and standard error. All absorbance measurements used were within the linear range of the instrument.

SUPPLEMENTAL DATA

Supplemental Data include synthetic protocols and compound characterization information, and procedures used to measure membrane potentials, and are available with this article online at http://www.chembiol.com/cgi/content/full/15/4/375/DC1/.

ACKNOWLEDGMENTS

We thank the NSF (CAREER award to S.O.K.) and the University of Toronto for their support of this work. We also acknowledge James Jonkman and the Advanced Optical Microscopy Facility at Princess Margaret Hospital for expert assistance with confocal fluorescence imaging.

Received: December 6, 2007
Revised: March 13, 2008
Accepted: March 17, 2008
Published: April 18, 2008

REFERENCES

Carreon, J.R., Stewart, K.M., Mahon, K.P., Shin, S., and Kelley, S.O. (2007). Cyanine dye conjugates as probes for cellular imaging. Bioorg. Med. Chem. Lett. *17*, 5182–5185.

Davis, S., Weiss, M.J., Wong, J.R., Lampidis, T.J., and Chen, L.B. (1985). Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. J. Biol. Chem. *260*, 13844–13850.

Derossi, D., Joliot, A.H., Chassaing, G., and Prochiantz, A. (1994). The third helix of the *Antennapedia* homeodomain translocated through biological membranes. J. Biol. Chem. *269*, 10444–10450.

Fantin, V.R., and Leder, P. (2006). Mitochondriotoxic compounds for cancer therapy. Oncogene *25*, 4787–4797.

Fernandez-Carneado, J., Gool, M.V., Martos, V., Castel, S., Prados, P., deMendoza, J., and Giralt, E. (2005). Highly efficient, nonpeptidic oligoguanidinium vectors that selectively Internalize into mitochondria. J. Am. Chem. Soc. *127*, 869–874.

Fischer, R., Fotin-Mleczek, M., Hufnagel, H., and Brock, R. (2005). Break on through to the other side—biophysics and cell biology shed light on cell-penetrating peptides. ChemBioChem *6*, 2126–2142.

Fotin-Mleczek, M., Fischer, R., and Brock, R. (2005). Endocytosis and cationic cell-penetrating peptides—a merger of concepts and methods. Curr. Pharm. Des. *11*, 3613–3628.

Frankel, A.D., and Pabo, C.O. (1988). Cellular uptake of the Tat protein from human immunodeficiency virus. Cell *55*, 1189–1193.

Futaki, S., Nakase, I., Tadokoro, A., Takeuchi, T., and Jones, A.T. (2007). Arginine-rich peptides and their internalization mechanisms. Biochem. Soc. Trans. *35*, 784–787.

Galluzzi, L., Larochette, N., Zamzami, N., and Kroemer, G. (2006). Mitochondria as therapeutic targets for cancer chemotherapy. Oncogene *25*, 4812–4830.

Goun, E.A., Pillow, T.H., Jones, L.R., Rothbard, J.B., and Wender, P.A. (2006). Molecular transporters: synthesis of oligoguanidinium transporters and their application to drug delivery and real-time imaging. ChemBioChem *7*, 1497–1515.

Green, K., Brand, M.D., and Murphy, M.P. (2004). Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes. Diabetes *53*, S110–S118.

Green, M., and Loewenstein, P.M. (1988). Autonomous functional domains of chemically synthesized human immunodeficiency virus. Cell *55*, 1179–1188.

Gump, J.M., and Dowdy, S.F. (2007). Tat transduction: the molecular mechanism and therapeutic prospects. Trends Mol. Med. *13*, 443–448.

Gupta, B., Levchenko, T.S., and Torchilin, V.P. (2005). Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv. Drug Deliv. Rev. *57*, 637–651.

Henriques, S.T., Costa, J., and Castanho, M.A. (2005). Re-evaluating the role of strongly charged sequences in amphipathic cell-penetrating peptides: a fluorescence study using Pep-1. FEBS Lett. *579*, 4498–4502.

Horobin, R.W., Trapp, S., and Weissig, V. (2007). Mitochondriotropics: a review of their mode of action, and their applications for drug and DNA delivery to mammalian mitochondria. J. Control. Release *121*, 125–136.

Joliot, A., and Prochiantz, A. (2004). Transduction peptides: from technology to physiology. Nat. Cell Biol. *6*, 189–196.

Lewin, M., Carlesso, N., Tung, C.H., Tang, X.W., Cory, D., Scadden, D.T., and Weissleder, R. (2000). Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. Nat. Biotechnol. *18*, 410–414.

Maiti, K., Lee, W., Takeuchi, T., Watkins, C., Fretz, M., Kim, D., Futaki, S., Jones, A., Kim, K., and Chung, S. (2007). Guanidine-containing molecular transporters: sorbitol-based transporters show high intracellular selectivity toward mitochondria. Angew. Chem. Int. Ed. Engl. *46*, 5880–5884.

Modica-Napolitano, J.S., and Aprille, J.R. (2001). Delocalized lipophilic cations selectively target the mitochondria of carcinoma cells. Adv. Drug Deliv. Rev. *49*, 63–70.

Murphy, M.P., and Smith, R.A. (2000). Drug delivery to mitochondria: the key to mitochondrial medicine. Adv. Drug Deliv. Rev. *41*, 235–250.

Petit, J.M., Maftah, A., Rantinaud, M.H., and Julien, R. (1995). Direct cardiolipin assay in yeast using the red fluorescence emission of 10-N-nonyl acridine orange. Eur. J. Biochem. *228*, 113–119.

Rosania, G.R. (2003). Supertargeted chemistry: identifying relationships between molecular structures and their sub-cellular distribution. Curr. Top. Med. Chem. *3*, 659–685.

Rosania, G.R., Lee, J.W., Ding, L., Yoon, H.S., and Change, Y.T. (2003). Combinatorial approach to organelle-targeted fluorescent library based on the styryl scaffold. J. Am. Chem. Soc. *125*, 1130–1131.

Ross, M.F., Filipovska, A., Smith, R.A., Gait, M.J., and Murphy, M.P. (2004). Cell-penetrating peptides do not cross mitochondrial membranes even when conjugated to a lipophilic cation: evidence against direct passage through phospholipid bilayers. Biochem. J. *383*, 457–468.

Rothbard, J.B., Jessop, T.C., Lewis, R.S., Murray, B.A., and Wender, P.A. (2004). Role of membrane potential and hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells. J. Am. Chem. Soc. *126*, 9506–9507.

Snyder, E.L., and Dowdy, S.F. (2004). Cell-penetrating peptides in drug delivery. Pharm. Res. *21*, 389–393.

Terrone, D., Sang, S.L., Roudaia, L., and Silvius, J.R. (2003). Penetratin and ated cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. Biochemistry *42*, 13787–13799.

 ©2008 Elsevier Ltd All rights reserved

Torchilin, V.P., Rammohan, R., Weissig, V., and Levchenko, T.S. (2001). Tat peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. Proc. Natl. Acad. Sci. USA *98*, 8786–8791.

Urban, B.W., Hladky, S.B., and Haydon, D.A. (1980). Ion movement in gramicidin pores: an example of single-file transport. Biochim. Biophys. Acta *602*, 331–354.

Wender, P.A., Mitchell, D.J., Pattabiraman, K., Pelkey, E.T., Steinman, L., and Rothbard, J.B. (2000). The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. Proc. Natl. Acad. Sci. USA *97*, 13003–13008.

Zhao, K., Zhao, G.-M., Wu, D., Soong, Y., Birk, A.V., Schiller, P.W., and Szeto, H.H. (2004). Cell-permeable pepide antioxidants targeted to inner mitochondrial membrane inhibit mitochondrial swelling, oxidative cell death, and reperfusion injury. J. Biol. Chem. *279*, 34682–34690.

 ©2008 Elsevier Ltd All rights reserved