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Multiple Triphenylphosphonium Cations Shuttle a Hydrophilic Peptide into Mitochondria

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Abstract: A variety of diseases are related to mitochondrial dysfunction. Hence, the ability to transport drugs to mitochondria that are otherwise cell impermeable would be of great therapeutic potential. Triphenylphosphonium (TPP) cations have been shown to accumulate in mitochondria when attached to small molecules. Here we report on the consequence of increasing the number of TPP moieties that are covalently linked to a model hydrophilic peptide Hemagglutinin A (HA). By extending the HA peptide with L-lysine amino acids to which the TPP's are covalently linked through the ε-amine, we have systematically synthesized the HA peptide with 0−3 TPP's. All peptides were subsequently labeled with FITC at the N-terminus. Cellular uptake and mitochondrial localization of the HA−TPP conjugates in HeLa cells were profoundly augmented with increasing number of TPPs, suggesting that this approach is applicable for the delivery of peptides. Furthermore, confocal microscopy demonstrated that the peptides localize to mitochondria. Importantly, all peptide conjugates did not show apparent toxicity at concentrations that are several orders of magnitude higher than those used for HA peptide delivery.

Keywords: Triphenylphosphonium (TPP) cations; mitochondria; delivery; lipophilic peptide

Introduction

The cell's key source of energy (ATP) is manufactured by oxidative phosphorylation exclusively in mitochondria. Accumulating evidence points to mitochondrial dysfunction as a critical factor in the emergence of cancer as well as several neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases.[1,2]

In recent years, several studies have shown the beneficial outcome of actively transporting therapeutic molecules to mitochondria.[2−18] Murphy and co-workers have shown that specific delivery of antioxidants to mitochondria protects these organelles from oxidative damage. In these studies, a single lipophilic triphenylphosphonium (TPP) cation was conjugated to antioxidants such as mitoquinone (MitoQ) and α-tocopherol for mitochondrial targeting,[15,19,20] although it

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(1) Scheffler, I. E. A century of mitochondrial research: achievements and perspectives. *Mitochondrion* **2001**, *1*, 3–31.

(2) Szewczyk, A.; Wojtczak, L. Mitochondria as a pharmacological target. *Pharmacol. Rev.* **2002**, *54*, 101–127.

(3) Stoyanovsky, D. A.; Vlasova, I. I.; Belikova, N. A.; Kapralov, A.; Tyurin, V.; Kagan, V. E. Activation of NO donors in mitochondria: Peroxidase metabolism of (2-hydroxyamino-vinyl)-triphenyl-phosphonium by cytochrome c releases NO and protects cells against apoptosis. *FEBS Lett.* **2008**, *582*, 725–728.

(4) Kyriakouli, D. S.; Boesch, P.; Taylor, R. W.; Lightowlers, R. N. Progress and prospects: gene therapy for mitochondrial DNA disease. *Gene Ther.* **2008**, *15*, 1017–1023.

(5) Hoye, A. T.; Davoren, J. E.; Wipf, P.; Fink, M. P.; Kagan, V. E. Targeting mitochondria. *Acc. Chem. Res.* **2008**, *41*, 87–97.

(6) Horton, K. L.; Stewart, K. M.; Fonseca, S. B.; Guo, Q.; Kelley, S. O. Mitochondria-penetrating peptides. *Chem. Biol.* **2008**, *15*, 375–382.

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should be noted that compounds with antioxidant activity (e.g., MitoQ) have been shown to elicit pro-oxidative activity, depending on experimental conditions.[21] Such TPP molecules have also been used as imaging agents for studying mitochondrial dysfunction.[22−26]

The accumulation of lipophilic cations in the mitochondrial matrix is driven by the electrochemical gradient that generates a negative charge in the matrix.[27] The mitochondrial membrane potential ($\Delta\Psi$mit) is typically 140−180 mV (negative inside)[9,28] in comparison to only 30−60 mV of the plasma membrane potential ($\Delta\Psi$p). According to Nernst equation, every ~60 mV increase in the $\Delta\Psi$ leads to a 10-fold increase in cation concentration in the mitochondrial matrix.[9] In addition, the mitochondrial membrane potential is significantly higher in cancer cells (e.g., 163 mV in colon carcinoma cells) than in normal epithelial cells (e.g., 104 mV in monkey kidney epithelial cells).[16,29] Such differences can account for a 10-fold increase of TPP molecules in the mitochondria of malignant cells.

However, molecules carrying a single TPP are limited in their cellular and mitochondrial uptake when possessing an overall hydrophilic character, as the case of TPMP (triphenylmethylphosphonium).[30]

Interestingly, even when two lipophilic TPP cations were linked by various alkyl chains (denoted PC_nP, where $n = 2$, 4, 5, 6 and 10) no significant cellular uptake was observed for all the shorter alkyl bridge linkers ($n = 2$, 4, 5 and 6) and $PC_{10}P$ uptake was not greater than TPMP.[31] Thus it seems that two positive charges are not sufficient to bring about cellular (and as a consequence mitochondrial) uptake

(7) Hickey, J. L.; Ruhayel, R. A.; Barnard, P. J.; Baker, M. V.; Berners-Price, S. J.; Filipovska, A. Mitochondria-Targeted Chemotherapeutics: The rational design of gold(I) N-heterocyclic carbene complexes that are selectively toxic to cancer cells and target protein selenols in preference to thiols. *J. Am. Chem. Soc.* **2008**, *130*, 12570–12571.

(8) Yamada, Y.; Akita, H.; Kogure, K.; Kamiya, H.; Harashima, H. Mitochondrial drug delivery and mitochondrial disease therapy - An approach to liposome-based delivery targeted to mitochondria. *Mitochondrion* **2007**, *7*, 63–71.

(9) Mukhopadhyay, A.; Weiner, H. Delivery of drugs and macromolecules to mitochondria. *Adv. Drug Delivery Rev.* **2007**, *59*, 729–738.

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

(11) Weissig, V.; Boddapati, S. V.; Cheng, S. M.; D'Souza, G. G. M. Liposomes and liposome-like vesicles for drug and DNA delivery to mitochondria. *J. Liposome Res.* **2006**, *16*, 249–264.

(12) Szeto, H. H. Mitochondria-targeted peptide antioxidants: Novel neuroprotective agents. *AAPS J.* **2006**, *8*, E521–E531.

(13) Wipf, P.; Xiao, J. B.; Jiang, J. F.; Belikova, N. A.; Tyurin, V. A.; Fink, M. P.; Kagan, V. E. Mitochondrial targeting of selective electron scavengers: Synthesis and biological analysis of hemigramicidin-TEMPO conjugates. *J. Am. Chem. Soc.* **2005**, *127*, 12460–12461.

(14) Weissig, V.; Cheng, S. M.; D'Souza, G. G. M. Mitochondrial pharmaceutics. *Mitochondrion* **2004**, *3*, 229–244.

(15) Asin-Cayuela, J.; Manas, A. R. B.; James, A. M.; Smith, R. A. J.; Murphy, M. P. Fine-tuning the hydrophobicity of a mitochondria-targeted antioxidant. *FEBS Lett.* **2004**, *571*, 9–16.

(16) Costantini, P.; Jacotot, E.; Decaudin, D.; Kroemer, G. Mitochondrion as a novel target of anticancer chemotherapy. *J. Natl. Cancer Inst.* **2000**, *92*, 1042–1053.

(17) Biswas, G.; Jeon, O.-Y.; Sirl Lee, W.; Kim, D.-C.; Kim, K.-T.; Lee, S.; Chang, S.; Chung, S.-K. Novel guanidine-containing molecular transporters based on lactose scaffolds: Lipophilicity effect on the intracellular organellar selectivity. *Chem.−Eur. J.* **2008**, *14*, 9161–9168.

(18) Murphy, M. P.; Smith, R. A. J. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 629–656.

(19) Cocheme, H. M.; Kelso, G. F.; James, A. M.; Ross, M. F.; Trnka, J.; Mahendiran, T.; Asin-Cayuela, J.; Blaikie, F. H.; Manas, A. R. B.; Porteous, C. M.; Adlam, V. J.; Smith, R. A. J.; Murphy, M. P. Mitochondrial targeting of quinones: Therapeutic implications. *Mitochondrion* **2007**, *7*, S94–S102.

(20) Kelso, G. F.; Porteous, C. M.; Coulter, C. V.; Hughes, G.; Porteous, W. K.; Ledgerwood, E. C.; Smith, R. A. J.; Murphy, M. P. Selective targeting of a redox-active ubiquinone to mitochondria within cells - Antioxidant and antiapoptotic properties. *J. Biol. Chem.* **2001**, *276*, 4588–4596.

(21) Doughan, A. K.; Dikalov, S. I. Mitochondrial redox cycling of mitoquinone leads to superoxide production and cellular apoptosis. *Antioxid. Redox Signaling* **2007**, *9*, 1825–1836.

(22) Yang, C.-T.; Kim, Y.-S.; Wang, J.; Wang, L.; Jiyun Shi, J.; Li, Z.-B.; Chen, X.; Fan, M.; Li, J.-J.; Liu, S. 64Cu-Labeled 2-(Diphenylphosphoryl)ethyldiphenylphosphonium cations as highly selective tumor imaging agents: Effects of linkers and chelates on radiotracer biodistribution characteristics. *Bioconjugate Chem.* **2008**, *19*, 2008–2022.

(23) Wang, J. J.; Yang, C. T.; Kim, Y. S.; Sreerama, S. G.; Cao, Q. Z.; Li, Z. B.; He, Z. J.; Chen, X. Y.; Liu, S. Cu-64-Labeled triphenylphosphonium and triphenylarsonium cations as highly tumor-selective imaging agents. *J. Med. Chem.* **2007**, *50*, 5057–5069.

(24) Madar, I.; Ravert, H.; DiPaula, A.; Du, Y.; Dannals, R. F.; Becker, L. Assessment of severity of coronary artery stenosis in a canine model using the PET agent F-18-fluorobenzyl triphenyl phosphonium: Comparison with Tc-99m-tetrofosmin. *J. Nucl. Med.* **2007**, *48*, 1021–1030.

(25) Kanai, A.; Zabbarova, I.; Amoscato, A.; Epperly, M.; Xiao, J.; Wipf, P. Mitochondrial targeting of radioprotectants using peptidyl conjugates. *Org. Biomol. Chem.* **2007**, *5*, 307–309.

(26) Cheng, Z.; Subbarayan, M.; Chen, X. Y.; Gambhir, S. S. Synthesis of (4-[F-18]fluorophenyl)triphenylphosphonium as a potential imaging agent for mitochondrial dysfunction. *J. Labelled Compd. Radiopharm.* **2005**, *48*, 131–137.

(27) Ono, A.; Miyauchi, S.; Demura, M.; Asakura, T.; Kamo, N. Activation-energy for permeation of phosphonium cations through phospholipid-bilayer membrane. *Biochemistry* **1994**, *33*, 4312–4318.

(28) Martin, J.; Mahlke, K.; Pfanner, N. Role of an energized inner membrane in mitochondrial protein import - Delta-psi drives the movement of presequences. *J. Biol. Chem.* **1991**, *266*, 18051–18057.

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

of the compounds when the alkyl chain is too short (i.e., not sufficiently hydrophobic).

Would multiple TPP moieties (with a cumulatively higher positive charge) have the propensity of shuttling hydrophilic molecules?

A recent study has also shown that conjugating a cell penetrating peptide (CPP, e.g. penetratin) to TPP did not result in the conjugate's mitochondrial uptake.[32] Hence, it is still unclear what is the potential of TPP molecules in leading peptides into mitochondria.

To this end we took a systematic approach to evaluate the capacity of TPP conjugation in mitochondrial delivery of a short peptide.

Here we explored the outcome of increasing the number of TPP molecules on a hydrophilic nonapeptide cargo as a means of improving its cellular uptake and, as a consequence, its mitochondrial accumulation. We have looked at the hemagglutinin A epitope, YPYDVPDYA (HA), as a model peptide, because this short peptide is hydrophilic, has negligible membrane permeability characteristics and is intracellularly stable as demonstrated by its fusion to numerous cellular proteins.

To circumvent any possible artifacts of its detection by virtue of anti-HA antibodies, we equipped it with a FITC label. Our results show a gradual increase in cellular and mitochondrial uptake of HA−TPP conjugates as a function of increasing the appended TPP molecules.

Experimental Section

Solvents and Reagents. Dry solvents (dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF), over molecular sieves) were purchased from Acros and used as received. All Fmoc amino acids and coupling reagents including *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU), *N*-hydroxybenzotriazole (HOBT), *N*,*N*′-dimethylaminopyridine (DMAP) and *N*,*N*′-diisopropylcarbodiimide (DIC) were purchased from GL Biochem (Shanghai). 4-Carboxybutyltriphenylphosphonium bromide, 6-aminohexanoic acid and fluorescein isothiocyanate (FITC) were purchased from Acros.

Peptide Conjugate Synthesis. Peptides conjugates were manually synthesized on a solid support using commercially available Wang resin (1.47 mmol/g, GL Biochem). All peptides were synthesized on a 0.2 mmol scale. For the first coupling to the resin, 10 equiv of Fmoc-L-Ala, 5 equiv of DIC and 0.1 equiv of DMAP were dissolved in dry DCM and stirred for 15 min in an ice bath followed by the addition of this solution to the preswelled (DCM) resin. The coupling reaction was continued with occasional shaking for 1 h. This coupling was repeated, followed by capping using 1 mL of acetic anhydride, 2 mL of pyridine and 2 mL of DCM. Further coupling reactions were performed using 4 equiv of Fmoc protected amino acid, 4 equiv of HBTU, 4 equiv of HOBT and 8 equiv of DIEA in dry DMF for 1 h. Coupling of amino acids following L-proline was performed twice. Deprotection of the Fmoc group was achieved using 20% piperidine in DMF (v/v) for 20 min.

The Ahx (6-aminohexanoic acid) linker was protected with Fmoc as previously described[33] and coupled by the same method as described above.

Trityl (trt) group on 1, 2, or 3 ε-amines of L-lysine (For 1−3 L-lysine amino acids, respectively) was deprotected using 3% TFA in DCM for 10 min and this procedure was repeated 2 additional times. Coupling of the TPP-acid (4-Carboxybutyltriphenylphosphonium bromide) on the free ε-amines (of 1, 2, or 3 L-lysines) was performed in the same way as described for Fmoc-protected amino acids. The coupling was repeated twice and monitored by the Kaiser test.[34]

Peptides were cleaved and deprotected in a solution of 88:5:5:2 TFA/water/phenol/triethylsilane for 2 h then precipitated with diethyl ether, spun-down by centrifugation and washed with diethyl ether. Peptides were purified by RP HPLC (Shimatzu LC 2010C, flow rate = 4 mL/min) on a C18 reverse phase column (Phenomenex, Jupiter 300A), using a linear gradient of 5 to 80% acetonitrile in 0.1% TFA over 35 min. Peptide conjugate peaks were monitored at 273 nm. The major peaks were collected, lyophilized and identified by ESI-MS (Figures S5−S8 in the Supporting Information). Purified peptides 0−3 were reinjected in an analytical RP-HPLC column (Figures S1−S4 in the Supporting Information). Estimated yields based on HPLC chromatograms were 55%, 37%, 37% and 50% for peptides 0−3, respectively.

Peptide labeling with FITC was performed in a DMF solution containing 1.5 equiv of FITC and 2 equiv of DIEA. Reaction mixtures were stirred for 48 h at room temperature in the dark under an inert atmosphere (argon). The solvent was then evaporated, and the resulting peptides were purified by RP HPLC by the same conditions and monitored at 495 nm (FITC absorption). The major peak was collected and lyophilized. The yield of the FITC coupling was 85−90% for all peptides. Peptide 0 was obtained with FITC either on

(30) Ross, M. F.; Prime, T. A.; Abakumova, I.; James, A. M.; Porteous, C. M.; Smith, R. A. J.; Murphy, M. P. Rapid and extensive uptake and activation of hydrophobic triphenylphosphonium cations within cells. *Biochem. J.* **2008**, *411*, 633–645.

(31) Ross, M. F.; Da Ros, T.; Blaikie, F. H.; Prime, T. A.; Porteous, C. M.; Severina, I. I.; Skulachev, V. P.; Kjaergaard, H. G.; Smith, R. A. J.; Murphy, M. P. Accumulation of lipophilic dications by mitochondria and cells. *Biochem. J.* **2006**, *400*, 199–208.

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

(33) Khandazhinskaya, A. L.; Kukhanova, M. K.; Jasko, M. V. New nonnucleoside substrates for terminal deoxynucleotidyl transferase: Synthesis and dependence of substrate properties on structure. *Russ. J. Bioorg. Chem.* **2005**, *31*, 352–356.

(34) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* **1970**, *34*, 595–598.

terminal amine or ε-amine of L-lysine (not resolved by HPLC) and was used in all experiments as a mixture.

Cell Culture. HeLa cells, maintained in DMEM medium containing 10% fetal calf serum, 1% penicillin−streptomycin, 2% glutamate, were incubated in a humidified atmosphere incubator with 5% CO_2 at 37 °C. Cells were routinely subcultured every three days. For FACS experiments, cells were grown to subconfluence and dissociated from the surface with a solution of 0.05% trypsin/0.53 mM EDTA. Cells were then plated in 8-wells at 15×10^3 cells/well 1−2 days prior to the FACS experiment. For uptake experiments, 1×10^5 cells/well were plated in a 12-well plate 1 day prior to the experiment.

Determination of IC_{50}. Cells, maintained in DMEM medium containing 10% fetal calf serum, were plated in a 96 well plate (∼1000 cells/well) and incubated 24 h in a humidified atmosphere incubator with 5% CO_2 at 37 °C. The 4 peptides (peptides 0−3), dissolved in ethanol, were added at different concentrations (0.1−100 μM) in eight wells and incubated for 72 h in 5% CO_2 at 37 °C.

Final ethanol concentration was 0.5% (v/v) and was added in wells without peptide conjugates as control. Next, 30 μL of MTT solution was added to each well and incubated for 30 min in 5% CO_2 at 37 °C. Medium was discarded, and 100 μL of DMSO was added. IC_{50} values were determined by measuring the absorbance at 540 nm on a plate reader.

Determination of Log *P*. Log *P* values were determined for all peptide conjugates by the following procedure.[6] In brief, an aliquot of 100 μL of 300 μM peptide conjugate in Tris buffer (50 mM, pH = 7.4) and 100 μL of 1-octanol were added to a 2 mL eppendorf. The eppendorfs were vigorously vortexed for 1 min, and phases were allowed to separate. Next, 25 μL of the octanol layer was removed and diluted to 100 μL with 3:1 methanol:Tris. The aqueous layer was diluted 40-fold (with Tris buffer), and then 25 μL of the diluted solution was removed and diluted to 100 μL with 3:1 methanol:octanol for a final composition of 3:1:1 methanol:octanol:Tris for both organic and aqueous layers. Three dilutions were prepared per layer and the absorbance was read at 495 nm on an Ultrospec 2100pro UV−vis spectrophotometer. The mean A_{495} of the three dilutions was calculated for each layer. Log *P* was then calculated according to log *P* = log[(A_{495} of octanol layer)/(A_{495} of aqueous layer)].

Flow Cytometry. After incubation with 50, 100, and 500 nM peptide (peptides 0−3) for 30 min, cells were removed from the surface of the plate with trypsin/ EDTA (250 μL/ well) for 2 min at 37 °C. The trypsinization was quenched with 2 mL of complete DMEM per well, and an additional 1 mL of PBS was added to each well. From this point on, the samples were maintained on ice until analysis. The samples were transferred from the wells to sterile tubes, pelleted by centrifugation (8 min at 1100*g*), and resuspended in 1 mL of PBS.

Cells were analyzed with a flow cytometer (FACS-calibur, Becton-Dickinson). CaliBRITE-3 beads (Becton-Dickinson) were used to calibrate the FACS. A minimum of 50,000 cells were analyzed per sample. Cells passed at a rate of ∼1,000/s, using saline as the sheath fluid. A 488 nm argon laser beam was used for excitation. Each cell population was gated based on forward light scatter and side light scatter FACS.

Confocal Fluorescence Microscopy. HeLa cells were cultured on coverslips. For colocalization studies, the culture medium was removed and cells were washed in PBS (pH 7.4). Cells were incubated with 10−100 nM peptide-conjugates (peptides 0−3) for 45 min. Mitotracker M7512 (Invitrogen) at a final concentration of 25 nM was added 30 min post peptide conjugate addition. Alternatively, the mitochondrial uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was added to HeLa cells (in PBS) followed by the addition of peptide-conjugate (50 nM) and Mitotracker (25 nM) 10 min after the addition of CCCP. Total incubation time was 45 min. Cells were washed three times with PBS, fixed with 3% paraformaldehyde for 15 min and washed again with PBS (×3). The cover glasses were placed on slides with a drop of fluorescent mounting medium (Dako). Images were taken with an Olympus Fluoview 300. The excitation wavelength for visualization of the FITC-labeled peptides was 488 nm, and emission spectra were collected between 505 and 550 nm. The excitation wavelength for visualization of Mitotracker M7512 was 579 nm, and emission spectra were collected with a long-pass 599 nm filter.

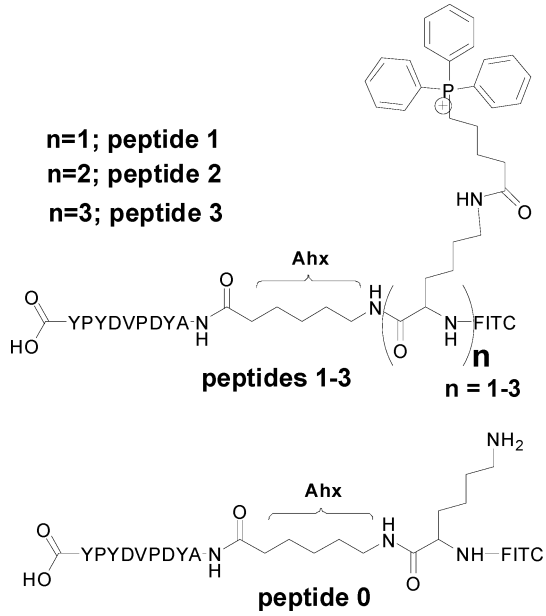
Results

Synthesis of Peptide−TPP Conjugates. All peptide conjugates were synthesized on the solid support (Wang resin) using standard N^{α}-Fmoc/*tert*-butyl peptide chemistry. The final structures of the various peptide conjugates are shown in Scheme 1.

After synthesizing the HA peptide (YPYDVPDYA), Fmoc-6-aminohexanoic acid (Ahx) was introduced in order to separate the peptide from the TPP units. Subsequently, 1, 2, and 3 L-lysine amino acids (Fmoc-L-Lys(Trt)-OH) were coupled to Ahx and on each ε-amine of L-lysine, 4-carboxybutyl triphenylphosphonium bromide was coupled in order to generate the peptide conjugates with 1, 2, and 3 TPP units (peptides 1−3). Next, the peptide−TPP conjugates were cleaved from the resin (by TFA treatment) and HPLC purified. Finally, peptides were reacted in solution with FITC for 48 h at room temperature. Peptide conjugates (FITC labeled) were HPLC purified (Figures S1−S4 in the Supporting Information) and further characterized by UV−vis spectroscopy and ESI-MS (Figures S5−S8 in the Supporting Information). The peptide conjugate with no TPP (peptide 0) was obtained by a similar synthetic approach by coupling one Fmoc-L-lysine to the Ahx spacer (Fmoc-Ahx), followed by peptide cleavage, HPLC purification and solution coupling with FITC. The synthetic route for all peptide conjugates is presented in Scheme 2. Peptide concentration was determined by UV−vis absorption of FITC (ε^{495} = 68,000).

Cellular Uptake of Peptide Conjugates (Peptides 0−3) in HeLa Cells. TPP conjugates that yield lipophilic compounds[15,18] internalize into cells by diffusion and subse-

Scheme 1. Chemical Structures of Peptide−TPP Conjugates (Peptides 1−3) and of Peptide-Conjugate without TPP (Peptide 0)[a]



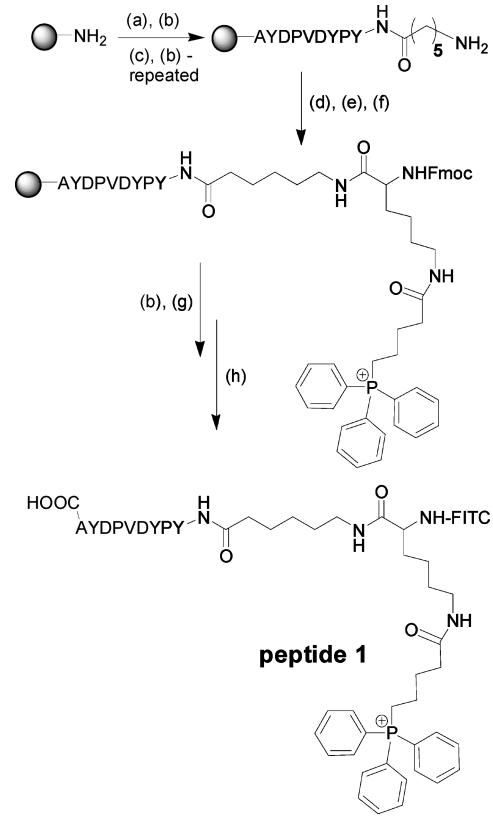
[a] All peptides are FITC-labeled.

quently localize into mitochondria. The HA peptide is hydrophilic with a total charge of −3 resulting from its carboxy C-terminus and two aspartic acid (D) side chains. Thus, such a peptide (either conjugated or not to the Ahx linker and lysine) is not expected to penetrate the cellular membrane. This was indeed the case, as can be seen for the HA conjugate that has no TPP (peptide 0). Fluorescence-activated cell sorting (FACS) analysis of HeLa cells treated with peptide 0 showed no cellular uptake (Figure 1, green trace). Interestingly, even after its conjugation to 1, 2, or 3 TPP molecules, we have found all peptide conjugates to be hydrophilic (see Table 1 for log *P* values).

In order to examine the effect of TPP on HA peptide cellular uptake, the various peptide conjugates were incubated at several concentrations (50 nM−0.5 μM) with HeLa cells (1×10^6 cells/well) for 30 min at 37 °C. Cellular uptake was then evaluated by FACS. As shown in Figure 1 (for 0.5 μM peptide conjugates), with increasing TPP moieties, a gradual increase in fluorescence was observed. This trend was consistent in all concentrations tested (Figures S9 and S10 in the Supporting Information).

To ensure that the observed fluorescence is due to peptide internalization and not a consequence of external membrane binding, we visualized peptide-conjugate uptake by confocal microscopy. The images (Figure S11 in the Supporting Information) clearly indicate that the peptide−TPP conjugates were internalized and that the FITC fluorescence increased with the number of TPPs. Interestingly, the peptides were excluded from the nucleus (Figure S11 in the Supporting Information).

Scheme 2. Description of the Synthesis of Peptide 1[a]



[a] Reagents and conditions: (a) DIC, DMAP, DCM, overnight; (b) 20% piperidine in DMF for 20 min; (c) Fmoc-L-amino acid or Fmoc-Ahx, HATU, HOBT, DIEA, 1 h; (d) Fmoc-L-Lys(trt)-OH, HATU, HOBT, DIEA, 1 h; (e) 3% TFA in DCM (10 min × 2); (f) 4-carboxybutyl triphenylphosphonium bromide, HATU, HOBT, DIEA, 1 h; (g) TFA:triethylsilane:phenol:H_2O (88:2:5:5), 2 h followed by HPLC purification; (h) FITC, DIEA in DMF, 48 h followed by HPLC purification.

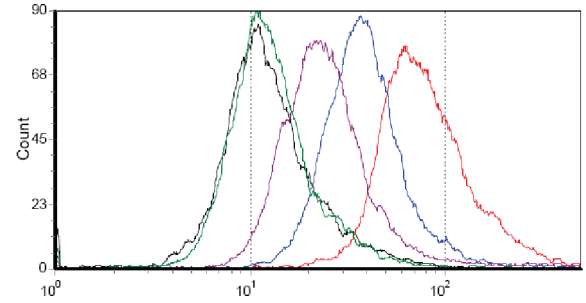


Figure 1. FACS analysis for cellular uptake into HeLa cells of peptide conjugates (0.5 μM) after a 30 min incubation period. Black and green traces = control (no peptide) and peptide without TPP (peptide 0), respectively. Purple, blue and red traces = peptide with one, two and three TPPs (peptides 1−3), respectively.

Peptide−TPP Conjugates Are Localized to Mitochondria of Intact Cells. To determine whether peptide−TPP conjugates traffic to the mitochondria in living cells, HeLa cells were incubated with the various peptide conjugates (50 nM) for 45 min and with Mitotracker (25 nM, M7512, Invitrogen); added 30 min after initial peptide−conjugate addition. Cells were thoroughly washed with buffer (PBS)

Table 1. Log *P* Values (Tris Buffer:1-Octanol) of Peptide Conjugates (Peptides 0−3) as Determined by UV−Vis Absorbance of FITC

	peptide 0	peptide 1	peptide 2	peptide 3
log *P*	−2.77 (±0.05[a])	−1.94 (±0.05)	−2.70 (±0.05)	−2.93 (±0.05)
net charge of peptide	−2	−2	−1	0

[a] Three UV−vis measurements were repeated for each peptide conjugate.

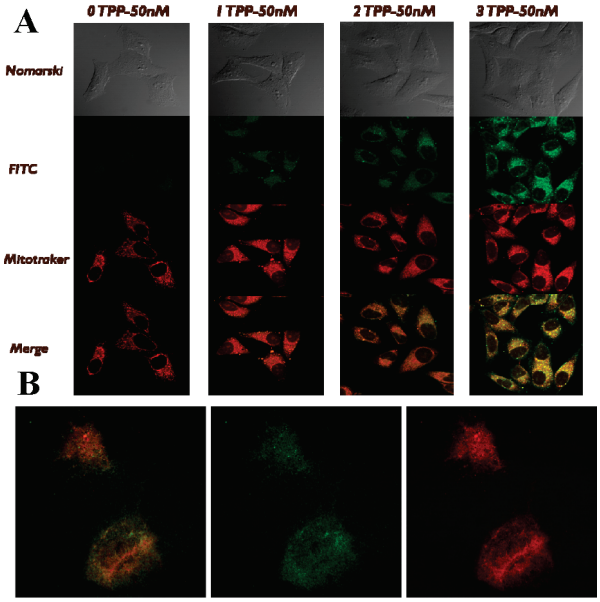


Figure 2. (A) Mitochondrial uptake of peptide conjugates (50 nM) as monitored by confocal microscopy. HeLa cells were treated with peptide conjugates (50 nM) followed by Mitotracker (25 nM). (B) Uptake of peptide 3 and Mitotracker after poisoning HeLa cells with the mitochondrial uncoupler (CCCP). Right panel: Mitotracker. Center panel: FITC-labeled peptide. Left panel: merge. Cells were treated with CCCP (5 μM), and after 10 min, peptide 3 (50 nM) and Mitotracker (25 nM) were added. Total incubation time = 45 min. Images were taken after mild fixation.

followed by gentle cell fixation with paraformaldehyde. Confocal microscopy demonstrated a clear colocalization of the peptide conjugates with the mitochondrial stain (Figure 2(A)). Importantly, the mitochondrial uptake of the various peptide conjugates was higher as the number of TPPs was increased. As expected, the peptide conjugate with FITC and no TPP moieties (peptide 0) had no significant uptake indicating that the FITC did not confer cellular uptake on its own.

To further verify mitochondrial localization of peptide conjugates, a mitochondrial uncoupler (CCCP = carbonyl cyanide 3-chlorophenylhydrazone) was added to cells prior to the addition of the peptide conjugate and Mitotracker. As shown in Figure 2(B), both peptide 3 and Mitotracker are diffused throughout the cell and not colocalized. This further emphasizes that the peptide conjugates are predominantly localized to mitochondria.

Peptide−TPP Conjugates Are Not Toxic to HeLa Cells. All peptide conjugates (peptides 0−3) were added to HeLa cells at several concentrations, and cell toxicity was determined by the MTT assay (Figure S12 in the Supporting Information). No apparent toxicity was observed for all peptide conjugates at concentrations of up to 100 μM (0.1−100 μM).

Discussion

We have shown a straightforward synthetic approach for modifying a hydrophilic peptide (HA) with one to three TPP molecules by extending the peptide with L-lysine amino acids to which the TPP unit is covalently linked through the ε-amine group. Such methodology could, in principle, be applied to any given bioactive peptide that is membrane impermeable. The effect of increasing the number of TPP units was evaluated by FACS and confocal microscopy and is consistent with an improved cellular and mitochondrial uptake of peptide conjugates into HeLa cells with increasing TPP units.

Although peptide conjugates are all hydrophilic, they internalize into mitochondria of living cells with increasing TPP moieties. These results are consistent with a mechanism of uptake that is charge-driven. Thus, the greater accumulation of the delocalized positive charges on the added TPP units provides an electrochemical driving force for improved entry of the peptide−TPP conjugates both through the cell's outer membrane and consequently through the mitochondrial membrane.

Such a charge-dependent mechanism of uptake has been recently proposed for mitochondria-penetrating peptides that are taken into mitochondria of several cancerous cell lines (HeLa, MRC-5 and MCF-7) although being relatively hydrophilic.[6]

Thus, a peptide such as HA, which has no cellular uptake and is hydrophilic (log $P = -2.93$) was internalized into HeLa cells and subsequently to HeLa mitochondria at a very low concentration (10 nM) when conjugated to 3 TPPs (see Figure S11 in the Supporting Information).

Furthermore, the fact that the peptide conjugates are nontoxic at concentrations of up to 100 μM suggests that they do not markedly disrupt the mitochondrial membrane potential, especially at the lower concentrations that were examined in this study (10−500 nM).

This approach can be potentially applied to bioactive peptides, such as the viral protein r1 (vpr1) derivative[35] that triggers a mitochondrial-dependent apoptotic response or

(35) Borgne-Sanchez, A.; Dupont, S.; Langonne, A.; Baux, L.; Lecoeur, H.; Chauvier, D.; Lassalle, M.; Deas, O.; Briere, J. J.; Brabant, M.; Roux, P.; Pechoux, C.; Briand, J. P.; Hoebeke, J.; Deniaud, A.; Brenner, C.; Rustin, P.; Edelman, L.; Rebouillat, D.; Jacotot, E. Targeted Vpr-derived peptides reach mitochondria to induce apoptosis of alpha(V)beta(3)-expressing endothelial cells. *Cell Death Differ.* **2007**, *14*, 422–435.

shepherdin[36] that elicits antiapoptotic activity by inhibiting mitochondrial HSP90 (TRAP-1) activity.

Conclusions

In this study we have shown that cellular uptake of a hydrophilic nonapeptide (HA) into mitochondria of HeLa cells is gradually improved with increasing the number of conjugated lipophilic cations (TPP). At a concentration as low as 10 nM the fully modified HA peptide (peptide 3) is found in HeLa cells.

Most importantly, the HA peptide lacking TPPs (peptide 0) has no apparent cellular uptake into HeLa cells, however, when conjugated to several TPPs, the HA conjugates localize to mitochondria of living cells. Such a strategy may be useful for the delivery of mitochondrial-effector molecules that are otherwise cellular and/or mitochondrial impermeable. Further elucidation of the specific cellular uptake is currently under investigation.

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Supporting Information Available: RP-HPLC chromatograms of purified peptide conjugates (peptides 0−3) and corresponding ESI-MS; FACS analysis, MTT assay and confocal microscopy images of peptide conjugates (peptides 0−3) in HeLa cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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(36) Gyurkocza, B.; Plescia, J.; Raskett, C. M.; Garlick, D. S.; Lowry, P. A.; Carter, B. Z.; Andreeff, M.; Meli, M.; Colombo, G.; Altieri, D. C. Antileukemic Activity of Shepherdin and Molecular Diversity of Hsp90 Inhibitors. *J. Natl. Cancer Inst.* **2006**, *98*, 1068–1077.