Novel cell-penetrating peptide targeting mitochondria

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ABSTRACT **Cell-penetrating peptides (CPPs) are short, nontoxic peptides with cationic and/or amphipathic properties able to cross the cellular membrane. CPPs are used for the delivery of a wide variety of cargoes, such as proteins, oligonucleotides, and therapeutic molecules. The aim of the present study was to synthesize unusually small novel CPPs targeting mitochondria based on the Szeto-Schiller peptide (SS-31) to influence intramitochondrial processes and to improve the biologic effects. All the peptides used were synthesized manually using 9-fluorenylmethyloxycarbonyl chemistry. In the first part of the study, HeLa 705, U87, and bEnd.3 cells were used as *in vitro* delivery model. Cells were incubated for 24 h at 37°C and 5% CO_2 with different concentrations of our peptides. Cell proliferation assay was performed to evaluate cell viability. Biologic effects such as mitochondrial membrane potential and antioxidant activity were evaluated. H_2O_2 was used as positive control. Uptake studies were performed using peptides conjugated with 5(6)-carboxyfluorescein (FAM). Fluorescent microscopy was used to determine presence and localization of peptides into the cells. Isolated mitochondria from pretreated cells and mitochondria treated after isolation were used to confirm the targeting ability of the peptide. Uptake of FAM alone was used as negative control. Microscopy studies confirmed the ability of peptides to penetrate cell. Localization analysis showed increase in uptake by 35% compared with SS-31. Mitochondrial CPP 1 (mtCPP-1) had no effect on mitochondrial membrane potential and prevented reactive oxygen species formation in bEnd.3 cells by 2-fold compared with SS-31. No cytotoxicity was observed even at high concentration (100 μM). These data suggest that mtCPP-1 is a mitochondrial CPP and protect mitochondria from oxidative damage due to its own antioxidant activities.—Cerrato, C. P., Pirisinu, M., Vlachos, E. N., Langel, Ü. Novel cell-penetrating peptide targeting mitochondria. *FASEB J.* 29, 4589–4599 (2015). www.fasebj.org**

Key Words: mitochondrial targeting · ROS reduction · mitochondrial membrane potential · antioxidant activity

The plasma membrane is a tightly controlled barrier designed to protect the cell from unregulated influx of bioactive molecules and ions, and in this way, the cell regulates its internal environment. Most drugs need to cross one or more cellular membranes to have any effect. Small molecules are able to cross the cellular membrane on their own but due to their physicochemica characteristics, many larger drugs cannot enter cells. A delivery system must be efficient, safe, and nontoxic. There are 2 kinds of delivery system, viral and nonviral. This study used a nonviral delivery system: cell-penetrating peptide (CPP). CPPs are short peptides with a sequence of up to 30 amino acids that are able to cross the cellular membrane and transport bioactive cargo into cells in a nontoxic and efficient way. These peptides are amphipathic, carry a positive charge, and show both hydrophilic and lipophilic properties.

A major breakthrough in the identification of this transporter goes back to the late 1980s when a series of short natural peptide sequences were identified that efficiently crossed the plasma membrane (1, 2). Full-length HIV-1 transcription transactivation protein and *Drosophila* Antennapedia homeodomain, known as Penetratin, received great attention when discovered due to their ability to cross the cellular membrane and to accumulate in the cellular nucleus (3). These discoveries were the basis for a new field focused on the use of CPPs as molecular carriers.

In the last 20 yr, chemists and biochemists have synthesized many peptides with modifications in the structure to improve the ability to cross the cell membrane, maintaining low toxicity and immunogenicity. There are more than 100 CPPs available with different amino sequences, physicochemical properties, and internalization mechanisms. Some CPPs are derived from natural proteins and others are artificial constructs engineered to have the important features of the natural molecules. Identification of cellular targets for treatment of different diseases required the development of a system able to delivery drugs into the target of interest. Different cargoes can be conjugated to CPPs, from small molecules such as small peptides and proteins, fluorophores, and small molecular drugs, to larger cargoes such as oligonucleotides, plasmids, and protein (4). Uptake and efficacy of several therapeutic compounds are improved by conjugation with CPPs, creating new opportunity to study the biologic process and developing new treatments for several diseases.

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; CPP, cell-penetrating peptide; $\Delta\psi_m$, mitochondrial membrane potential; Dmt, 2,6-dimethyl-L-tyrosine; DLS, dynamic light scattering; FAM, 5(6)-carboxyfluorescein; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; Fmoc, 9-fluorenylmethyloxycarbonyl; mtCPP-1, mitochondrial cell-penetrating peptide 1; ROS, reactive oxygen species; SS, Szeto-Schiller; TMRE, tetramethylrhodamine methyl ester

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CPPs are used as one of the most useful strategies to delivery molecules/drugs inside specific cellular organelles such as nucleus and mitochondria. Cellular nucleus can be targeted using nuclear localization sequences, short cationic sequences with 10 amino acids in length. The most promising field of application of nuclear localization sequences conjugated with CPPs is for cancer treatment. These sequences are widely used to achieve nuclear delivery for a variety of DNA-damaging agents or nucleic acids for gene therapy (5). Another important target for the delivery of molecules within the cell is the mitochondria. This target is reached using designed peptides, rather than natural signal sequences (6). Mitochondria are a crucial target for drug therapy due to their role in the pathology of cancer, neurodegenerative diseases, and other diseases where reactive oxygen species (ROS) are linked with disease such as cardiovascular disease and diabetes.

The short Szeto-Schiller (SS) peptides developed by Szeto and Schiller are the most promising mitochondria-targeted antioxidants. SS peptides are tetrapeptides designed with alternating aromatic residues and basic amino acids (7). The antioxidant action of SS peptides in scavenging ROS and inhibiting LDL oxidation was attributed to the tyrosine or 2,6-dimethyl-L-tyrosine (Dmt) residues, and to the position of this residues in the sequence, while the replacement of Dmt with phenylalanine resulted in complete loss of antioxidant activity (8). Tyrosine can scavenge oxyradicals, forming relatively unreactive tyrosyl radicals, which can be followed by radical–radical coupling to create dityrosine, or react with superoxide to form tyrosine hydroperoxide (9). Dmt is more effective than tyrosine in scavenging ROS because it bears much structural similarity to vitamin E. Zhao *et al.* (8) showed that cells treated with the SS peptides, after induction of oxidative stress by *tert*-butyl hydroperoxide, had decreased levels of mitochondrial ROS and halted the progression of apoptosis. SS peptides are taken up into cells in an energy-independent nonsaturable manner. The uptake is really fast, and they concentrate preferentially in mitochondria compared with the cytosolic compartment. The mechanism of uptake into mitochondria is still unclear. These aromatic-cationic peptides, unlike coenzyme Q (Mito Q), are not taken up due to mitochondrial membrane potential ($\Delta\psi_m$) but many studies showed that they are also concentrated in depolarized mitochondria (10).

In the present study, we designed and synthesized a series of peptides able to cross both the plasma and the mitochondrial membrane. We analyzed mitochondrial uptake of CPPs by isolated mitochondria and fluorescence microscopy. Furthermore, we investigated the effects of mitochondrial CPP 1 (mtCPP-1) on oxidative stress and on $\Delta\psi_m$.

MATERIALS AND METHODS

Design, synthesis, purification, and analysis of mitochondria CPPs

All the peptides were designed based on the sequence and characteristics of SS-31 peptide. Peptides were synthesized manually by solid phase peptide synthesis using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry (11). Standard Fmoc solid phase peptide synthesis was performed in polypropylene syringes equipped with polytetrafluoroethylene filter using a Rink-amide Chemmatrix resin (PCAS BioMatrix, Québec, Canada). The resin was swollen in dichloromethane for 10 min. At each coupling step, we used Fmoc-protected D- or L-amino acids dissolved in *N,N*-dimethylformamide mixed with 6-chloro-1-hydroxybenzotriazole and *O*-(6-chlorobenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate, used as coupling reagents, and *N,N*-diisopropylethylamine for 30 min (Supplemental Table 1). The Fmoc group was removed by treatment with piperidine (20% v/v) in *N,N*-dimethylformamide (20 min). Each coupling/deprotection step was evaluated by qualitative ninhydrin (triketohydrindene hydrate) test. After coupling of the last amino acid, the crude peptide was cleaved for 4 h in a mixture of 95% trifluoroacetic acid, 2.5% H_2O, and 2.5% triisopropylsilane, precipitated in cold diethyl ether and purified by reversed-phase high performance liquid chromatography on a BioBasic C-8 column (Thermo Fisher Scientific, Göteborg, Sweden) using a 20–100% gradient of acetonitrile in water with addition of 0.1% trifluoroacetic acid to both solvents. The purified peptides were lyophilized and the molecular mass of peptides was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry on a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA), using α-cyano-4-hydroxycinnamic acid as matrix.

Cell viability

Cell viability was determined by conventional 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) toxicity assay (Roche Diagnostics Scandinavia AB, Bromma, Sweden) according to the manufacturer's instructions. Briefly, U87 (human primary glioblastoma cell line), HeLa 705 (a human cervical carcinoma cell line), and bEnd.3 (mouse brain endothelial cell line) cells were seeded (1×10^4 cells/well) into 96-well plates, 24 h prior to experiments. The cells were treated with peptides at different concentrations and incubated for 24 h in complete medium—DMEM with GlutaMax (Thermo Fisher Scientific) supplemented with 0.1 mM nonessential amino acids, 10% fetal bovine serum, 200 U/ml penicillin, and 200 μg/ml streptomycin (Invitrogen, Stockholm, Sweden). H_2O_2 at 200 and 400 μM for 2 h was used as positive control. WST-1 cell proliferation reagent was added to each well at final dilution 1:10. After 4 h incubation, absorbance was measured at 450 nm on Sunrise microplate absorbance reader (Tecan, Männedorf, Switzerland). The same assay was performed to evaluate the viability after insult with H_2O_2. Briefly, HeLa 705 cells were seeded and treated as described above. After 24 h incubation of mtCPP-1, H_2O_2 at 200 μM was added for 2 h to induce an oxidative stress. WST-1 cell proliferation reagent was added to each well as described above and incubated for 4 h, then the absorbance was measured. Cell viability of the treated cells was expressed as viability in percentage of untreated cells *versus* cells treated with mtCPP-1 or H_2O_2.

$\Delta\psi_m$ assay

$\Delta\psi_m$ was evaluated using the fluorescent probe tetramethylrhodamine methyl ester (TMRE) (mitochondrial potential membrane assay kit; Abcam, Cambridge, United Kingdom). TMRE is a cellular positively charged permeant dye that readily accumulates in active mitochondria due to its relative negative charge. Depolarized and/or nonfunctional mitochondria have decreased membrane potential and fail to sequester TMRE. Briefly, U87, bEnd.3, and HeLa 705 cells were seeded into 96-well plates 24 h before the treatments. Cells were treated with mtCPP-1 for 24 h at different concentrations as previously described. H_2O_2 (200 μM) was used as a positive control. TMRE (400 nM) was added to the complete medium, and cells were incubated for 30 min at 37°C, 5% CO_2, protected from light. The medium containing TMRE

was aspirated. The cells were washed once with 0.2% bovine serum albumin (BSA) in PBS to remove background fluorescence from the cell culture medium, and 100 μl/well of 0.2% BSA in PBS was added. The plate was read on a fluorescence reader (peak excitation = 549 nm, peak emission = 575 nm). The same assay was performed to evaluate the ability of mtCPP-1 to restore a physiologic $\Delta\psi_m$ after insult with H_2O_2. Briefly, cells were plated and treated with mtCPP-1 at different concentrations as previously described. After 2 h, cells were insulted by adding H_2O_2 200 μM and incubated for 24 h at 37°C and 5% CO_2. TMRE was added to the medium in each well to a final concentration of 400 nM. Cell medium was aspired after 30 min and cells were washed once with 0.2% BSA in PBS and replaced with 100 μl/well of 0.2% BSA in PBS. The $\Delta\psi_m$ was measured by fluorescence reader. $\Delta\psi_m$ of treated cells was expressed as a percentage of the $\Delta\psi_m$ of treated cells *vs.* H_2O_2-treated and/or -untreated cells.

ROS production detection assay

The ROS production was determined by using the fluorescent probe MitoSOX Red mitochondrial superoxide indicator (Molecular Probes; Life Technologies, Eugene, Oregon, USA). MitoSOX Red reagent is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits red fluorescence. According to the manufacturer's protocol, a vial of MitoSOX reagent was dissolved in 13 μl of DMSO to make a 5 mM MitoSOX reagent stock solution. The 5 mM stock solution was diluted in HBSS/Ca/Mg to obtain a 5 μM MitoSOX reagent working solution. Briefly, HeLa 705 and bEnd.3 cells were seeded in 96-well plates at a concentration of 1.5×10^4 cells/well containing 100 μl of complete medium and allowed to recover for 24 h. Cells were treated with 5 μM mtCPP analogs for 24 h. MitoSOX reagent 5 μM was added to the cells, and they were incubated for 10 min at 37°C and 5% CO_2, protected from light. After the staining with MitoSOX, the medium was aspired and the cells were washed once with 0.2% BSA in PBS to remove background fluorescence, and finally 100 μl/well of 0.2% BSA in PBS was added. The plate was read with a fluorescence reader (Flex Station II; Molecular Devices, Sunnyvale, CA, USA) with settings suitable for MitoSOX (excitation = 510 nm, emission = 580 nm). The same assay was performed to evaluate the ability of mtCPP-1 to restore normal levels of ROS after insult by adding H_2O_2. Briefly, HeLa 705 cells were seeded and treated as described above. Cells were treated with mtCPP-1 for 2 h and were insulted with H_2O_2 (final concentration 200 μM); then cells were incubated for 22 h at 37°C, 5% CO_2. MitoSOX reagent 5 μM was added to each well and cells were incubated for 10 min. The medium was aspirated, cells washed once with 0.2% BSA in PBS, and fresh 0.2% BSA in PBS (100 μl) was added to each well. ROS levels were measured using the fluorometer device (Flex Station II; Molecular Devices). The ROS production in treated cells was expressed as a percentage of the amount of ROS with or without the H_2O_2 stimulation.

Measurement of mitochondrial uptake of 5(6)-carboxyfluorescein_mtCPP-1 in isolated mitochondria

To evaluate mitochondrial uptake of mtCPP-1, mitochondria from HeLa 705 cells were extracted according to the instructions from the Mitochondria Isolation Kit for cultured cells (Thermo Fisher Scientific, Waltham, MA, USA). It was essential to start with an appropriate amount of cultured cells to obtain a visible and experimentally manageable pellet of mitochondria. Briefly, for the mitochondrial uptake study, according to the method used by Zhao *et al.* (8), isolated mitochondria from 20×10^6 cells were incubated for 4 h at 37°C and 5% CO_2 in 300 μl of isolated mitochondrial buffer (10 mM Tris hydrochloride, pH 6.7, 0.15 mM $MgCl_2$, 0.25 mM sucrose, 1 mM PMSF, 1 mM DTT) containing 5(6)-carboxyfluorescein (FAM)_mtCPP-1 at a final concentration of 5 μM. Uptake was stopped by centrifugation (12,000 *g*, 5 min at 4°C), and the mitochondrial pellet was washed twice and suspended in PBS (300 μl). The solution containing the mitochondria was transferred into 96-well black plate (100 μl volume each well). Mitochondrial uptake of mitochondria treated with FAM alone was determined using the same procedure. FAM dissolved in PBS at a final concentration of 5 μM (same concentration used for the treatment with peptides) was used as negative control. Fluorescence at 494/519 nm (excitation/emission) was measured every 2 s in a semi–high throughput screening fluorescence reader (FlexStation II; Molecular Devices) to quantify the peptides into the isolated mitochondria. PBS values were record as baseline. The mean of the values from the baseline recording was set to zero using SoftMax Pro 4.8 software (Molecular Devices).

Fluorescence microscopy

The digital images were obtained with an inverted fluorescence microscope LSM Pascal (Carl Zeiss GmbH, Jena, Germany) at 2048 × 2048 pixels. Images were taken using ×10 and ×40 dry objective lens and ×63 oil immersion objective lens, and optical section was <1 μm. Fluorescence was excited for Hoechst 33342, FAM by the 346 and 494 nm line of an argon laser, and the emission at 497 and 519 was recorded, respectively, for the nuclear and peptide stains. To localize mitochondria, cells were loaded with 200 nM TMRE, which distributes into negatively charged cellular compartments, for 20 min at 37°C. TMRE fluorescence was excited by a laser at 535 nm and recorded at 575 nm. A blue pseudocolor was applied to visualize the nuclear stain. Green and red pseudocolors were applied to visualize the localization of peptide into cells/mitochondria. All the recording were performed at controlled temperature (35 to 37°C). Images were analyzed with the software program ImageJ (National Institutes of Health, Bethesda, MD, USA).

Dynamic light scattering

Dynamic light scattering (DLS) was used to determine the hydrodynamic mean diameter of mtCPP-1. The peptide was dissolved in DMEM (Invitrogen, Sweden) supplemented with 10% fetal bovine serum. Measurements were carried out using a Zetasizer Nano ZS apparatus (Malvern Instruments, Malvern, United Kingdom). Samples were assessed in disposable low-volume cuvettes. All data were plotted as size distribution by intensity.

Circular dichroism measurements

Circular dichroism (CD) was used to determine the secondary structure of the mtCPP-1 in Milli-Q H_2O (Millipore, Billerica, MA, USA). CD spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics Ltd., Leatherhead, United Kingdom) at 20°C. Wavelengths between 190 and 260 nm were recorded, using a bandwidth of 0.5 nm. A quartz cuvette with an optical path length of 1 mm was used, requiring ~300 μl of sample. The temperature was adjusted using a TC 125 (Quantum Northwest, Liberty Lake, WA, USA) temperature control. The background spectra of the Milli-Q H_2O solution were subtracted from the peptide spectra. Spectra were collected and averaged over 10 measurements.

ATP assay

The ATP measurements were performed on HeLa 705 cells. ATP was determined by use of the ATP determination assay.

The quantitative determination of ATP was assessed using recombinant firefly luciferase and its substrate D-luciferin. The assay was based on luciferase's requirement for ATP in producing light. All other reagents, $MgSO_4$, tricine, DTT, coenzyme A, EDTA, sodium azide, and ATP were from Sigma-Aldrich (Stockholm, Sweden). For the luciferase assay, a standard reaction solution was prepared containing distilled water, 20× reaction buffer (500 mM tricine buffer, pH 7.8, 100 mM $MgSO_4$, 2 mM EDTA, and 2 mM sodium azide), 0.1 M DTT, 10 mM D-luciferin, and firefly luciferase 5 mg/ml stock solution. ATP was diluted serially in the standard reaction solution and a standard curve was generated. To assess the intracellular ATP assay, HeLa 705 cells were seeded 24 h before the treatment with the peptide in white-walled 96-well plates. Cells were treated for 24 h and cell medium was removed. Then, 20 μl of cell lysis buffer was added and incubated for 10 min at room temperature. Then, standard reaction solution was added and immediately light emissions were acquired for ~30 s using a GLOMAX 96 microplate luminometer (Promega, Stockholm, Sweden).

Statistical analysis

All values are represented as means ± SD. Statistical significance of the data was assessed by paired Student's *t* test or 1-way ANOVA as appropriate, performed using GraphPad Prism software version 6.00 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant.

RESULTS

Design, synthesis, and characterization of mitochondria CPPs

A new series of short peptides targeting mitochondria were synthesized using the solid-phase synthesis protocol described previously. Positive charge and lipophilic character were the 2 main properties used to design the peptides. These 2 properties are known to be important for the passage across both the plasma and mitochondrial membranes. mtCPP-1 is a tetrapeptide, and its sequence was designed to display at least 3 characteristics known to be important to target the mitochondria: positive charge, lipophilicity, and alternating aromatic residues and basic amino acids. The release of protons from the mitochondrial matrix to the inner membrane space generates a negative potential (−150/−180 mV) in mitochondrial inner membrane (12). The negative potential works as attractive point or the use of lipophilic cations to deliver redox agents into mitochondrial matrix. Lipophilicity is an important condition to obtain selective peptide targeting mitochondria and to allow partitioning of the peptides through the lipid bilayer (13). It was suggested by Zhao *et al.* (8) that alternating aromatic and cationic residues is possible to obtain motifs effective for mitochondrial delivery. Thus, we synthesized peptides containing cationic and hydrophobic residues that would provide electrostatic driving force for uptake through the charged plasma and mitochondrial membranes, while preserving the lipophilic character to facilitate passage through the latter. Peptides containing either 4 or 5 residues were synthesized and labeled with and without FAM (**Table 1**).

The synthesis of the designed peptides is based on:

1. Dmt residue in the second position of the sequence to increase antioxidant activity. The methyl groups of tyrosine can easily access, react, and stabilize the radical and prevent the reaction with other molecules.
2. L-arginine, D-arginine, L-ornithine, and D-ornithine (o) were selected to provide positive charge; D-amino acid in either the first or third position to minimize amino peptidase degradation and increase stability in serum.

TABLE 1. *Designed peptides library*

Name	Sequence	MW (g/mol)	Net charge at pH 7
SS-31	D-Arg–Dmt–Lys–Phe–NH_2	639.40	+3
mtCPP-1	D-Arg–Dmt–Orn–Phe–NH_2	625.38	+3
mtCPP-2	D-Orn–Dmt–D-Orn–Phe–NH_2	583.34	+3
mtCPP-3	D-Arg–Dmt–Orn–Trp–NH_2	664.41	+3
mtCPP-4	D-Arg–Dmt–D-Arg–Trp–NH_2	706.40	+3
mtCPP-5	D-Arg–Dmt–D-Arg–Dmt–NH_2	725.78	+3
mtCPP-6	D-Arg–Dmt–Orn–Orn–Phe–NH_2	757.54	+4
mtCPP-7	D-Orn–Dmt–Orn–Phe–NH_2	583.34	+3
mtCPP-8	D-Arg–Dmt–Arg–Phe–NH_2	667.37	+3
mtCPP-9	D-Arg–Dmt–D-Arg–Phe –NH_2	667.37	+3
FAM_SS-31	FAM–D-Arg–Dmt–Lys–Phe–NH_2	980.33	+2
FAM_mtCPP-1	FAM–D-Orn–Dmt–Orn–Phe–NH_2	1063.09	+2
FAM_mtCPP-2	FAM–D-Orn–Dmt–D-Orn–Phe–NH_2	1023.34	+2
FAM_mtCPP-3	FAM–D-Arg–Dmt–Orn–Trp–NH_2	1023.34	+2
FAM_mtCPP-4	FAM–D-Arg–Dmt–D-Arg–Trp–NH_2	939.27	+2
FAM_mtCPP-5	FAM–D-Arg–Dmt–D-Arg–Dmt–NH_2	939.27	+2
FAM_mtCPP-6	FAM–D-Arg–Dmt–Orn–Orn–Phe–NH_2	1021.05	+3
FAM_mtCPP-7	FAM–D-Arg–Dmt–Orn–Phe–NH_2	982.01	+2
FAM_mtCPP-8	FAM–D-Arg–Dmt–Arg–Phe–NH_2	1065.44	+2
FAM_mtCPP-9	FAM–D-Arg–Dmt–D-Arg–Phe–NH_2	1096.21	+2

Name, sequence, molecular weight, and theoretical net charge at pH 7 of the synthesized peptides. D-Arg, *d*-arginine; Orn, ornitine; D-Orn, *d*-ornitine; NH_2, C-terminal amidation.

3. Phenylalanine and tryptophan residues were used to impart lipophilicity.
4. Amidation at C terminus protected against hydrolysis.

Previous studies on arginine-based peptides indicated that high levels of cellular uptake could be achieved through the inclusion of cationic residues (14). When protonated, the basic amino acids facilitate charge-driven uptake through the plasma membrane, which exhibits a potential gradient that will cause electrophoresis in cationic species from the extracellular space into the cell (15, 16). Cationic residues in the sequences should, in principle, allow passage through both membranes due to the energized interface of mitochondrial membrane. However, the conjugation of a CPP to a lipophilic cation, a small-molecule mitochondrial localizer, did not facilitate its uptake into mitochondria, and this observation was taken as evidence that this class of peptides could not permeate the inner mitochondrial membrane (17, 18). As overall lipophilicity was shown to be an important factor for obtaining selective mitochondrial localization for small molecules (19), it is expected that a high degree of lipophilicity must also be incorporated into the sequence to allow partitioning of the peptides through the lipid bilayer.

CD and DLS analysis of mtCPP-1

Physical/chemical properties of the novel mitochondrial CPP were characterized. CD was measured to evaluate secondary structures of mtCPP-1 in Milli-Q H_2O. At all the different concentrations (50 μM, 100 μM, and 1 mM), the peptide had an unordered secondary structure with irregular CD spectra (**Fig. 1**). Prediction *via* Chou-Fasman algorithm (20, 21) and *via* basis spectra are shown in **Table 2**. A similarity in hits based on the frequency of particular reference spectrum among the 25 nearest neighbors compared with mtCPP-1 in the range of 180–240 nm is presented in Supplemental Fig. 1.

To confirm the absence of aggregates in the formulations, DLS studies were performed. These assessments were conducted using DMEM in the presence of serum at 37°C, as the formulations for the cells' treatment.

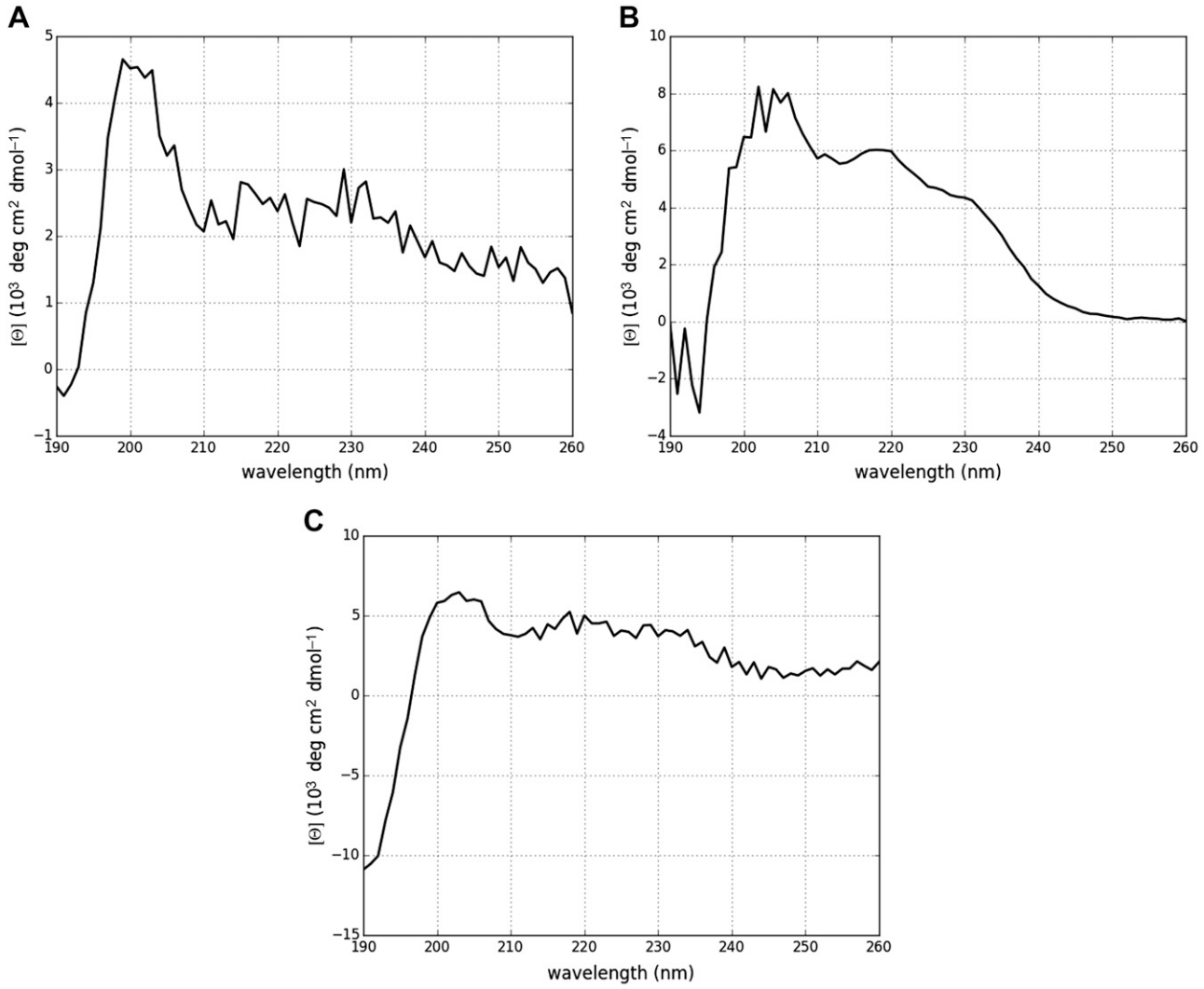

Figure 1. Examination of the secondary structure using CD. CD spectra of 50 μM (*A*), 100 μM (*B*), and 1 mM (*C*) of mtCPP-1. Measurements were performed dissolving the peptide in Milli-Q H_2O_2.

TABLE 2. *CD analysis*

Concentration (μM)	Helix	B-strand	Irregular
Prediction *via* Chou-Fasman algorithm			
50	0.00	0.00	1.00
100	0.00	0.00	1.00
1000	0.00	0.00	1.00
Prediction *via* basis spectra			
50	0.00	0.73	0.49
100	0.00	0.64	0.50
1000	0.00	0.78	0.45

Prediction in percentage of the characteristics secondary structures *via* Chou-Fasman algorithm and *via* basis spectra for mtCPP-1 in Milli-Q H_2O (final concentration 50, 100, and 1000 μM).

Proteins contained in the serum scattered light resulting in a population of particles (~100 nm) independent from mtCPP-1. Although the proteins of serum can affect such measurements, these conditions are the physiologic ones to study the peptide size. For DLS, we have compared the populations detected in serum-containing medium alone and after addition of mtCPP-1 (data not shown). It was possible to distinguish the constant protein aggregates (inherent to the serum) and a completely independent population associated to the peptide (**Fig. 2**). The Z-average of the peptide at all the different concentrations resulted to have a diameter of ~10 ± 6 nm.

Effects of mtCPPs on the cell viability

The cytotoxicity of the new series of peptides was evaluated in human and mouse cells by conventional WST-1 assay. The viability of 3 different cell lines was analyzed after treatment with the designed peptides. HeLa 705, U87, and bEnd.3 cells were treated with peptides and compared with untreated cells (**Fig. 3** and Supplemental Fig. 2). Different concentrations of mtCPP-1 were chosen for the treatment (0.5, 5, 50, 100 μM). Viability was analyzed after 24 h

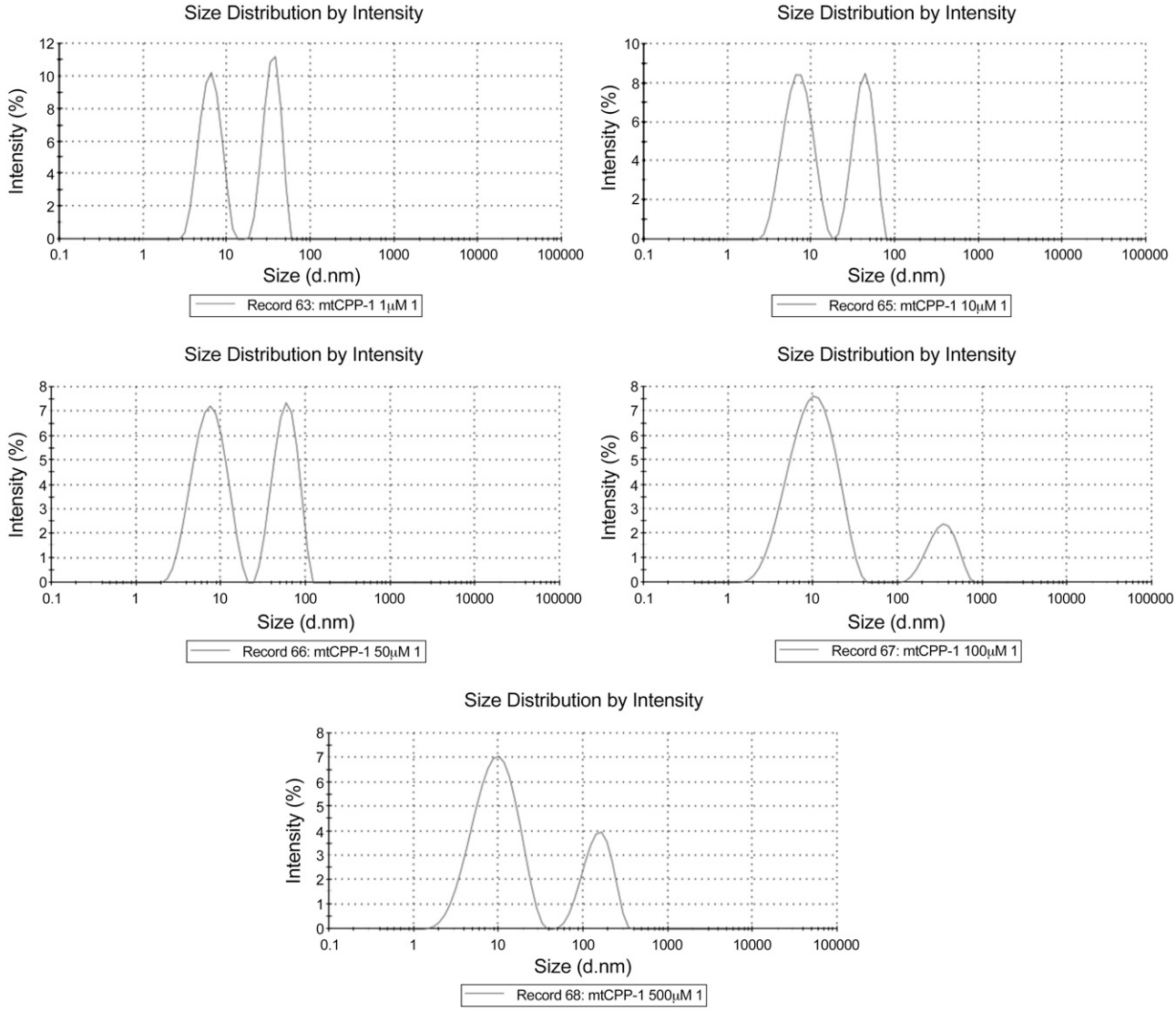

Figure 2. DLS. Average diameter of the peptide at different concentrations (1, 10, 50, 100, and 500 μM). Particle size distribution was assessed by dynamic light scatter. The figure shows representative curves of the populations' distribution in relation to intensity of the scattered light.

incubation with peptides. Treatments with H_2O_2 200 and 400 μM were chosen as positive controls. mtCPP-1 did not show any toxic effects in all the different cell lines at all the concentrations; furthermore, at the highest used concentration (100 μM), the viability was reduced to 90%. HeLa 705 cells treated with H_2O_2 showed viability decreased by 35% compared with the untreated cells (Fig. 3).

Detection of mitochondrial superoxide levels

Analysis of fluorescence intensity of MitoSOX (normalized *vs.* untreated cells) demonstrated decrease of superoxide levels in bEnd.3 cells treated with all the designed peptides at 5 μM (Fig. 4). Spectrofluorometric data plotted in the graph show a decreased intensity for 3 peptides (mtCPP-1, -8, -9). Quantitative measurements of the mean fluorescence intensity shown to have a significant 2-fold decrease (MitoSOX fluorescence intensity) after 24 h treatment with mtCPP-1 at 5 μM final concentration compared with fluorescence intensity of cells treated with SS-31 (Fig. 4). As a positive control, a solution containing antimycin A (final concentration 200 μM), MitoSOX (5 μM) in DMEM, was used to induce and register the highest ROS levels. Instead, ROS levels of untreated cell (in physiologic state) were considered as the lowest ROS levels.

Analysis of mtCPP cellular uptake and localization

Cellular uptake and localization of the peptides were then investigated. To demonstrate that mtCPPs are CPPs, they were conjugated with FAM. HeLa 705 and bEnd.3 cells were treated with FAM_mtCPPs (final concentration 5 μM) and incubated for 4 h at 37°C, then imaging was performed using epifluorescence microscope. mtCPPs

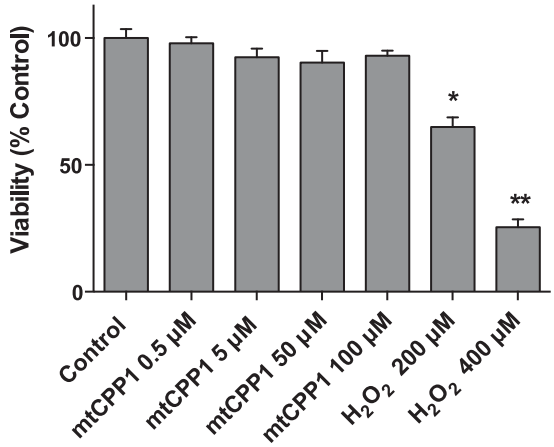

Figure 3. Cell viability assay on HeLa 705 after treatment with mtCPP-1 at different concentrations. HeLa 705 cells were treated with mtCPP-1 for 24 h. H_2O_2 200 and 400 μM was chosen as positive control. mtCPP-1 treatments did not show toxicity on HeLa 705 cells. Data shown represent means of a minimum of 3 individual experiments; the SE for each data point is shown. The cell viability was quantified by conventional WST-1 assay. $^*P < 0.05$, $^{**}P < 0.001$ compared with nontreated cells (mtCPP-1 0 μM).

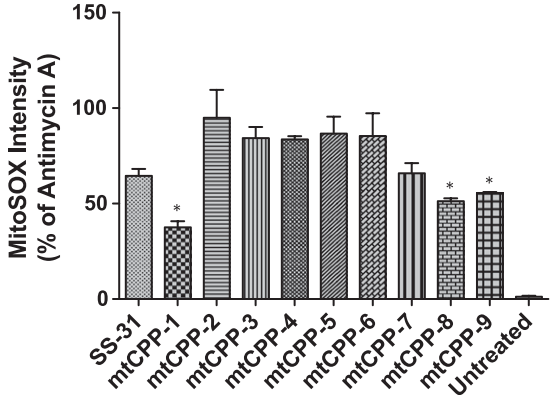

Figure 4. Detection of ROS levels in bEnd.3 cells. Peptides treatment decreases mitochondrial superoxide formation in bEnd-3 cells. Quantitative data expressing fluorescent intensity of MitoSOX following peptides exposure measured by spectrofluorometric analysis. Data presented as average ± SD, $n = 3$. $^*P < 0.005$ *vs.* SS-31 + MitoSOX.

were taken up into cells after 4 h of treatment. To verify the ability of the peptide to target the mitochondria, the red fluorescence dye TMRE, which specifically localizes into active mitochondria, was used. To compare the cellular localization between the original peptide and the new one, cells were treated also with FAM_SS-31. HeLa 705 cells internalized FAM_mtCPP-1, as shown in **Fig. 5**. HeLa 705 cells were also incubated with TMRE and FAM_mtCPP-1. The merged images showed the mitochondria localization stained with TMRE and the peptide labeled with FAM. Overall, that mtCPP-1 targeted mitochondria was proved using isolated mitochondria. The SS-31 peptide was used as model of CPPs targeting mitochondria. Different extent of mitochondrial localization for all the peptides was observed with fluorescence microscopy (data not shown). The evaluation of relative fluorescence of both FAM_mtCPP-1 and FAM_SS-31 showed similar intracellular distribution. Moreover, HeLa 705 cells took up more FAM_mtCPP-1 than FAM_SS-31, as shown in Fig. 5 and from the relative quantification based on the fluorescence intensity (Fig. 6).

Peptide treatment did not induce apoptosis

HeLa 705 and bEnd.3 cells were treated at the optimal concentration (5 μM) for 4 h, and the results were assessed by epifluorescence microscopy. Photographs at different orders of magnitude were taken and apoptosis was not observed. Morphologic changes were not observed in nuclei of cultured HeLa 705 (data not shown) and bEnd.3 cells (Supplemental Fig. 3) after staining with Hechst 33342.

Mitochondrial uptake of FAM_mtCPP-1

Subsequently, mitochondrial uptake of mtCPP-1 was examined in isolating mitochondria from treated cells. **Figure 7*A*** shows the fluorescence intensity of isolated mitochondria after incubation of HeLa 705 cells with FAM_SS31 and FAM_mtCPP-1 (5 μM) for 24 h. FAM_mtCPP-1 was taken

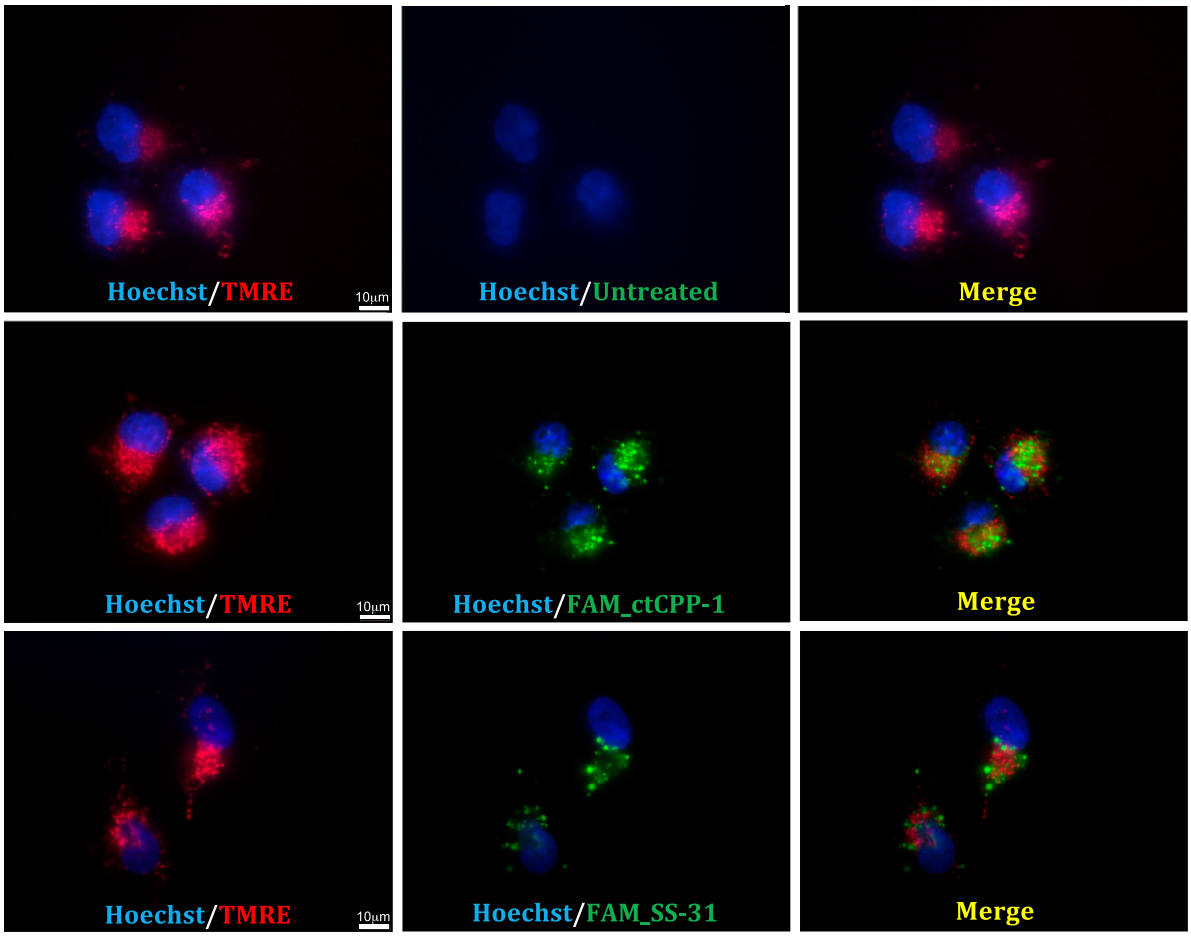

Figure 5. Cellular uptake and localization of FAM_mtCPP-1 and FAM_SS-31. Cells were incubated with peptides for 4 h before imaging; 200 nM TMRE was introduced 30 min before images were acquired. Hoechst 33342 was added at 1 μg/ml for 10 min before images were acquired. Untreated cells were kept under the same condition as the treated cells without any peptide treatment. Images shown in green correspond to the peptides labeled with FAM, final concentration of 5 μM; those shown in red correspond to TMRE; and those shown in blue correspond to Hoechst 33342. Merged images shown the extent of mitochondrial localization for the peptides.

up by cells and accumulated into mitochondria 1.8-fold more than FAM_SS-31. Moreover, to confirm the ability of the peptide to target mitochondria without being influenced by fluorophores, isolated mitochondria were treated with FAM_mtCPP-1 and FAM alone for 4 h. FAM alone was used as positive control at the same peptide concentration. Relative FAM fluorescence intensity was associated to the amount of peptide conjugated with FAM or FAM alone inside the mitochondria. Isolated mitochondria treated with FAM_mtCPP-1 showed fluorescence intensity 8-fold higher than mitochondria treated with FAM alone (Fig. 7*B*).

mtCPP-1 protects changes in $\Delta\psi_m$

The effect of mtCPP-1 to prevent $\Delta\psi_m$ changes is shown in **Fig. 8**. Cells were treated with H_2O_2 to induce ROS production and treated with mtCPP-1 at different concentrations to evaluate the ability to protect mitochondria. mtCPP-1 at 5 μM was able to rescue the $\Delta\psi_m$ as in untreated cells. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was used as positive control. It is a potent mitochondrial oxidative phosphorylation uncoupler (IC_{50} = 20 nM) that disrupts ATP synthesis by transporting protons across mitochondrial inner membranes and depolarized $\Delta\psi_m$. The fluorescence intensity recorded for the cells treated with FCCP was very low (~30%).

Cellular ATP levels are not altered by treatment with mtCPP-1

To determine the effect of mtCPP-1 and compare with the effect of SS-31 on ATP levels, the luminescence intensity of firefly luciferin-luciferase was measured in cultured HeLa 705 and bEnd.3 cells. ATP levels in cells treated with mtCPP-1 (final concentration 5 μM) were approximately the same as that of untreated cells (**Fig. 9**). ATP levels were decreased in HeLa 705 cells exposed to SS-31 (Fig. 9*A*). As positive controls H_2O_2, antimycin A, and FCCP were used.

DISCUSSION

This study was focused on mitochondrial target antioxidant peptides. Mitochondrial impairment results in overproduction

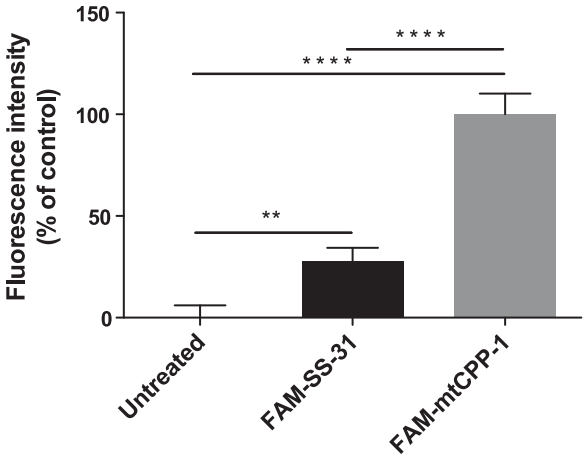

Figure 6. Relative FAM intensity quantification of FAM_mtCPP-1 and FAM_SS-31 in HeLa 705 cells. Relative fluorescence quantification in HeLa 705 cells after treatment with mtCPP-1 and SS-31 both conjugated N terminally with FAM at 5 μM final concentration. Control cells were incubated with only complete medium. $^{**}P < 0.01$; $^{****}P < 0.0001$.

of ROS, giving rise to onset of diseases characterized by different clinical symptoms. Antioxidants may be harmful because sometimes high-dose supplementation is necessary to result in pro-oxidant effects (22). Mitochondrial ROS are generated as normal by-products of oxidative metabolism. Approximately 3% of mitochondrial oxygen consumed is not completely reduced (23); those leaky electrons can easily interact with molecular oxygen to generate ROS, such as superoxide anion (24). This knowledge increased exponentially due to the number of studies on mitochondrial functions and resulted in a wide range of strategies to develop mitochondrial targeted antioxidants and drug delivery strategies into this compartment. A range of natural and synthetic molecules showed great antioxidant activity in *in vitro* studies, but these molecules did not prove to be particularly effective in clinical trials (25, 26). This limit could be due to physical and chemical characteristics that obstruct antioxidants, keeping them from reaching the relevant sites of free radical generation. In 2000, a new class of cell-penetrating antioxidant peptides targeting mitochondria was synthesized by Schiller and coworkers (27). Many publications showed and confirmed that SS peptides are the most promising tool applicable in the treatment of all diseases and impairments associated with oxidative injury such as neurodegenerative diseases. The present study provides a series of new short CPP targeting mitochondria designed and manually synthesized based on the SS-31 peptide. There are no CPPs with amino acid sequences shorter than 4 amino acids long. mtCPP-1 is a water-soluble tetrapeptide, with a theoretical positive net charge. The sequence of mtCPP-1 was designed based on a peptide found in the literature and displaying 2 important properties to be able to cross both the plasma and mitochondrial membranes: positive charge and lipophilic character.

HeLa 705, U87, and bEnd.3 cell lines were chosen as cellular models for the evaluation of toxic effects of mtCPP peptides. Interestingly, mtCPP-1 did not exhibit toxicity even at 100 μM. mtCPP-1 did not perturb $\Delta\psi_m$ and cause ROS overproduction in exposed cells. As shown in the literature, loss of $\Delta\psi_m$ and oxidative stress are part of a vicious circle that gives rise to increased mitochondrial permeability transition (28). mtCPP-1 gradually increased ROS and at the same time increased $\Delta\psi_m$, but the increase never reached statistical significance. The design of mtCPP sequences was focused on alternating aromatic residues and basic amino acids to have aromatic cationic peptides. mtCPPs always had Dmt in the second position and eventually antioxidant activity was investigated. The Dmt amino acid has antioxidant properties, due to methylation of the phenolic ring, and is able to scavenge oxyradicals, forming relatively unreactive tyrosyl radicals, which can be followed by radical-radical coupling to give dityrosine or scavenging by glutathione and/or ascorbate (8, 29).

mtCPP-1 was easily dissolved in water and was rapidly taken up by cells. The uptake mechanism of mtCPP-1 has not been studied yet but is under investigation. One possible hypothesis is that the potential gradient across the mitochondrial inner membrane is used by peptides to get

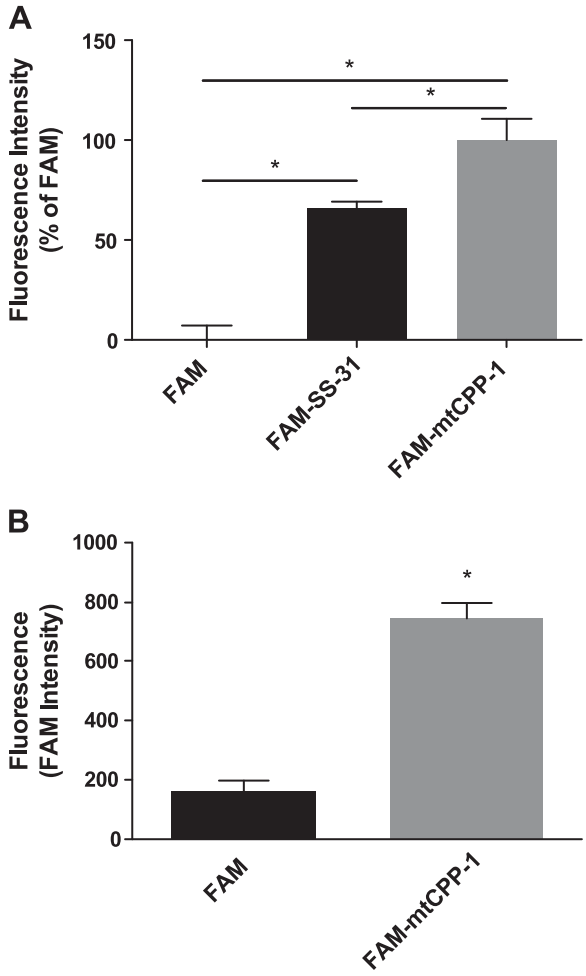

Figure 7. *A*) Relative fluorescence intensity in isolated mitochondria. Relative fluorescence of FAM_SS-31 and FAM_mtCPP-1 in mitochondria isolated after treating HeLa 705 cells for 24 h. *B*) Fluorescence intensity in isolated mitochondria from HeLa 705 cells directly treated with FAM alone (black bar) or with FAMmtCPP-1 (gray bar). Data shown represent means with SE. $^{*}P < 0.05$.

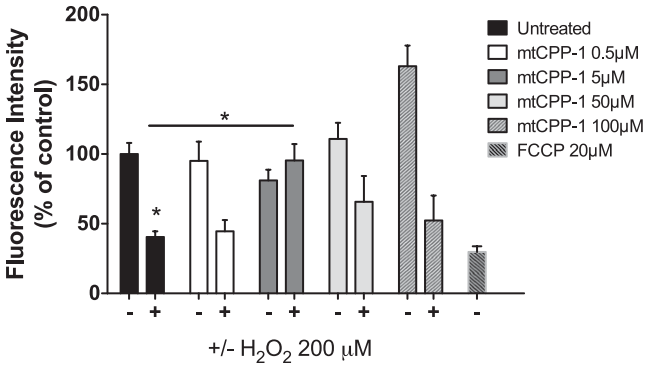

Figure 8. $\Delta\psi_m$. TMRE fluorescence intensity measured in HeLa 705 cells treated with mtCPP-1 at different concentrations with and without H_2O_2 for 24 h at 37°C and 5% CO_2. $\Delta\psi_m$ was measured with TMRE $\Delta\psi_m$ assay. $^*P < 0.05$.

into mitochondrial organelle due to the theoretical 3^+ net charge. As shown by Zhao and coworkers (8), the uptake of SS peptides were decreased by only 10 to 15% in mitochondria that were depolarized by FCCP; because mtCPP-1 is based on SS-31 sequence, a similar mechanism to get inside the cell may be present. Fluorescence microscopy confirmed rapid uptake of fluorescent mtCPP-1 in living cells. The intracellular distribution pattern of the fluorescent FAM_mtCPP-1 was shown to be similar to the mitochondrial fluorescent dye that is taken up into active mitochondria in a potential-driven manner, suggesting that mtCPP-1 peptide was targeting mitochondria.

The SS-31's ability to target mitochondria and to concentrate in mitochondria (1000-fold more in this organelle than in the cytosol compartment) has been documented (8). As shown by fluorescence microscopy and the relative quantification based on the fluorescence intensity, the amount of mtCPP-1 into the cells was higher than SS-31, underlining its great potential to target mitochondria. The ability of mtCPP-1 to breach the cellular membrane and accumulate into mitochondria was confirmed by an uptake study on isolated mitochondria. To evaluate mitochondrial uptake, mtCPP-1 was conjugated to the membrane impermeant dye, FAM. FAM_mtCPP-1 was taken up within 4 h by isolated mitochondria, confirming that this organelle is the target of mtCPP-1. Moreover, the higher relative fluorescence of FAM_mtCPP-1 on isolate mitochondria underscores the ability of mtCPP-1 to easily deliver molecules inside specific compartments. The ATP measurements confirmed that the treatment with mtCPP-1 in HeLa 705 and bEnd.3 cells did not exert any influence on ATP production. The uptake mechanism of this peptide is under investigation. mtCPP-1 could be tested as a mitochondrial delivery machine for treatment of inherited or spontaneous mutation in mtDNA.

CONCLUSIONS

Here we reported a series of novel mitochondrial CPPs (mtCPP-1 to -9), which consist of 4 or 5 natural and modified amino acids with C-terminal amidation, based on the SS-31 peptide. We investigated the ability of mtCPPs to enter into different cell lines and specifically target mitochondria. All the peptides were tested for cytotoxicity and none of them were toxic, even at high concentrations (100 μM). The mitochondrial functionality was not affected by the treatment with any peptide at the optimal concentration (5 μM), as confirmed by $\Delta\psi_m$ and ATP measurements. Three peptides (mtCPP-1, -8, and -9) were able to prevent ROS formation with lower mitochondrial superoxide levels into cells compared with the original peptide. mtCPP-1 showed a 2-fold reduction in ROS levels, which can be attributed to the peptide's ability to act as a ROS scavenger, limiting the ROS production into mitochondria and consequentially the cytosolic levels. mtCPP-1 is a small peptide, easy and fast to synthesize, and completely soluble in water. Microscopy and studies in isolated mitochondria indicated that the peptide was taken up in mitochondria; moreover, it showed antioxidant properties and is potentially conjugable to different cargo molecules. Thus, mtCPP-1 characteristics and properties may make it a candidate as a mitochondrial transporter, advantageous for therapeutic delivery of molecules to this organelle. FJ

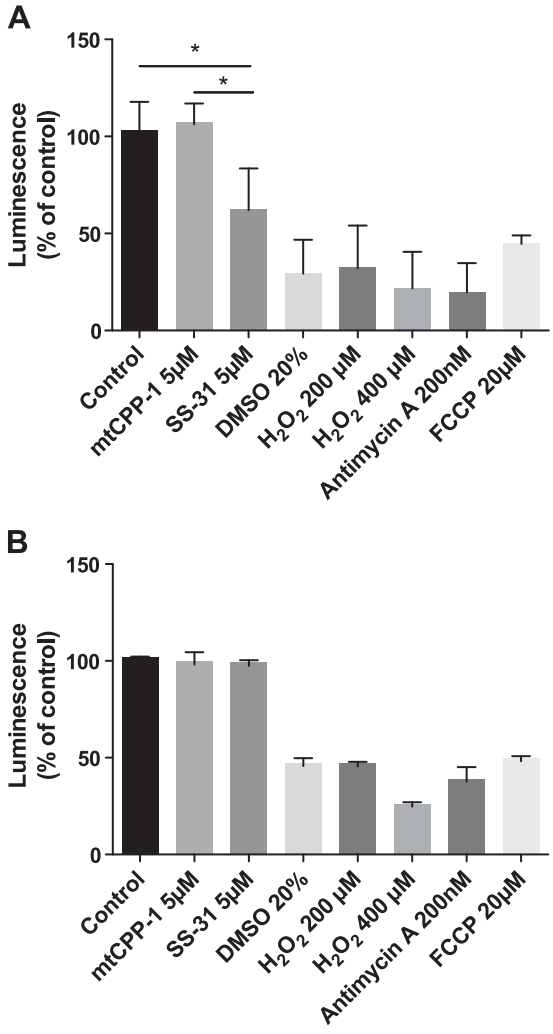

Figure 9. ATP measurement. ATP levels as measured by firefly luciferin–luciferase luminescence intensity at 24 h after treatment with peptides or positive controls. The luminescence level was normalized to the untreated cells (control) level in each individual well ($n = 3$ wells). $^*P < 0.03$.

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Novel cell-penetrating peptide targeting mitochondria

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