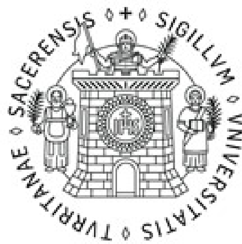

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Direttore della scuola: Prof. Andrea Piana

INDIRIZZO IN NEUROSCIENZE

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XXVII CICLO

Rational design and applications of a new Cell-penetrating Peptide targeting Mitochondria

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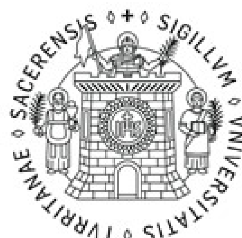

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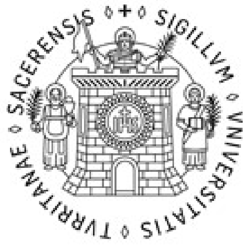
La presente tesi è stata sviluppata nell'ambito della scuola di dottorato in Scienze Biomediche dell'università degli studi di Sassari, a.a. 2011-12 – XXVII ciclo, finanziata dalla borsa di studio fornita dal P.O.R. SARDEGNA F.S.E 207-2013. Obiettivo competitività regionale e occupazione, Asse IV capitale umano, Linea di Attività 1.3.1



University of Sassari

University of Stockholm

My PhD was carried out during three years at the Department of Clinical and Experimental Medicine of the Medical School of the University of Sassari and for a period of ten months at the Department of Neurochemistry ant toxicology, University of Stockholm. The collaboration between these two groups contributed to developing of following thesis.

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ABBREVIATIONS

ACN	Acetonitril
ALS	Amyotrophic lateral sclerosis
Arg	Arginine
BBB	Blood brain barrier
CME	Clathrin- mediated endocytosis
CPPs	Cell Penetrating Peptides
CvME	Caveolae–mediated endocytosis
DA	Dopamin
D-Arg	D-arginine
DIC	1,3-diisopropylcarbodiimide
DIPEA	N,N-Diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DTT	Dithiolthreitol
EDT	1, 2-Ethanedithiol
EDTA	Ethylenediaminetetraacetic
FAM	Carboxyfluorescein
FCCP	Carbonyl cyanide 4-(trifluorometoxy)phenylhydrazone)
FDRA	Friedreich’s Ataxia
Fmoc	9-Fluorenylmethyloxycarbonyl
GFP	Green fluorescent protein
GPx	Gluthatione peroxidase

GSH	Glutathione
GSSG	Glutathione disulfide
HOBt	N-hydroxybenzotriazole
IMM	Inner mitochondria membrane
IMS	Inner membrane space
Lys	Lysine
MALDI	Matrix-assisted desorption ionization
MP	Macropinocytosis
MPT	Mitochondrial permeability transition
NAC	N-Acetyl-L-Cysteine
NLS	Nuclear Localization Sequences
NMP	*N*-Methyl-2-pyrrolidone
NS	Nervous system
OMM	Outer mitochondrial membrane
PBS	Phosphate buffered saline
PD	Parkinson's disease
PMSF	Phenylmethanesulfonyl fluoride
ROS	Reactive oxygen species
RP-HPLC	Reversed phase-High-performance liquid chromatography
SNpc	Substantia nigra pars compacta
SOD	Superoxido dismutase
SPPS	Sintesi peptidica in fase solida
Tboc	Di-tert-butyl dicarbonate
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane

TMRE	Tetramethylrhodamine methyl ester
TOF	Time of flight
TPP^+	Triprenylphosphonium ion
UV	Ultraviolet

INTRODUCTION

Literature Overview

Cellular oxidative stress is implicated in a wide array of cellular dysfunctions that give rise to onset of clinical disorders as ischemia-reperfusion injury **(Takizawa et al. 2011)**; neurodegenerative disease ***(Aoun et al., 2013)***; diabetes; infiammatory diseases; drug induced toxicity ***(Rivas, 2010).*** Over the years, different antioxidant approaches has been assessed but most of them did not show appreciable positive effects. The cellular membrane is a stumbling-block hard to overcome for most of natural and synthetic antioxidants, because of that their applicability window were strongly restricted. The efficacy of any drugs or gene therapy is related to two properties: ability of crossing cellular membranes and delivering a bioactive molecule on a specific cellular organelle. The cellular membrane of eukaryotic cell acts as a buckler that protects the cell from unregulated flow of bioactive molecules, ions and unwanted substances, in this way cell regulates the internal environment. Small molecules are able to cross the cellular membrane on their own instead of the larger drugs that because of their physicochemical characteristics are not capable to get into the cells and they need a special "help" as a delivery system. Delivery system must be efficient, safe and healthy. Mainly, there are two kind of delivery system: viral and non viral ***(Lajoie et al., 2015)***. This tesis is about one of the most novel non viral delivery system: cell penetrating peptide (CPPs). CPPs are short peptides sequences consisting up to 30 amino acids able to cross the cellular membrane and transport bioactive cargo into cells in an efficient and non toxic way. These short peptides have a positive charge, they are amphipathic and show both hydrophilic and lipophilic properties. A major breakthrough on CPPs date back in 1980s and early 1990s, when a series of short natural peptides sequences able efficiently cross the plasma membrane were identified ***(Green et al., 1988)***. Over the years more CPPs was discovered. Full length HIV-1 transcription transactivation (Tat) protein and *Drosophilia Antennapedia* homeodomain, known as Penetratin,

received a great deal of attention when their ability to cross cellular membrane and accumulate into cellular nucleus was demonstrated ***(Derossi et al., 1994)***. Those discoveries served like a cornerstone for a new subfield focused on the use of CPPs as molecular transporters: that was the beginning of molecular drug delivery strategy. Day by day, chemists and biochemists developed many variations of peptide structures in order to improve crossing activity, keeping low toxicity and immunogenic effects. As of today, hundreds of CPPs are available. They show different amino sequence, physicochemical properties and several mechanism of internalization. Some CPPs has been obtained from natural sequences ***(Vivès et al., 1997)***, while others from artificial constructs engineered ***(Pujals et al., 2008)*** to keep and exalt the important features of the molecules designed by nature. The identification of cellular targets for treatment of different disease states required the development of an efficient system able to delivery drugs into a target of interest. Different cargoes can be conjugated to CPPs as fluorophores, small molecular drugs, larger cargoes such as oligonucleotides, plasmids or proteins ***(Fawell et al., 1994).*** Uptake and efficacy of several therapeutic compounds are improved by CPPs conjugation, opening new opportunities to study biological process and making the treatment of several diseases more controlled and less toxic. The key of success in delivery strategy it depends on delivering drugs into specific target associated with the onset of particular disorder. Several studies demonstrated the mitochondrial involvement in the occurrence or worsening of the most disabling diseases. In view of all this mitochondria are interesting intracellular target for drug delivery. Nowadays, nucleus and mitochondria are great targeted. Cellular nucleus is targeted by Nuclear Localization Sequences (NLS), short cationic sequences, 10 amino acids in length ***(Goldfarb et al., 1986)***. These sequences are widely used to achieve nuclear delivery for a variety of DNA damaging agents or nucleic acids for gene therapy ***(Cartier et al., 2002).*** NLS find in cancer disease treatment the most promising field of application**.** Between NLS, simian virus 40 (SV40) showed high levels of cell permeability and low toxicity, for this reason has been applied in a number of studies to drive uptake of DNA for non-viral gene

therapy ***(Sing et al., 1998)***. This thesis is focused on mitochondrial targeting peptide. Mitochondrion is an organelle structurally discernable to the others cellular organelles being characterized by two membranes that underline pivotal cellular functions played by mitochondria. A lot of studies are focused on this organelle and it is of gaining attention in pharmaceutical and medical research since has been confirmed that it is involved in several diseases showing a great diversity of clinical appearance ***(Saraste, 1999)****.* Literature shows several examples of CPPs targeting mitochondria, using artificial, rather than natural signal sequences ***(Mahon et al., 2007)***. Mitochondrion is an important target for drug therapy given its role in the pathology of cancer, neurodegenerative diseases, and others where reactive oxygen species are linked with pathological conditions ***(Dai et al., 2014)***. To date, the short SS peptide developed by Szeto and Schiller ***(Schiller et al., 2000)*** are the most promising mitochondria-targeted antioxidants. SS-compounds are tetrapeptides and have been designed alternating aromatic residues and basic amino acids (aromatic-cationic peptides). They are characterized by tyrosine (tyr) or dimethyltyrosine (Dmt) residues in order to increase antioxidant activity, the presence of D-amino-acid in either the first or second position minimizes aminopeptidase degradation and amidation of C-terminus to protect against hydrolysis ***(Szeto, 2006)*.** The antioxidant action of SS peptides can be attributed to the Tyr or Dmt residues. The sequence position of the Tyr or Dmt residue is not important in scavenging ROS and reducing LDL oxidation ***(Zhao et al., 2004).*** All of SS peptides show 3^+ net charge at physiologic pH and they can get into cells in an energy-independent non saturable manner. The uptake is really fast, several studies showed that SS-20 peptide is taken up into cells in less than 30 minutes ***(Zhao et al., 2004)***. The SS peptides target preferentially the inner mitochondrial membrane, indeed they concentrate in mitochondria 1,000-fold more compared with the cytosolic concentration ***(Zhao et al., 2004)*.** The mechanism of uptake into mitochondria is still unclear. SS-peptide probably make use of their own positive charge to get into mitochondria but a lot of studies have been showed that these peptides can cross as well as membranes of depolarized mitochondria ***(Doughan et***

al., 2007). To date, SS peptides are the most important targeting mitochondria of all know CPPS, since they are potent in reducing intracellular ROS and as well as preventing cell death. The first part of this thesis will start from a short description of mitochondria, structure and underline hub role on cellular metabolism and functions, why is important to preserve the right functionality of this organelle and why it is a promising target for drug delivery. After that, CPPs and characteristics of most important CPPs targeting mitochondria will be discussed. The last part of the thesis will be focused on the synthesis and application of a new short antioxidant cell penetrating peptide targeting mitochondria, outlining its applicability on drug delivery strategy and on antioxidant treatments, obtained results and future aspects finalize this thesis.

Mitochondria

Organization and functions

Mitochondria are located in the cytoplasm of eukaryotic cells and play a pivotal role in cellular metabolism. Mitochondrion is fundamental in the generation of metabolic energy and it is responsible for most of the useful energy derived. Into mitochondria, the breakdown of carbohydrates and fatty acids is converted to ATP by the process of oxidative phosphorylation. Mitochondria are the main organelle for the synthesis of ATP under normal aerobic condition. The last oxidation step for fats and carbohydrates takes place in the mitochondria. The complicated structure of mitochondria is fundamental to perform these functions. During oxidation of fats and glycolysis, electrons are transferred from bioenergetic substrates to nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). The high energy electron reduced forms, NADH and $FADH_2$, is processed by a complex carrier system called electron chain reaction (ETC) ***(Cooper, 2000)***. Briefly, two electrons and two protons combine with ½ O_2 to produce H_2O. In the same time, protons are pumped from the mitochondrial matrix into inner membrane space (IMS) generating potential energy across the membrane, which is used to drive ATP synthesis. In short, all mitochondrial compartments are involved in ATP synthesis through a complex multistep process. In addition to this critical metabolic role mitochondria store calcium for cell signaling activities, generate heat, mediate cellular growth and death ***(Scheffler, 1999)***. Both number and size of mitochondria varies widely by organism and tissue type, but usually size range from 0.5 to 10µm and number range from a few hundreds to thousands each cell, it depending on both of the energy demands and cell type ***(Scheffler, 1999).*** Organization and function of mitochondria is widely studied. Mitochondria are rod-shaped, surrounded by double membrane, it consisting of four distinct compartments: the outer mitochondrial membrane (OMM), the inner membrane space (IMS), the inner mitochondrial membrane (IMM) and the mitochondrial matrix, each of these

components plays distinct functional roles. IMM separates the mitochondrial matrix from the intermembrane space, it forms numerous folds (***cristae)*** that extend into the interior (or matrix) of the organelle ***(Fig. 1)***. The *cristae* greatly increase the total surface area of the IMM. The IMM includes all the most important complexes that mitochondria need to fulfil their functions: all the complexes of the electron transport system, the ATP synthetase complex and transport proteins complex. The IMS is the smallest component of mitochondrion and it is located between the IMM and OMM. The IMS provides a redox active space, necessary environment to oxidize metabolic residues **(Riemer, 2011)**. The IMS can exchange proteins, lipids, metal ions, and various metabolites with other cellular compartments, as OMM, allowing mitochondrial metabolism to adapt to cellular homeostasis. In particular, the biogenesis and activity of the respiratory chain is controlled by various proteins of the IMS ***(Vögtle, 2012)***. OMM is in direct contact with cellular cytoplasm. OMM is freely permeable to small molecules and contains special protein called porin that form channels allowing the free diffusion of molecules smaller than 6000 Daltons ***(Lin et al., 2014)***. OMM shows enzymes involved in the elongation of fatty acids, oxidation of epinephrine (adrenaline), and the degradation of tryptophan. Latest studies showed that apoptosis, longevity control are regulated by protein of OMM ***(Ran, 2014)***. The mitochondrial matrix is the space between *cristae*. The matrix contains the mitochondrial genetic system as well as the enzymes responsible for the central reactions of oxidative metabolism.

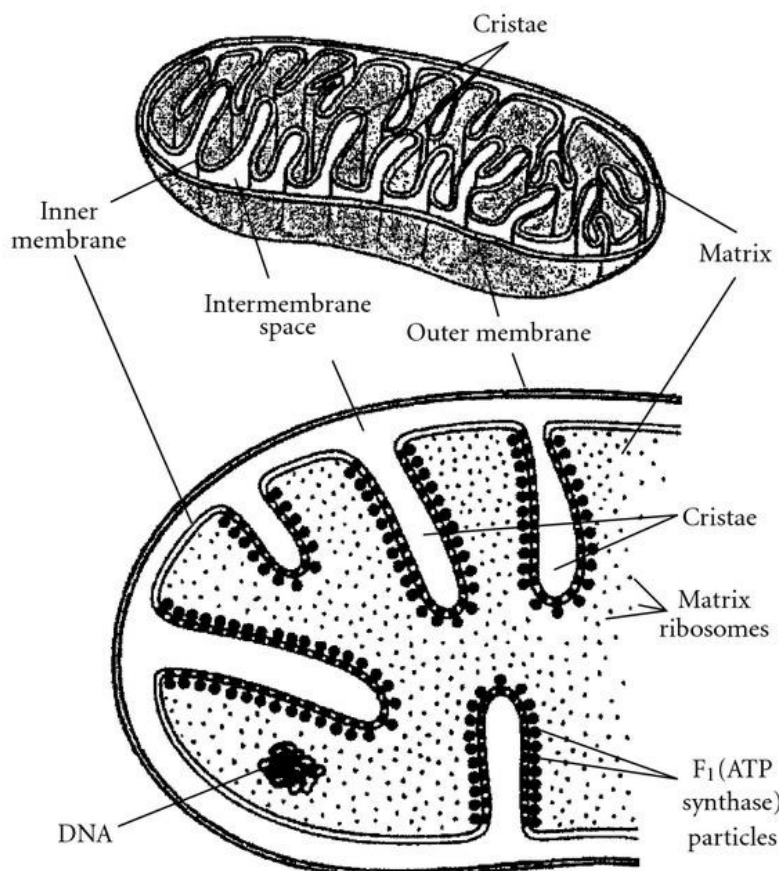


Fig. 1. Mitohondrial structure. Adapted from Freitas Jr., Basic Capabilities, Landes Bioscience, Austin, Tex, USA, 1999

Mitochondrial electron transport chain

Mitochondrial electron transport chain is bound to inner mitochondrial membrane and mainly consists of five complexes called Complexes I through V ***(Fig. 2)***. Complex I, called also NADH-ubiquinone oxidoreductase, transports two electrons from NADH to the mitochondrial matrix and to coenzyme Q within the membrane. Complex I accepts electrons from NADH and it acts like a bridge between glycolysis, tricarboxylic acid cycle (TCA), fatty acid oxidation, and electron transport chain. Its complete structure is not been totally understood, our knowledge of the structure come mainly from electron microscopy and biochemistry. Its characteristic L-shape,

or boot, can be dissociated in two sub-complexes knows as sub-complexes 1α and sub-complexes 1β, containing 23 and 17 subunits respectively ***(Grigorieff, 1999).*** The ankle of "boot" is thought to protrude from the membrane so as to be predominant in the aqueous phase and contains the binding site for NAD(H) and the input electron transfer chain. The foot (the hydrophobic protein) is membrane-linked. Many inhibitors and several iron sulfur centers are localized on it as well as a catalytic site where reduction of ubiquinone occurs. Many disease conditions are associated to this complex, including leber hereditary optic neurophaty, melas syndrome, Altzheimer's disease and Parkinson's disease ***(Meyers, 2013. Gaweda-Walerych, 2013)***.

Complex II, also known as succinate-coenzyme Q reductase or succinate dehydrogenase, is the only membrane-bound component of the Krebs cycle and in addition functions as a member of the electron transport chain in mitochondria and in many bacteria ***(Cecchini, 2003).*** Complex II acts as link between the TCA and electron transport chain. It is the only TCA cycle enzyme that is an integral membrane protein. Complex II oxidizes succinate to fumarate reducing FAD to $FADH_2$. The structure of complex II is well investigated, has a mass of 124 kD and composed of two hydrophilic subunits, a flavoprotein and an iron-sulfur protein, and two hydrophobic subunits linked to membrane.

Complex III, also named Cytochrome reductase, is a multisubunit transmembrane protein acceptor of electrons from reduced Coenzyme Q and uses them to reduce the second mobile electron carrier Cytochrome C. For each coenzyme Q fully oxidized, complex III moves four hydrogen ions outward from the matrix to the mitochondrial intermembrane space ***(Bolsover, 2011)***.

Complex IV, or Cytochrome C oxydase, is the terminal enzyme of the respiratory chain. It is a transmembrane protein and consists of 13 polypeptide subunits, 3 of which are encoded by mitochondrial DNA. The Complex IV moves an electron from each of four Cytochrome C and reduces one oxygen molecule to two molecule of water and moves four hydrogen ions from the matrix to the intermembrane space,

contributing to generate a differential transmembrane difference of proton that the ATP synthase will use to synthesize ATP.

ATP synthase, called also complex V, catalyzes ATP-Pi exchange, and ATP, GTP, and ITP hydrolysis. The synthesis of ATP from ADP and phosphate is driven across the membrane by a flux of protons gradient generated by IMS electron transfer ***(Galante et al., 1979)***. The ATP synthase catalyses a reversible reaction for this reason ATP hydrolysis generates a proton gradient by a reversal of this flux.

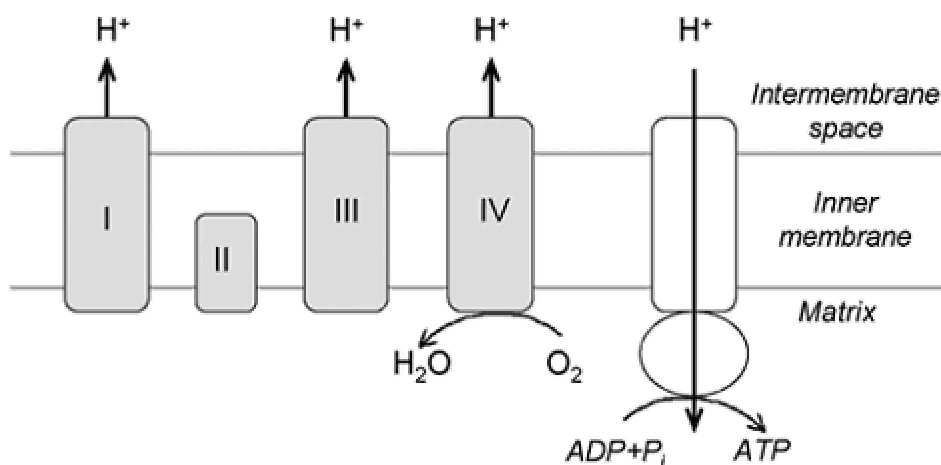


Fig. 2. Electron transport chain and ATP synthesis on the mitochondria inner membrane. Adapted from Szeto H.H. The AAPS Journal 2006; 8 (3) Article 62

Mitochondrial Membrane Potential

The optimum mitochondrial membrane potential (ΔΨm) is critical for preserving important cellular functions and mitochondrial processes as well keeping the physiological function of respiratory chain. The Δψm controls ATP synthesis, generation of ROS, mitochondrial calcium sequestration, import of proteins into the mitochondrion and mitochondrial membrane dynamics ***(Dai et al., 2014)***. Conversely, Δψm is controlled by ATP utilization, mitochondrial proton conductance, respiratory chain capacity and mitochondrial calcium. Depolarization might be found in oxidative stress conditions as consequence of mitochondrial calcium overload ***(Joshi et al., 2011)***. A significant loss of ΔΨm renders cells depleted of energy with subsequent increased mitochondrial membrane permeability. Hyperpolarization might be related to ATPase inhibition, inadequate supply of ADP, increased supply of NADH and apoptosis due to oxidative stress. The most used method for driving compound to mitochondria uses of the potential gradient across the mitochondrial inner membrane. As a result of moving proton and electron through mitochondrial electron transport complexes, a negative potential from 150 to 180 mV is generated across the IMM. Lipophilic cations may therefore accumulate 100-to 1000-fold in the mitochondrial matrix. A number of studies showed increased uptake into mitochondria in TPP^+ and VitE -conjugated lipophilic sequence ***(Murphi et al., 2000. Jauslin et al., 2003)***. The uptake failed in depolarized mitochondrial membrane potential ***(Dhanasekaran et al., 2004)***, because of that the utility of TPP^+-conjugated antioxidants is limited in model of neurodegenerative disease where mitochondrial membrane potential is impaired. To overcoming this problem in 2004 a new class of small cell permeable peptide antioxidants targeting mitochondria was described by Zhao and coworkers ***(Zhao et al., 2004).*** This class of CPPs is described in specific chapter of this thesis.

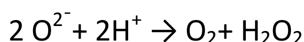
Mitochondrial generation of ROS

Mitochondria are small cellular organelle but consume around 85% of cellular oxygen to run the oxidative phosphorylation. As a consequence of mitochondrial metabolism, around 2% of oxygen is turned on superoxide anion (O_2^-) ***(Chance, 1979)***. The amount of O_2^- production is subordinated to mitochondrial metabolic state and mitochondrial potential. Superoxide anion is not able to cross cellular membranes, but is converted to hydrogen peroxide (H_2O_2) by mitochondrial matrix enzyme MnSOD or by CuZnSOD in the intermembrane space. H_2O_2 is more stable than O_2^- and can diffuse out of the mitochondria into the cytosol ***(Szeto, 2006)***. H_2O_2 can be readily converted on water by mitochondrial glutathione peroxidase or catalase. Moreover, H_2O_2 can reacts with ferrous iron and resulting, through Fentom reaction, in highly reactive hydroxyl radicals ($OH^•$). As discussed above mitochondria are a major source of reactive oxygen species and superoxide is constantly generated during normal respiration by healthy mitochondria. Complex I, Complex II and Complex III are mainly involved in ROS production. Complex I transfers electrons from NADH to coenzyme Q, at the same time protons pass from the matrix to the intermembrane space and the anion superoxide is generated during movement of charge by complex I. Complex II reduces Coenzyme Q and is also responsible for production of low levels of superoxide anion. Complex III is responsible for increased ROS production in a state of decreased electrons transfer. In 1966, Jensen and colleagues understood the pivotal role mitochondria in ROS producing ***(Jensen, 1976)***, but to date, in spite of great knowledge of mitochondrial metabolism, a lot of pathway are still unclear. It is important to understand that ROS production is inevitable and useful process. Many studies showed the implication of ROS in important cellular pathway such as autophagy, signal transduction and immune function ***(Chen, 2007. Niess, 1999)***.

Mitochondrial Antioxidants

Excess of ROS can be highly dangerous and damage cellular components as protein, lipid and DNA, leading to death of cell. Mitochondria and cells in general posses different defense system to avoid excess of ROS. These antioxidant network system can be rated as enzymatic (superoxide dismutase, catalase) or non enzymatic systems (glutathione).

Superoxide Dismutase (SOD) is widely spread between living organism. All oxygen-metabolizing cells ***(Gregory, 1974)***, many anaerobic bacteria ***(Hewitt, 1975)*** also fungi ***(Rapp, 1973)*** own SOD able to run the dismutation of superoxide radical to H_2O_2. Superoxide is converted to hydrogen peroxide (H_2O_2) by two types of intracellular superoxide dismutase (SOD) under physiological conditions: Cu/Zn-SOD in the cytosol and Mn-SOD in the mitochondrial matrix. Subsequently, the produced H_2O_2 is catalyzed into water and molecular oxygen by catalase or gluthatione peroxidase (GPx)



$2\ O^{2-} + 2H^{+} \rightarrow O_2 + H_2O_2$

Catalase (CAT, oxidoreductase, EC1.11.1.6) is an enzyme found in all aerobic organism and some anaerobic organisms ***(Brioukhanov et al., 2006)***. Catalase is located in cellular and subcellular compartements ***(Roels, 1976)*** and in mitochondria matrix ***(Radi, 1991)***.The primary function of catalase enzymes is the rapid breakdown of hydrogen peroxide into water and safe oxygen.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Glutathione (L-y-glutamyl-L-cysteinyl-glycine; GSH,) is a tripeptide synthesized in cytosol. Cysteine and glycine are linked by peptide bond and the carboxyl group of the glutamate side-chain to the amine group of cysteine by gamma peptide linkage. Glutathione has been discovered in animals, plants and fungi ***(Penninckx, 2000)*** and one of the most important antioxidant in our body, it protects against free radicals,

H_2O_2 and reactive nitrogen species (RNS), it is involved in many cellular functions as cell signaling, protein function, gene expression, cell differentiation/Proliferation, and its depletion is correlated with many disease as neurodegenerative disease. GSH is not required by diet but every cells of animal organism can synthesize it, so GSH is distributed overall the human body and levels vary according to organs and tissues and subcellular compartments ***(Wu, 2004)***. GSH is synthesized in the cytosol then delivered in different cellular compartments; 12% of total cellular GSH is located in mitochondria. The antioxidant action of molecule of GSH is related to thiol groups of cysteine by serving as an electron donor. In presence of H_2O_2, GSH is oxidized by Glutathione peroxidase (GPx) to GSH disulfide (GSSG), which is then regenerated as GSH by the reaction with GSSG reductase (GSSG red) ***(Drigen, 2002)***.

Oxidative stress

Generation of ROS is a physiological consequence of cellular metabolism and useful tool to different biological pathways. Cells own different antioxidant systems in order to maintain right concentration of ROS. The physiological production of ROS is not dangerous, but a spatiotemporal imbalance between ROS production and ROS defense systems the starting point of cellular impairments. The reduction of antioxidant systems lets to oxidation of membrane phospholipids, proteins, and nucleic acids and this condition, named oxidative stress, can lead to necrotic or apoptosis cellular death ***(Zamzami et al., 1997)***. Oxidative stress has been associated to many diseases, including cancer, renal disease, neurodegenerative and cardiovascular disease ***(Hroudová 2014)***. Several studies showed increased levels of ROS in diabetes type 1 and type 2. At the moment, the relations between increased ROS and diabetes is not clear but seems that high level of reactive species of oxygen contribute to insulin resistance, the basis of diabetes ***(Rösen, 2001)***. Mitochondria are the major site of ROS, generated as byproducts of the electron transport chain. Moreover, mitochondria are continuously exposed to ROS and because of that particularly susceptible to oxidative damage. Mitochondrial DNA has been shown to undergo oxidative damage. In addition to lipid peroxidation, protein oxidation and nitration results in altered function of many metabolic enzymes in the mitochondrial matrix as well as in the electron transport chain. A particularly relevant protein that loses function upon oxidation is SOD, which would further compromise antioxidant capacity and lead to further oxidative stress ***(Szeto, 2006)***. In addition, the excess of ROS seems to be involved in cytochrome *C* release from mitochondria. Cytochrome *C* is normally bound to the inner mitochondrial membrane linked to cardiolipin. Cytocrom *C* participates supporting function of ATP synthesis. High levels of ROS lead to peroxidation of cardiolipin, then to the dissociation of Cytochrome C release through the OMM into the cytosol. Cytochrome *C* in the cytoplasm triggers the activation of caspase-9, which triggers the caspase cascade and ultimately leads to apoptosis ***(Liu, 1996)***. Mitochondria are

critical regulators of cell death and a key feature of neurodegeneration, and they play important role in cell processes, signaling pathways, calcium homeostasis, cell cycle regulation, apoptosis, ROS production, and thermogenesis rendering this organelle an important target for the delivery of radical scavengers. Achieving successful mitochondrial drug delivery could produce enhanced treatments for mitochondria-related disorders and also advance our knowledge of the roles that mitochondria play in cellular biology.

Cell-Penetrating Peptides

Classification

Over the last two decades, many different short peptide sequences able to transport diverse types of cargo molecules across cellular membrane have been identified. The continuous development of this field indicates that chemical space is rich in peptide sequences that exhibit high levels of cellular uptake. There are different ways to classify CPPs, on according to their origin, such as their charge, function, hydrophobicity or amphypathicity. One way to classify is to subdivide them into protein-derived, chimeric and synthetic or designed CPPs *(Table. 1).*

Protein–derived CPPs were the first type of CPPs discovered, Penetratin and Tat belong to this category. The basic domain of HIV-Tat, which is sufficient for cell penetration, and the penetratin peptide, residues 43–58 derived from the third helix of Antennapedia protein homeodomain from Drosophila, are arguably the most studied CPPs. Both of them are still being used to this date and have in many cases been further modified to obtain new CPPs ***(Saleh et al., 2010)***.

Synthetic CPPs are entirely designed, this class ranges from simple polypeptides such as poly–argynine ***(Futaki et al., 2007)*** to more complicated synthetic sequences including the model amphipatic peptide ***(Oehlke et al., 1998)***. Synthetic CPPs are still less common than protein derived or chimeric CPPs.

Chimeric CPPs are combinations of protein–derived and synthetic sequences. Transportan is typical examples of chimeric CPPs. Transportan is a 27 amino acid-long peptide. The sequence of Transportan was designed using the natural amino terminus of the neuropeptide Galanin and the carboxyl terminus of Mastoparan by means of a lysine. ***(Pooga et al., 1998)***.

CPPs	Origin	Sequence	Ref.
Tat	HIV-1transactivator protein	GRKKRRQRRRPPQ	Vivès et al.1997
EB1	Chimeric	LIRLWSHLIHIWFQNRRLWKKK	Lundberg et al. 2007
SAP	Designed	VRLPPPVRLPPPVRLPPP	Pujals et al. 2008
R_9	Designed	RRRRRRRRR	Futaki et al.2001

TABLE 1. Example of common CPP by classification

CPPs uptake mechanism

A numbers of investigations have been conducted to elucidate how CPPs get into the cells ***(Duchardt et al., 2007)***. Most of naked peptides CPPs use endocytosis mechanism but a variety of uptake mechanisms appear to be operative in different systems, and in some cases, the mechanism is cell-type or cargo-specific ***(Mueller et al., 2008)***. For example, in 2003, Fittipaldi and coworkes showed that TAT uses a lipid raft mediated endocytosis when conjugated to a protein ***(Fittipaldi et al., 2003)*** and clathrin–dependent endocytosis if conjugated to a fluorophore ***(Richard et al., 2005*)**. The uptake mechanism was initially considered direct, non–endocytic and receptor independent, but later studies showed CPPs can access the cell by two distinct routes: energy-dependent vesicular mechanisms, collectively referred to as endocytosis, or via a direct process involving translocation of the lipid bilayer, especially at high concentrations of peptide, also the same peptide can be taken via endocytic and direct pathways ***(Fig.3) (Duchardt et al., 2007).***

Endocytosis

Endocytic mechanism is an energy dependent form of cellular uptake, is a regulated process used by cells to internalize solutes and fluids in the extracellular matrix ***(Yamada et al., 2008)***. This mechanism is run by interactions between peptide and cell membrane constituents, such as negatively charged glycosaminoglycans and

phospholipids. In addition, when linked to larger cargos, CPPs can utilize certain receptors in order to improve their uptake, such as class A scavenger receptors ***(Lindberg et al., 2013)***. Endocytosis is commonly divided in phagocytosis and pinocytosis. Phagocytosis has generally not been associated with CPPs and is reserved only for specialized cells, such as macrophages, monocytes, dendritic cells and neutrophiles; is a complex process used to engulf large particles ***(Aderem et al., 1999)*** such as bacteria or dead cells, it has been recently shown that a phagocytic process occurs in particles larger than 0.5 µm and is influenced by particle shape ***(Aderem et al., 1999)***.

Pinocytosis can be further classified in diverse pathways: clathrin-mediated endocytosis (CME), caveolae–mediated endocytosis (CvME), macropinocytosis (MP), clathrin and caveolin independent endocytosis ***(Hillaireau et al., 2009)***. Pinocytosis occurs in all cell types. The exact mechanisms of each of these pinocytic processes differ with regard to vesicle structure and the protein machinery utilized, they all share a common outcome: extracellular molecules are encapsulated into lipid vesicles, which are internalized after resealing of the plasma membrane.

Clathrin-mediated endocytosis

CME is the most well studied endocytic pathway. This mechanism starts with formation of vesicles, size around 100-150 nm in diameter and coated with a complex of proteins mainly consisting of clathrin. Vesicles are formed in specialized regions of the plasma membrane called clathrin coated pills. The mechanism of their formation is characterized by several steps. Primarily, the enzyme GTPase dynamin drives the invagination of the plasma membrane, as the invagination gets deeper turns in vesicles ***(Takey et al., 2001)***. Afterwards, endocytosed vesicles move from the surface of plasma membrane to deeper region of cell, during moving, vesicles turn into endosomes, pH around 6, to lysosomes, pH around 5, where the cargo is enzymatically degraded ***(Luzio et al., 2009)***.

Caveolae–mediated endocytosis

CvME, also called as lipid raft-mediated endocytosis, is a cholesterol, dynamin-dependent and receptor-mediated pathway ***(Nichols, 2003)***. It is characterized by endosomes formed from non-clathrin coated plasma membrane but consists of thecholesterol-binding protein caveolin and a cholesterol and glycolipid bi-layer. The fission of the caveolae from membranes is run by dynamin. Caveolae are approximately 50–80 nm in diameter. Their composition and function are highly cell-type dependent. Caveosomes are not degraded by acidic pH, therefore the cargo can be directly driven to the Golgi and/or endoplasmatic reticulum, avoiding normal lysosomal degradation ***(Bengali et al., 2007)***.

Macropinocytosis

MP is a mechanism of uptake able to take relatively large amounts of non-specific substances. MP usually occurs in macrophages and cancer cells, it is characterized by formation of actin–driven membrane protrusions which collapse into and fuse with the plasma membrane **(Hillaireau et al., 2009)**. The size of vesicles, called macropinosomes, is around 200 nm-5 µm in diameter. The fate of macropinosomes is still unclear and seems to be cell type dependent. Futaki and colleagues reported arginine-rich CPPs are preferentially taken by the cells via macropinocitosis ***(Futaki et al., 2007)***.

Clathrin and caveolin independent endocytosis

This pathway is less studied than the others mechanism of uptake. Endocytosis occurs in cells depleted of both CME events and caveolin in cholesterol dependent manner, implying distinct endocytic pathways that require specific lipid compositions for internalization ***(Doherty et al., 2009)***. Clathrin and caveolin independent endocytosis can be further divided into dynamin-dependent and independent routes. The formed vesicles have different size, heterogeneous shape and did not show a protein coat ***(Kirkham et al., 2005)***.

Direct translocation

Latest studies suggest that the translocation of polycationic CPPs across biological membranes occurred via an energy-independent cellular process, controlled by cholesterol and membranous protein ***(Pae et al., 2014)***. Direct translocation is characterized by destabilization of cellular membrane in an energy and temperature-independent manner ***(Bechara et al., 2013)***. Different models have been proposed to explain this kind of mechanism: inverted micelle formation, adaptive translocation and pore formation. Inverted micelle formation starts with an intussusception of cellular membrane due an electrostatic interactions and subsequent interaction of hydrophobic residues with the membrane core. Micelle origin from reorganization of neighbouring lipids at cellular surface, CPP is encapsulated by micelle and will be release inside after disruption of micelle ***(Derossi et al., 2002)***. Adaptive translocation seems to be exclusive for arginine-rich CPPs. Guanidium groups of arginines form bidentate hydrogen bonds with the phospholipid headgroups on the cell membrane: in this way CPPs get inside the cell. The pore formation model allows the passive diffusion of CPPs across the plasma membrane. This mechanism of uptake is mainly used by arginine and lysine rich CPPs. The attraction between the side chain of amino acid and the phospholipid headgroups of the distal layer leads to the formation of a transient pore ***(Morciano et al., 2014)***

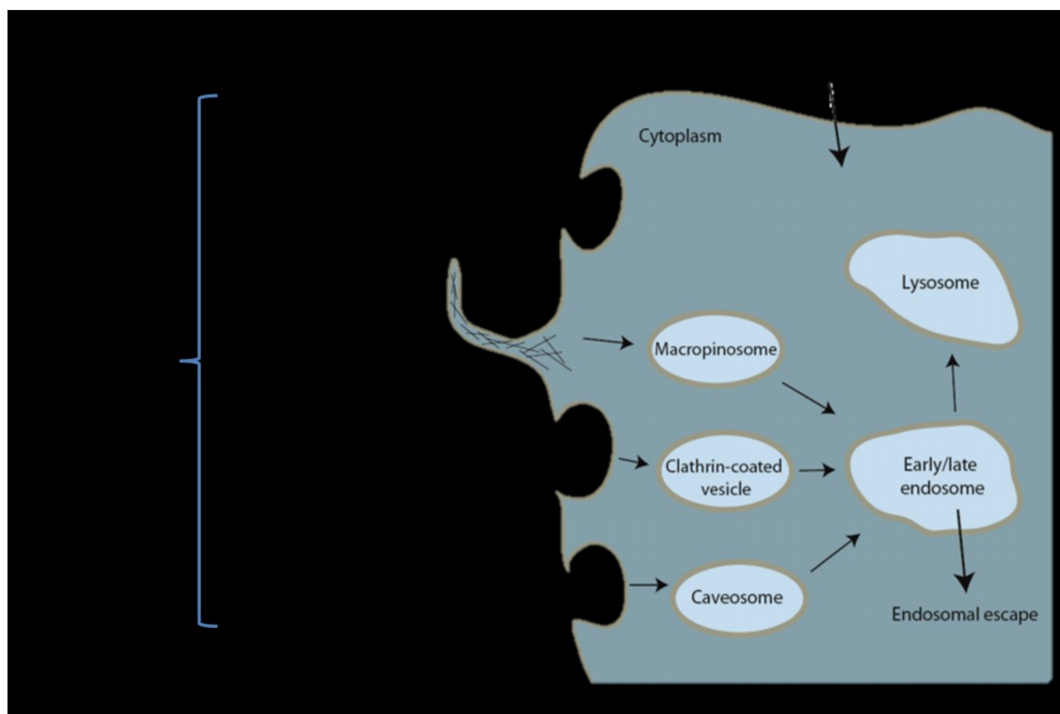


Fig.3. *Mechanism of uptake across the plasma membrane*

CPPs in drug delivery strategy: Applications and organelle specific delivery

Identification of cellular targets for treatment of different diseases required the development of a successful system able to delivery drugs into a target of interest. Some therapeutics exhibited excellent properties on *in vitro* studies, on the other hand on *in vivo* model the utilization was limited by their physicochemical characteristics. Uptake and efficacy of several therapeutic compounds are improved by CPPs conjugation, creating new opportunity to study biological process and making the treatment of several diseases more controlled, less toxic ***(Veldhoen et al. 2006)***. Different cargoes such as small molecules, imaging agents ***(Rao et al. 2007)***, small molecular drugs and larger cargoes such as oligonucleotides ***(Meade et al. 2007)***, plasmids and protein ***(Morris et al. 2001)*** can be conjugated to CPPs ***(Fawell et al.. 1994) (Fig.4)***. CPPs are actually one of the most important biological tools used to delivery drugs inside specific cellular organelle.

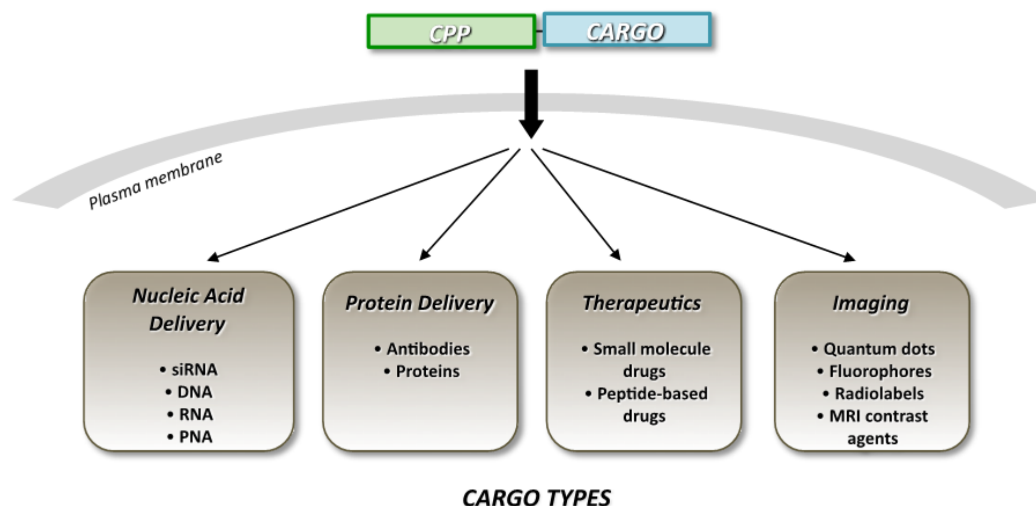


Fig.4. Applications of cell-penetrating peptidies as molecular devilery machine. Modified from Stewart et al., 2008

Nuclear Localization Sequences

Signal peptides are an effective strategy for organelle-specific targeting, used by cellular machinery to identify newly translated peptides and traffic them to the correct destination in the cell. As the storehouse of genomic DNA, the nucleus is a desirable target and the necessary destination for agents used in gene therapy ***(Cartier et al., 2002)***. Between Nuclear Localization Sequences (NLS) are cellular penetrating peptides targeting nucleus. These short cationic sequences, 10 amino acids in length, are widely used to achieve nuclear delivery for a variety of DNA damaging agents or nucleic acids for gene therapy ***(Cartier et al., 2002)***, between NLS, Simian virus 40 (SV40) showed high levels of cell permeability and low toxicity, because of that has been applied in a lot of studies to drive uptake of DNA in nonviral gene therapy ***(Singh et al., 1998)***. Other strategy adopted to delivery DNA inside the nucleus is encapsulating in polymer nanospheres or phage particles with NLS peptide displayed on the exterior ***(Akuta et al., 2002*)**. L. Benimetskaya and coll. used NLS to delivery Antisense oligonucleotides and block translation of Bcl-2 and

PKC-a in prostate and bladder carcinoma cells ***(Benimetskaya et al., 2002)***. Gold nanoparticles, carboplatin-based anti-cancer therapeutics, and green fluorescent protein (GFP) can be driven and localized inside the nucleus by NLS ***(Tkachenko et al., 2003. Wagstaff et al., 2007)***. At today, many studies show an improvement in nuclear localization and demonstrate an improved transfection efficiency ***(Ludtke et al., 1999)***

Mitochondria-Target Antioxidants

Literature shows several examples of CPPs targeting mitochondria, using artificial, rather than natural signal sequences ***(Mahon et al., 2007)***. Mitochondrion is an important target of drug therapy due to its role in the pathology of cancer, neurodegenerative diseases and other diseases dealing with reactive oxygen species. Oxidative stress is the beginning or a consequence of several pathological conditions. Mitochondria are often the organelles where oxidative stress starts or main target of oxidative stress, therefore the mitochondrion represents candidate of significant interest for organelle-specific exogenous molecules. Hindering oxidative stress with delivering of antioxidant has been found to be effective in many animal models of diseases associated with oxidative damage ***(Chao, 2014. Kim, 2014)***. Sometimes, administration of antioxidants have exacerbated the oxidative condition instead of leading to significant benefits, this conflicting action is named antioxidant paradox ***(Halliwell, 2000)***. Some antioxidants beside their antioxidant action can have a prooxidant action especially in the presence of metals such as iron (Fe) which starts Fenton reaction ***(Murakami et al., 2007)***. High hopes and expectations *in vitro* about promising antioxidants have turned into delusions when the same results were not obtained *in vivo* studies. Up to now, there are literally dozens of completed or ongoing clinical trials using such antioxidants as vitamin E, epigallocatechin gallate (EGCG), resveratrol, curcumin, pramipexole, latrepirdine, ubiquinone, lipoic acid, idebenone, Ginkgo biloba, and N-acetylcysteine ***(Mecocci et al., 2012)***. Unfortunately, only a handful of these trials have yielded

positive results, since several showed a negative association between antioxidant supplementation and positive outcomes. A significant challenge to mitochondrial drug delivery is the impervious structure of the hydrophobic inner membrane. Some antioxidants do not penetrate cellular or mitochondrial membranes therefore they are not effective against intracellular ROS, others are very lipophilic and tend to be retained in cell membranes ***(Dixon et al. 2007)***. Many efforts have been done in order to develop an ideal antioxidant and there is still a long way to go and crucial questions remain to be answered. Does the "ideal" antioxidant really exist? Several research groups are looking for this answer. The ideal antioxidant should be cell-permeable and able to target mitochondria therefore it can protect against oxidative damage and prevent mitochondrial impairmet. Latest results showed that two properties are important for passage across both the plasma and mitochondrial membranes: positive charge takes advantage of the potential gradient to get into mitochondria, lipophilic character in order to allow partitioning of the molecule through the lipid bilayer ***(Rosania et al., 2003)***.

TPP^+-conjugated

Several mitochondria-targeted antioxidants have been developed and are currently undergoing preclinical testing. In the past, the most common strategies used for delivering compounds into mitochondria have relied on the conjugation of known redox agents to triprenylphosphonium ion (TPP^+) ***(Adlam et al., 2005)***. It is an example of a cationic hydrophobic molecule with delocalized charge that can cross the mitochondrial membrane without a transporter. Another strategy is aromatic-cationic tetrapeptides that selectively target the inner mitochondrial membrane without relying on mitochondrial potential. TPP^+ has been conjugated to lipophilic antioxidants such as coenzyme Q (MitoQ) ***(Skulachev et al., 2009)***, plastoquirone (SkQ1) ***(Smith et al., 2012)*** and Vitamin E (MitoVitE) ***(Murphy et al.,2000. Sheu et al., 2006)***. They showed an increased uptake in comparison to the same molecule without carrier and improved action in reducing intracellular ROS, preserving

reduced thiols, and reducing oxidative cell death ***(Kelso et al., 2001)***. In particular, MitoVitE was reported to be 800-fold more potent than idebenone protecting against GSH depletion in cultured fibroblast from patients with Friedreich's Ataxia (FDRA). Furthermore, MitoVitE is 350-fold more potent than trolox (water soluble Vitamin E analog) ***(Szeto, 2006)***. Intraperitoneal and intravenous administrations of TPP^+ were used in mice and the times of uptake and distribution were controversial. Uptake was obtained in the liver 1 hour after intraperitoneal injection, but was not detected in the brain and in the heart after 20 hours. TPP^+ conjugated to MitoVitE was detectable in the heart 4 days after administration while levels in the brain were still really low ***(Smith et al., 2003)***. These TPP^+-conjugated were evaluated on ischemia-reperfusion injury but not yet on models of neurodegenerative diseases. The utility and applicability of TPP^+ is also limited by their mechanism of uptake. They need a normal mitochondrial potential to get this organelle, but all the neurodegenerative diseases are associated with abnormal mitochondrial potential. Furthermore, study on isolate mitochondria demonstrated that concentrations of TPP^+ greater than 20 µM lead to wasting of IMM potential ***(Smith et al., 1999)***.

Choline Esters of Glutathione and N-Acetyl-Cysteine

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) with strong antioxidant activity, detoxifying ROS and preventing thiol oxidation. Mitochondria are not capable to synthesize glutathione. The synthesis of glutathione occurs in the cytoplasm and transported into mitochondria by dicarboxylate and 2-oxoglutarate carriers ***(Chen et al., 2008)***. N-Acetyl-L-Cysteine (NAC) arises from the amino acid L-cysteine, it exhibits antioxidant activity ***(Xue et al., 2011. Reliene et al., 2004)*** as well is also used to provide cysteine for glutathione synthesis. Both of them are really useful in protecting mitochondria against oxidative damage. Using the same approach as TPP^+-conjugated described above, Sheu and co-workers prepared choline esters of glutathione and NAC to increase the uptake of glutathione and

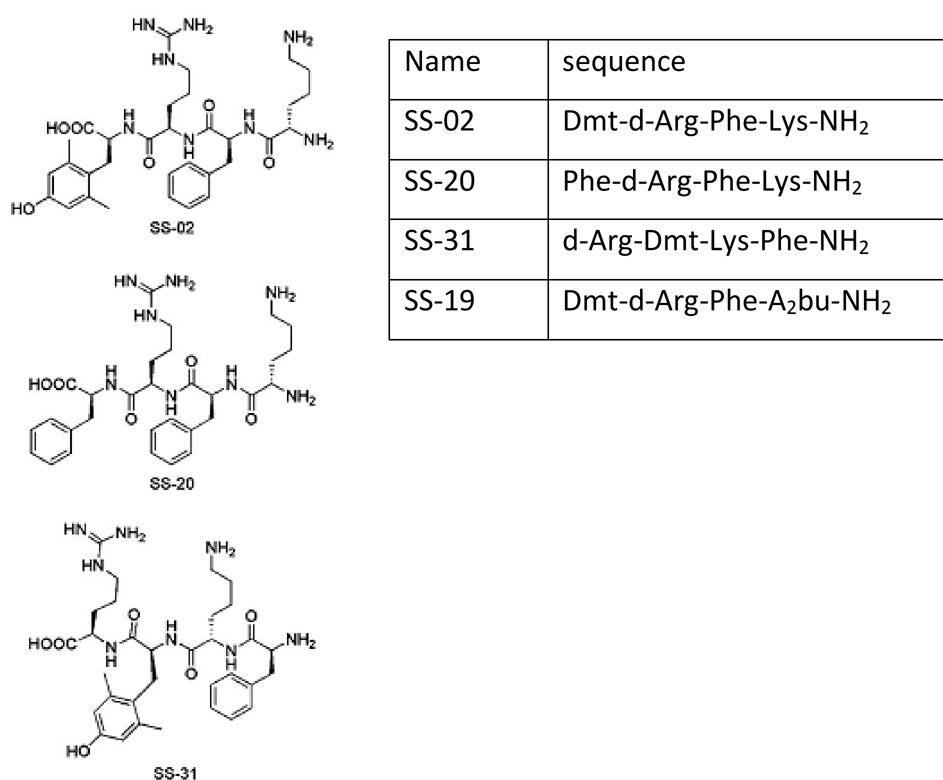
NAC into mitochondria ***(Sheu et al., 2005)***. These compounds make use the negative internal potential of mitochondria, which leads and regulates their concentration in mitochondria. Ester of Glutathione is a membrane/lipid permeable derivative of GSH that is used to restore the GSH pool within cells subjected to cysteine and/or GSH depletion. Preliminary *in vitro* studies demonstrated that they are able to avoid depolarization due to hydrogen peroxide in neonatal rat ventricular myocytes and striatal neurons, but to date but *in vivo* animal studies are not yet accessible ***(Szeto et al., 2006)***.

Szeto-Schiller Peptides

Date back in 2000, synthesis of the short Szeto-Schiller peptides (SS-peptides), the most promising mitochondria-targeted antioxidant peptide ***(Schiller et al., 2000)***. The Szeto-Schiller (SS) compounds are tetrapeptides ***(Fig. 5)*** designed for alternating aromatic residues and basic amino acids (aromatic-cationic peptides), they are characterized by tyrosine (tyr) or dimethyltyrosine (Dmt) residues in order to increase antioxidant activity and the presence of D-amino-acid in either the first or second position to minimize aminopeptidase degradation, amidation of C-terminus to reduce hydrolysis from C-terminus ***(Schiller, 2006)***. Scavenge activity of SS-peptides was proved first *in vitro* using luminol chemiluminescence, then antioxidant properties of SS-peptides were further established by inhibition of fatty acid peroxidation and low density lipoprotein (LDL) oxidation ***(Zhao et al., 2004)***. The antioxidant action of SS-peptides is dose dependent manner and can be attributed to Tyr or Dmt residues can scavenge H_2O_2, $OH^{\cdot}$ and $ONOO^{\cdot}$. The sequence position of the Tyr or Dmt residue is not important in scavenging ROS and inhibiting LDL oxidation but replacement of Dmt with phenylalanine (Phe) resulted in complete loss of antioxidant activity ***(Zhao, 2004)***. Tyr, or Dmt, can scavenge oxyradicals forming relatively unreactive tyrosyl radicals, which can be followed by radical-radical coupling to give dityrosine, or react with superoxide to form tyrosine

hydroperoxide ***(Hazel et al., 2006)***. Dmt is more effective than tyrosine in scavenging of ROS because bears much structural similarity to vitamin E, indeed both have the methylated phenol structure. Zhao and colleagues showed that upon induction of oxidative stress by tertbutylhydroperoxide (tBHP), cells treated with the SS-peptides decreased levels of mitochondrial reactive oxygen species and halted the progression of apoptosis ***(Zhao et al., 2004)***. All of SS peptides show 3^+ net charges at physiologic pH and they are taken up into cells in an energy-independent non saturable manner. The uptake is really fast, several studies showed that SS-20 peptide is taken up into cells in less than 30 minutes and can freely pass through the plasma membrane in both directions ***(Zhao, 2004)***. The SS-peptides targeting preferentially the inner mitochondrial membrane, indeed SS-31 and SS-02 are taken 1000-fold and 10,000-fold respectively, in liver and brain mouse mitochondria ***(Zhaoet al., 2004. Zhao et al., 2005)*** and concentrate in mitochondria 1,000-fold more than the cytosolic concentration ***(Zhao, 2004)***. The mechanism of uptake into mitochondria is not self limiting but how it works is still unclear. During uptake of SS-peptides there is not a well defined formation of vesicles, typical of an endocytosis uptake. The uptake of these aromatic-cationic peptides is not dependent on mitochondrial membrane potential, a study showed that they are also concentrated in FCCP depolarized mitochondria ***(Doughan, 2007)***. Experience has taught us that talking about drug development to cross the blood brain barrier (BBB) is the maximum impediment for new drugs. Studies have shown ability of SS02 to get mouse brain in 5 minutes after intraventricular injection (Gifford A., 2004, unpublished data). Between SS-peptides, the short SS-31 peptide developed by Szeto is the most promising mitochondria-targeted antioxidants ***(Zhao, 2004)*** its antioxidant potentialities were confirmed against different types of adverse treatments in both *in animal* ***(Huang et al., 2013)*** and *in vitro* models ***(Zhao et al., 2013)***. A number of studies confirmed the potential applicability of SS-peptides on different pathological conditions. Both SS-02 and SS-31 impeded myocardial stunning when administered upon reperfusion after 30 minutes ischemia in the *ex vivo* guinea pig heart ***(Wu et al., 2002)*** and SS-31 reduced infarct volume when

administered to mice after acute cerebral ischemia ***(Cho et al., 2005).*** SS-31 was evaluated in animal models of neurodegenerative disease, PD ***(Yang et al., 2009)*** and Amyotrophic lateral sclerosis (ALS) ***(Petri et al., 2006)***. Nowadays, SS-peptides are the most promising approach with targeted delivery of antioxidants to mitochondrial organelle. Their extraordinarily potent in protecting against oxidative cell death is already proven by a number of publications. Moreover, they have an excellent pharmacokinetic profile and they are easily "druggable", small, easy and fast to synthesize, readily soluble in water and resistant in human serum until to six months to ***(Schiller et al., 2000)***.



Name	sequence
SS-02	Dmt-d-Arg-Phe-Lys-NH_2
SS-20	Phe-d-Arg-Phe-Lys-NH_2
SS-31	d-Arg-Dmt-Lys-Phe-NH_2
SS-19	Dmt-d-Arg-Phe-A_2bu-NH_2

Fig. 5. *Structre and sequence of SS-peptidesAdopted from Sheu et al., 2006*

GOAL OF THE THESIS

Mitochondria are subcellular organelles involved in pivotal metabolic cellular pathways. Mitochondria developed a special membrane structure and network of antioxidant systems to help preserve its functions. A number of studies showed that the onset or the exacerbation of many diseases is caused by mitochondrial impairment ***(Szarka et al., 2014)***. Mitochondrion is an expecially interesting organelle for drug therapy given its role in the pathology of cancer, neurodegenerative diseases, and other diseases characterized by oxidative stress ***(Weissig et al., 2004)***. Drugs could be necessary for both inhibiting mitochondria in order to kill cancer cells as well as to protect the cells from oxidative damage and to repair dysfunctions. Over the years different strategies have been developed in order to get access to mitochondria, but its complex structure was often a tough hurdle to overcome so gaining access to this organelle could be difficult. Lately, the most useful strategy is based on cell penetrating peptide targeting mitochondria. Nowadays a novel class of small cell-permeable peptide antioxidants reported a great deal of attention. The structural motif of these peptides, named Szeto-Schiller peptides (SS-peptide), is characterised on alternating aromatic residue and basic amino acids ***(Szeto, 2006)***. This thesis was focused on the synthesis of a new short cell penetrating antioxidant peptide able to cross cellular membrane and target mitochondria. Gaining access to mitochondria means make easier treatments on it. The final purpose was to use antioxidant actions of this peptide to protect mitochondria against oxidative stress and due of its "druggable" properties use it as new tool on drug delivery strategy.

MATHERIAS AND METHODS

Solid phase Peptide Synthesis (SPPS)

A Peptide is a chemical compound consisting of amino acids condensed with each other through a peptide bond or amide bond between α-carboxyl group of residue and the α-amino group of next amino. Peptides have a maximum of 50 amino acids ***(Jones, 1991)*** and the term protein describes molecules with more than 50 amino acids. Peptides have a wide range of applications in medicine and biotechnology, for this reason solid phase peptide synthesis (SPPS) plays today a pivotal rule in the area of development of new therapeutic strategies, allowing the chemical synthesis of peptides and small proteins. SPPS has been used for the first time by Merrified ***(Merrefield, 1973)***, starting a revolutionary approach to the chemical synthesis of polypeptides. The basic principle of SPPS is the stepwise addition of protected amino acid to a growing peptide chain bounded by its C-terminal carboxylic acid by a covalent bond to a solid, stable and inert resin particle ***(Fig. 6)****.* By-products and excess reagents may be removed easily by filtration and washing. Peptide Synthesis starts from Carboxyl-Terminus (COOH-Terminus, or C-terminal end) to Amino-Terminus (NH_2-Terminus, or N-terminal end), adding one by one activated amino acids. Activation of the carboxyl component of amino acids is based on formation of active esters. The activation makes more electrophilic carboxyl group, because of that α-carboxyl group of the amino acids is activeted to facilitate nucleophilic attack by the α-amino group of the previously coupled amino acid. Peptide bond is assisted by presence of coupling reagents, called also auxiliary nucleophiles. The newest coupling reagents belong to uranium and phosphonium salt. The use of these reagents has been reported to be more convenient and superior. Some of the most commonly used coupling reagents are BOP, PyBOP, TBTU, HBTU, HATU, TFFH, PYBrOP, TOTU ***(Hudson, 1988)*****.** Coupling and activation of amino acids are really important step, on account of this the amount of reagents is at least 10 times more

than amount of resin. Good outcome of reaction depends on availability of only a single nucleophile during acylation reaction, therefore is necessary to block those functional groups that must not participate in the peptide bond formation. In peptide synthesis the α-amino group are protected with temporary protecting groups, which are cleaved after each coupling reaction, and the functional groups of the amino acid side chains are protected with permanent protecting groups, which are cleaved after the synthesis is completed. In SPPS two main strategies are used: Boc /Benzyl-based strategy, Fmoc/*tert*-butyl-based strategy. ***Tert*-butyloxycarbonyl** (Boc) was first applied in 1950s; this group is stable towards most nucleophiles and bases, it is cleaved by acid *e.g.* Trifluoroacetic Acid (TFA) in Dichloromethane (DCM) or others strong acids *e.g.* HBr in TFA. This strategy is based upon the graduated acid lability of the side-chain protecting groups. In Boc/benzyl-based SPPS a number of side reactions may be caused by repetitive acid treatments during the synthesis and the use of a strong acid for the final deprotection. In order to avoid side reactions generated by Boc strategy, a new protocol based on orthogonal 9-fluorenylmethyl-oxycarbonyl (Fmoc) protecting group was developed by Carpino and Han. ***(Carpino, 1972*)**. The Fmoc group is stable to acid, but is clave by base, *e.g.* piperidine in DMF, or tert-butyloxycarbonyl. Fmoc-based SPPS method is now the method of choice for the routine synthesis of peptides. Semi permanent protection groups for functional amino acid side chains are removed when synthesis ends. There are different protecting group strategies that allow in selecting amino acid being protected on the base of amino acids side chains. Cleavage and final deprotection are really important steps in peptide synthesis. The cleavage cocktails frequently used is a mixture of TFA and scavengers. The SPPS involves numerous repetitive steps. After loading the first amino acid, the desired peptide sequence is assembled in a linear fashion from the C-terminus to the N-terminus, alternanting deprotection and coupling of amino acids until the desired sequence is obtained. This technique has made the synthesis of peptides faster: averagely, each amino acid is added every hour, just the time required in order to complete the reaction.

In a final step, the peptide is released from the resin and the side-chain protecting groups concomitantly removed.

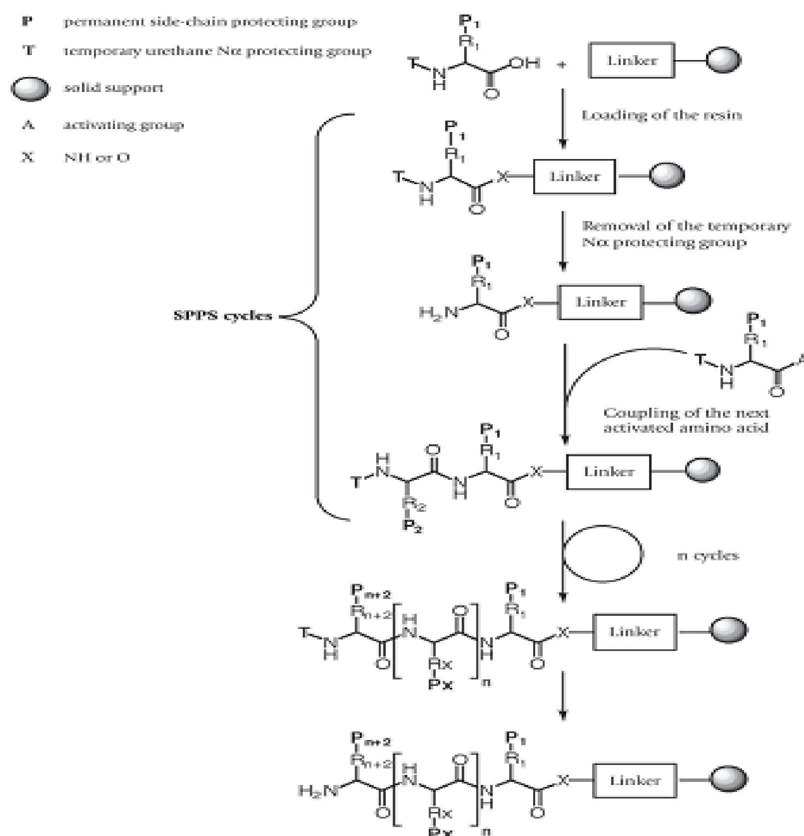


Fig.6. *General scheme of solid phase peptide synthesis, SPPS. Adapted from Amblard et all., Peptides synthesis and application. Vol. 298. Humana press 2005*

Manual Synthesis of MitPep-peptide and SS31 peptide

All the peptides were synthesized manually by solid phase peptide synthesis using Fmoc-chemistry protected amino acids and 1,3-diisopropylcarbodiimide (DIC):1-(HOBt) as coupling agents ***(Soomets et al., 2005)***. SPPS can be performed in classical glass reaction vessels that can be made by glassblowers or purchased from manufacturers, alternatively syringes equipped with PTFE or glass frits may also be used. For the peptide synthesis Fmoc-Rink-Amide-MBHA-resin was usually used. Before starting the solid phase synthesis, the resin was swollen in an adequate solvent such as DCM or DMF. Couplings were performed with Fmoc-protected D or L-amino acids and tree activators 1H-benzotriasole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), N-hydroxybenzotriazole (HOBt) and N,N-diisopropylethylamine (DIPEA) in N,N-dimethyl formamide (DMF) for 45’ each coupling *(Table. 2)*, the Fmoc group was removed with piperidine (20% v/v) in DMF (20min). The success in each coupling and deprotection step were evaluated by qualitative Kaiser test. Typically compared to the resin, 2–10 times excess of activated amino acid is used. This excess allows a high concentration of reactants (typically 60–200 mM) to ensure appreciable amount of products ***(Muriel et al., 2006)***. The final cleavage from the resin/deprotection of the peptides was performed to minimize the by-products formation resulting from Rink amide resin at high concentrations of trifluoroacetic acid (TFA). The peptide was deprotected from permanent groups and detached from the resin and by adding trifluoroacetic for peptide lacking cysteine acid (TFA) 95% (v/v), water 2.5%(v/v), triisopropylsilane (TIS) 2,5% (v/v); for peptides containing cysteine or metionine TFA 94% (v/v), water 2,5% (v/v), 1,2-Ethanedithiol (EDT) 2,5%(v/v), TIS 1% (v/v), both mixture per 100 mg of resin. Cleavage and deprotection are one of the crucial steps in peptide synthesis, is not a simple reaction, but a series of competing reactions, it takes at least 3 hours at room temperature, under gentle stirring. To induce peptide precipitation, the cleavage mixture dropwise was added to cold (-20) ether in a 50-ml falcon tube,

about 40 ml ether for 3 ml cleavage cocktail, centrifuge at 7000 rpm for 5 minutes. Then slowly and carefully ether solution was decanted to a waste container avoiding shaking up the precipitate, was repeated two times to get rid of residual TFA and scavengers. The crude precipitate was left under fume hood until ether evaporated completely. The crude peptide was obtained in solid form through lyophilization of the acetic acid extract. A fluorescent analog containing 5,6-carboxyfluorescein (FAM) was prepared for mitochondrial and cellular uptake studies

Compound	MW	Equivalents	mmol
Peptide resin		1	0.065
Fmoc-amino acid		4	0.26
HCTU,0.5M in DMF	413.17	4	0.26
6-Cl-HOBt 0.5M in NMP	169.57	4	0.26
DIPEA, 5.84M neat	129.3	8	0.52

TABLE. 3. *Relative amount used during synthesis of peptides*

Purifiction of peptides by HPLC and mass evaluation by MALDI-TOFF

The crude peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a BioBasic C-8 column (Thermo Scientific, Sweden). The stationary phase was porous silica, covalently bound to a non–polar compound such as octadecyl silane (C18) or octysilane (C8). The convenient mobile phase for the separation of peptides was a gradient elution system where the composition of the solvent was continuously changed by a gradient programmer. All solvents used in HPLC systems must be of specific grade to keep save column and allow the use of a highly sensitive detection system. Routinely the crude peptides were purified employing an acetonitrile (ACN)/water mixture with a gradient of 0.1% TFA. Crude

peptide was lyophilized, briefly, to dilute the correct peptide fraction in few millilitres of MQ water and freeze at -80°C for 24 h, then make holes into the cap of the tube with a syringe needle. Thereafter the tube of freezed peptide with solution was put into vacuum machine overnight. Each peptide was at least 98% pure as assessed by analytical reversed-phase HPLC. Molecular mass of peptides was determined by a matrix–assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI–TOFF) (Voyager-DE STR, Applied Biosystems), the values were compared with theoretical mass. According to the Applied Biosystems guidelines, saturated solution of α-cyano-4-hydroxycinnamic acid was chosen for the matrix (10 mg/ml in 50:50 ACN/water mix with 0.1% TFA content). MALDI is an ionization technique based. In this technique the sample is crystallized together with a matrix which is irradiated by a UV–laser beam. The matrix is ionized by the laser and some of its charges achieve the analyte, generating molecular ions of the analyte, in the same time protecting it from direct ionization by the laser. The time of flight detector evaluates the spent time for the analyte ion to reach a detector at known distance. This time depends on the mass-to-charge ratio of the particle and might be used to get the mass of the ion.

Cell culture

HeLa 705, cervical human cellular line was chosen to evaluate biological effects and uptake of MitPep. HeLa cells are important tool on biological science and medical research, they are the first immortal human cells ever grown in culture, many scientific landmarks since then have used HeLa cells, including cloning ***(Leid et al., 1992)***, gene mapping ***(He et al., 2014)***, in vitro fertilization ***(Ashizawa et al., 1992)***, drug delivery strategy ***(Bracht et al., 2014)***. HeLa 705 cells (human cervical cancer cell line) were cultured as subconfluent monolayers in 75cm^2 cell culture flask. HeLa 705 in Dulbecco's Modified Eagle Medium (DMEM) (lifetechnologies) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% non-essential amino acid (GIBCO) and

1% Penicillin-Streptomycin (life technologies), kept in a humidified incubator at 37° C, 5% CO_2. Cells grown to subconfluence were enzymatically dissociated from the surface with a solution of 0.05% trypsin/0.53mM EDTA (ethylenediaminetetraacetic). To evaluate viability, mitochondrial membrane potential and ROS production, cells were plated at 1 X10^4 cells/well in 96 well plates 24h prior to the experiment. To isolate mitochondria 7X10^6 cells were plated in 275 cm^2 cell culture flask 3 days prior to experiment. For cellular uptake experiments and microscopy studies cells were plated at 7,5X10^4 cells were plated in glass bottom disches. For fluorescence studies was used a fluorescent analog FAM-MIP1 conjugated.

Cell viability assay

Cell viability was determined by conventional WST-1 assay (Roche Diagnostics Scandinavia AB, Sweden). WST-1 reagent is designed to be used for the quantification of cell proliferation using 96 well-plate format, it is a colorimetric assay based on reductases activity of mitochondrial succinato deidrogenase. A tetrazolium salt is reduced to formazan dye by active mitochondria, the amount of dye is correlated to the number of cell with active succinato deidrogenase. Briefly, HeLa 705 cells were plated at a density 1X10^4 cells/well in 96-well plates and allowed to grow for 24 h before treatment with MIP1. The concentration of MIP1 stock solution was 1 mM in MilliQ water, different working solutions were prepared. Cells were treated at the final concentrations 0.5-5-50-100 µM in 100 µl of complete medium for 24 h and H_2O_2 200 µM for 24h was used as positive control. Cell proliferation reagent WST-1 was added to each well at final dilution 1:10, in this case 10µl/100µl. After 4 h incubation the absorbance was measured at 450 nm on Sunrise microplate absorbance reader (Tecan, Switzerland). The same assay was performed in order to evaluate the viability after H_2O_2 treatment. Briefly, HeLa 705 cells were plated and treated as described above. Cells were insulted by

two different hydrogen peroxide conditions: treatment of MIP1 for 30' or 2 h before to receive H_2O_2 200 µM, then kept in a humidified incubator at 37° C, 5% CO_2 for 24 h. Cell proliferation reagent WST-1 was added to each well as described above, after 4 h substrate reaction the absorbance was determined at 420 nm. Cell viability of treated cells was expressed as a percentage of the viability of cells MIP1 or H_2O_2 untreated.

Mitochondrial membrane potential assay

Mitochondrial membrane potential was evaluated using the fluorescent probe TMRE (tetramethylrhodamine methyl ester) (TMRE, mitochondrial potential membrane, assay kit. Abcam). TMRE is a cell permeant, positively-charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have a reduced mitochondrial membrane potential and fail to sequester TMRE. Briefly, HeLa 705 cells were plated in 96 well plates 24h prior to receive treatments. The day after cells were treated with MIP1 for 24h at the same concentrations described above, H_2O_2 200 µM was used as positive control. Cells were loaded with TMRE 400 nM and were kept at 37°C-5% CO_2, protected from light. After 30' of TMRE staining, media was aspired and cells were washed once with 0.2% BSA in normal PBS to remove background fluorescence from the culture media, 100µL/well of 0.2% BSA in normal PBS was added. The plate was read on a fluorescence reader with settings suitable for TMRE (more details see below). The same assay was performed in order to evaluate the ability of MIP1 to restore a normal mitochondrial membrane potential after insult of H_2O_2. Briefly, HeLa 705 cells were plated and treated with MIP1 at the same concentrations described above, after 2 h cells were insulted by H_2O_2 200 µM, kept for 24 h of incubation at 37°C-5% CO_2. TMRE was added to each well at concentration described above. After30', media was aspired and cells were washed once with 0.2% BSA in normal PBS and replaced with 100µL/well of 0.2%

BSA in normal PBS. Semi-high throughput screening (HTS) fluorescence reader (FlexStation II, Molecular Devices) was used and Fluorescence at 518/580 (ex/em) was registered in order to measure the production of ROS by mitochondria. Fluorescence at 549/575 (ex/em) (TMRE-tetramethylrhodamine, ethyl ester) was registered for interrogating mitochondrial membrane potential in live cells. $\Delta\Psi m$ polarizations were observed on increasing TMRE relative fluorescence unit, and decreasing TMRE relative fluorescence unit were associated to depolarization. After an initial 20 seconds baseline recording, 10 µl of reference chemical dilutions (prepared at 10 times the final well concentration) was transferred automatically by the FlexStation II to plate wells (6 wells per reference chemical concentration) and fluorescence was read for another 220 seconds. The mean of the values from the baseline recording was set to zero, and the size of CMP change was quantified as area under the curve, using the SoftMax® Pro 4.8 software (Molecular Devices). Mitochondrial membrane potential on treated cells was expressed as a percentage of the mitochondrial membrane potential on untreated cells or H_2O_2 treated cells.

ROS production assay

Mitochondria's ROS production was measured by fluorescent probe MitoSOX Red mitochondrial superoxide indicator (Invitrogen Detection technologies). 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 ***(Invitrogen Detection technologies)***. As manufacture protocol suggests, one vial of MitoSox reagent was dissolved in 13 µL of dimethylsulfoxide (DMSO) to make a 5 mM MitoSOX reagent stock solution, the 5 mM MitoSOX reagent stock solution (prepared above) in HBSS/Ca/Mg to make a 5 µM MitoSOX reagent working solution. Briefly, HeLa 705 cells were plated in 96 well plates 24h prior to receive treatments. The day after cells were treated with FAM-MIP1 for 24 h at the same concentrations previously described. Cells were loaded with MitoSOX

5 µM and were kept for 10' of incubation at 37°C-5% CO_2, protected from light. After MitoSOX staining, media was aspired and cells were washed once with 0.2% BSA in normal PBS to remove background fluorescence from the culture media, 100µL/well of 0.2% BSA in normal PBS was added. The plate was read on a fluorescence reader (more details see below). The same assay was performed in order to evaluate the ability of MIP1 to save normal levels of ROS in insulted cells by H_2O_2. Briefly, HeLa 705 cells were plated and treated as described above. After 2 h treatment of MIP1, cells were insulted by H_2O_2 200 µM and kept for 24 h of incubation at 37°C-5% CO_2. MitoSOX was added to each well at concentration described above. After 10', media was aspired and cells were washed once with 0.2% BSA in normal PBS and replaced with 100µL/well of 0.2% BSA in normal PBS. The absorbance was measured by fluorescence reader. Semi-high throughput screening (HTS) fluorescence reader (FlexStation II, Molecular Devices) was used and Fluorescence at 518/580 (ex/em) was registered in order to measure the production of ROS by mitochondria. Fluorescence at MitoSOX (Ex: 510nm, Em: 580nm) was registered for interrogating levels of ROS in live cells. More amount of ROS were observed on increasing MitoSox relative fluorescence unit, and decreasing MitoSox relative fluorescence unit were associated to decreased oxidant species. After an initial 20 seconds baseline recording, 10 µl of reference chemical dilutions (prepared at 10 times the final well concentration) was transferred automatically by the FlexStation II to plate wells (6 wells per reference chemical concentration) and fluorescence was read for another 220 seconds. The mean of the values from the baseline recording was set to zero, and the size of CMP change was quantified as area under the curve, using the SoftMax® Pro 4.8 software (Molecular Devices). Amount of fluorescence in treated cells was expressed as a percentage of the amount of fluorescence in MIP1 or H_2O_2 untreated cells.

Measurement of mitochondrial uptake of (FAM) 5(6)carboxyfluorescein-MIP1 conjugated on isolated mitochondria

In order to evaluate mitochondrial uptake of MIP1, mitochondria from HeLa 705 cells were extracted following the instruction of Mitochondria Isolation Kit for cultured cells (Thermo Scientific, Pierce, USA). It was essential to start with an appropriate amount of cultured cells to obtain a visible and experimentally manageable pellet of the mitochondrial fraction. Briefly, according to the method by Zhao K. et al. (Zhao et al., 2004) for uptake study isolated mitochondria from 20 106 cells were incubated in 0.3 ml buffer (10 mM TrisHCl pH 6.7, 0.15 mM MgCl2, 0.25 mM sucrose, 1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 mM Dithiolthreitol (DTT) containing 5 µM of fluorescent polyanionic probe FAM-conjugated to MIP1, time of treatments was 4 h at RT. Uptake was stopped by centrifugation (12,000 X g for 5' at 4° C), the mitochondrial pellet was washed twice and resuspended in 0.3 ml of phosphate buffered saline (PBS). Mitochondrial suspension was split into three rates, 100µl volume each and transferred in 96 plates black well. Mitochondrial uptake of FAM was determined using same procedure. Fluorescence at 494/519 nm (ex/em) (FAM) was registered every 2 seconds in a semi-high throughput screening (HTS) fluorescence reader (FlexStation II, Molecular Devices) to quantifying the peptides into the cells or into the isolated mitochondria. After initial 20 seconds baseline recording, 10 µl of reference chemical dilutions (prepared at 10 times the final well concentration) was transferred automatically by the FlexStation II to plate wells and fluorescence was read for another 220 seconds. The mean of the values from the baseline recording was set to zero, and the size of CMP change was quantified as area under the curve, using the SoftMax® Pro 4.8 software (Molecular Devices). Amount of fluorescence in FAM-MIP1 complex treated cells was expressed as a percentage of the amount of fluorescence in FAM treated cells

Cellular uptake and intracellular localization of MIP1 Peptide

FAM-MIP1 uptake in HeLa 705 cells was carried out as previously described ***(Zhao et al., 2004)***. Cells were incubated with FAM labeled MIP1 (5 µM) for 4 h at 37° C and imaging was performed. In order to identify MIP1 intracellular localization, a final concentration of 5 µM FAM labeled MIP1 or SS31 was incubated with HeLa 705 cells for 4 h at 37° C. To demonstrate mitochondrial localization, TMRE (100 nm) mitochondrial dye was added to the medium 4 h after and nuclear dye Hoechst 33358 (50 nm) 15' after TMRE. The cells were washed three times with PBS. Imaging was performed on living cells using Leica DM/IRBE 2 epi-fluorescence microscope controlled by micro manager ***(Edelstein et al., 2010)*** with a 63 × 1.4 NA oil immersion objective. Emission were collected between 494 and 519 nm (FAM-5(6)-Carboxyfluorescein), 549 and 575 nm (TMRE red fluorescence), 352 and 461 nm (Hoechst 33358-Pentahydrate (bis-Benzimide). Cells were manually segmented and region intensities were quantified using Fiji (ImageJ) ***(Schindelin et al., 2012)***.

Statistical Analisys

All data were expressed as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism software v. 5.00. Comparisons between two values were performed using an unpaired Student t test

RESULTS

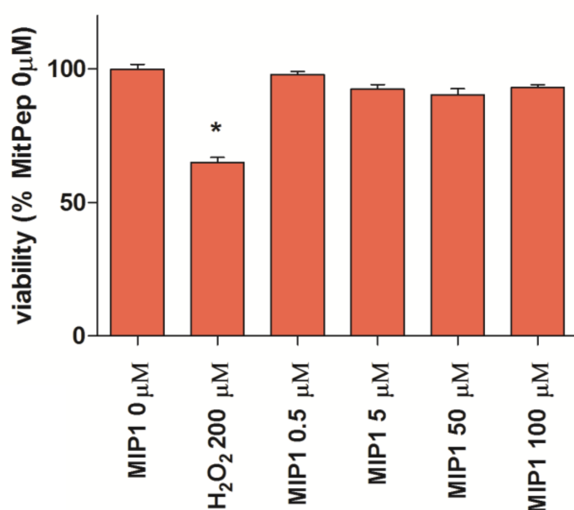
Design of MIP1 peptide

Using the solid-phase synthesis protocol described above, a new short peptide was synthesized. MIP1 is a tetrapeptide, its sequence was designed to display at least three characteristics known as important to targeting mitochondria: positive charge, lipophilic character and alternating aromatic residues and basic amino acids. The release of protons from the mitochondrial matrix to the intermembrane space generates a negative potential (-150/-180Mv) in mitochondrial inner membrane ***(Dai et al., 2014)***. The negative potential works as attractive point on the use of lipophilic cations to deliver redox agents into mitochondrial matrix. Lipophilicity is an important condition in order to obtain selective peptide targeting mitochondrial and to allow partitioning of the peptides through the lipid bilayer ***(Rosania, 2003)***. It was suggested by Zhao and colleagues ***(Zhao et al., 2004)*** that alternating aromatic and cationic residues indicate that this motif is an effective one for mitochondrial delivery. The synthesis of MIP1 was carried out after evaluation of data previously published in this field **(Horobin, 2007. Szeto, 2006)** and the following rules were kept :

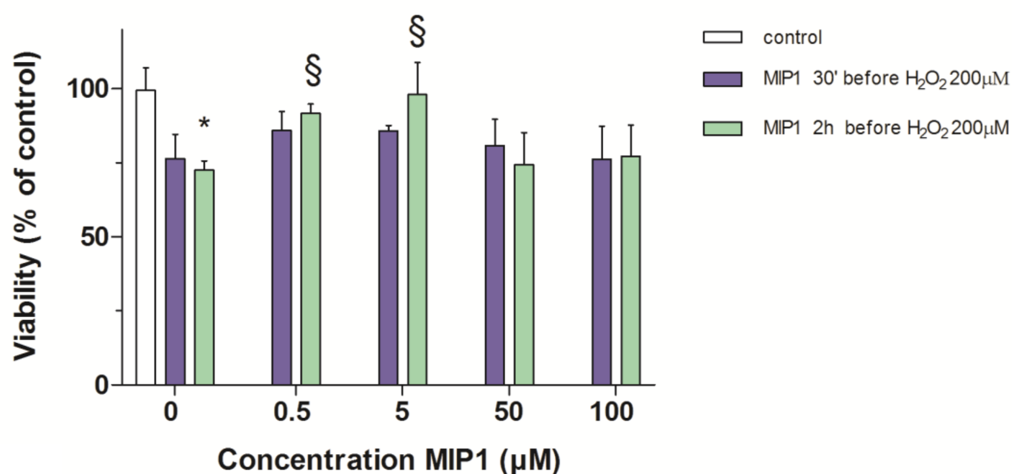
1. tyrosine (tyr) or dimethyltyrosine (Dmt) residues in the first or second position of sequence to increase antioxidant activity.
2. D-amino-acid in either the first or second position to minimize aminopeptidase degradation and increase stability in serum.
3. Amidation of C-terminus to protect against hydrolysis.

Effects of MIP1 peptide on the cell viability of HeLa 705 cells

The toxic effect of MIP1 peptide was evaluated on HeLa 705 cells using conventional WST-1 assay ***(figure. 7-A)***. Different concentrations of MIP1 were chosen as treatment (0.5-5-50-100 µM). Viability was calculated after 24h of MIP1 incubation and H_2O_2 200 µM for 24h was chosen as positive control. MIP1 did not show toxic effects on viability of HeLa 705 cells at concentrations used even at higher used concentration (100 µM) viability was still around 90% compared to non treated cells; on the contrary positive control group showed 35% of decreased viability in comparison with no treated cells. The same viability assay was used to appreciate eventual protective effect of MIP1 against H_2O_2 damage. HeLa 705 cells received MIP1 pretreatments at the concentrations described above, after 30' or 2h they were insulted by H_2O_2 200 µM. Hydrogen peroxide resulted in a significant reduction of cell viability but in pretreated cells H_2O_2 damage was decreased by antioxidant activity of MIP1. Protective effects were seen with MIP1 doses of 0.5 and 5 µM. As shown in **figure 7-B**, hydrogen peroxide treatment reduced cell viability up to 77% compared to control group, pre-treatment of 30' with MIP1 at 0.5 and 5 µM restored it at 85%. Cell viability was restored at 90% after 2h pretreatment of MIP1 with dose of 5 µM. MIP1 showed protective action from 0.5 to 5 µM, on the contrary high used concentration did not show defensive properties against hydrogen peroxide insult



A



B

Fig.7. Effect of MIP1 peptide (0.5-5-50-100 μM) on HeLa 705 cells viability in absence or presence of hydrogen peroxide. Cell viability was quantified by conventional WST-1 assay

(A). HeLa 705 cells were treated with MIP1 for 2 h, H_2O_2 200 μM was chosen as positive control of toxicity, MIP1 treatments did not show toxicity on HeLa 705 cells. *$p < 0.05$ compared to MIP1 0μM ***(B)*** HeLa 705 cells were treated as the same concentrations described above, H_2O_2 200 μM insult was added 30' or 2h after MIP1 treatments. Pretreatment with MIP1 (0.5-5 μM) attenuated the reduction in cell viability. Maximum protection was seen in cells pretreated with MIP1 at 5 μM 2h before H_2O_2 200 μM.*$p < 0.5$ compared to MIP1 0 μM. § $p < 0.05$ compared to MIP1 0 μM 2h before H_2O_2 200 μM

Data shown represent means of a minimum of three trials, standard error for each data point is shown.

Effects of MIP1 peptide on mitochondrial membrane potential of HeLa 705 cells

Mitochondrial membrane potential was determined by fluorescent probe TMRE, HeLa 705 human cervical cancer cell line was chosen as cellular model. Eventual membrane potential impairment was evaluated after 24h treatments of MIP1 peptide (0.5-5-50-100 µM); H_2O_2 200 µM for 24 h as positive control of toxicity was used to obtain a significant membrane depolarization. Results showed that treatments of MIP1 did not perturbed mitochondrial membrane potential**.** Mitochondrial membrane potential after MIP1 treatments at 0.5 and 5 µM was between 80 and 95 % of the control value. On the other hand cells treated with 100 µM of MIP1showed a significant reduction of mitochondrial membrane potential ***(Fig. 8.)***. To demonstrate that reduction in mitochondrial generated ROS can protect against mitochondrial dysfunction, was examined the effect of MIP1 on mitochondrial depolarization-H_2O_2-induced. Hydrogen peroxide caused a depolarization of mitochondrial potential 45 % of $\Delta\psi m$ compared to control. Pretreatments of MIP1 at 0.5 and 5 µM showed a significant increasing of fluorescence intensity compared to value of hydrogen peroxide fluorescence. In particular, pretreatment of MIP1 at 5 µM, maintained a potential up to 92% of control value (MIP1 0 µM). Concentrations of MIP1 at 100 µM did not show protective action ***(Fig 8.)***.

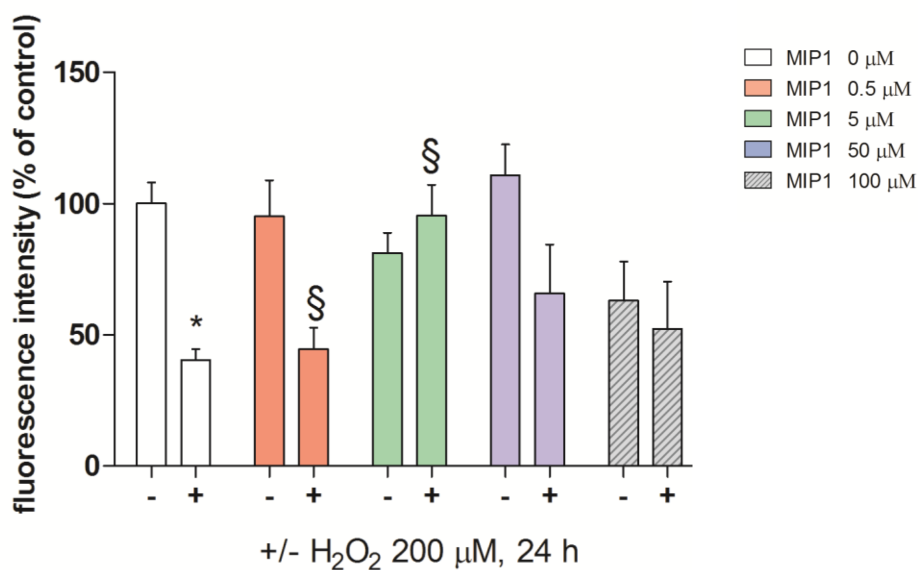


Fig.8. Effect of MIP1 peptide (0.5-5-50-100 μM) on HeLa 705 cells mitochondrial membrane potential in absence or presence of hydrogen peroxide. Mitochondrial membrane potential was quantified by the fluorescent probe TMRE

MIP1 24 h treatments did not perturb mitochondrial membrane potential on HeLa 705 cells. ***(first bar of each group)***. In the second bar of each group H_2O_2 200 μM treatment was added 2h after MIP1 treatments. Pretreatment with MIP1 (0.5-5 μM) preserved a normal mitochondrial membrane potential. Maximum protective action was seen in cells pretreated with MIP1 at 5 μM 2h before H_2O_2 200 μM.*$p < 0.5$ compared to Mip1 at 0μM. § $p < 0.05$ compared to MIP1 at 0 μM 2h before H_2O_2 200 μM

Effects of MIP1 peptide on ROS production in HeLa 705 cells

Reactive Oxygen species (ROS) production was determined using fluorescent probe MitoSOX Red mitochondrial superoxide indicator. HeLa 705 human cervical cancer cell line was chosen as cellular model. Eventual ROS production was analyzed after 24h treatments of MIP1 peptide (0.5-5-50-100 µM); treatments of H_2O_2 200 µM for 24 h were used as positive control. In order to evaluate an eventual antioxidant activity, cells were pretreated with MIP1 for 2 h then H_2O_2 200µM was added for 24h. H_2O_2 treatment increased ROS production up to 135% of control value ***(Fig. 9)***, lower mitosox fluorescent intensity was appreciated in MIP1 at 0.5 and 5 µM pretreated group**.** In particular, treatments of MIP1 0.5 µM and 5 µM decraesed of 10% and 20% the MitoSox fluorescence intensity compared to H_2O_2 treatment. Higher concentrations of MIP1 showed a decreasing protective action, 50 and 100 µM did not protect against H_2O_2 ***(Fig. 9).***

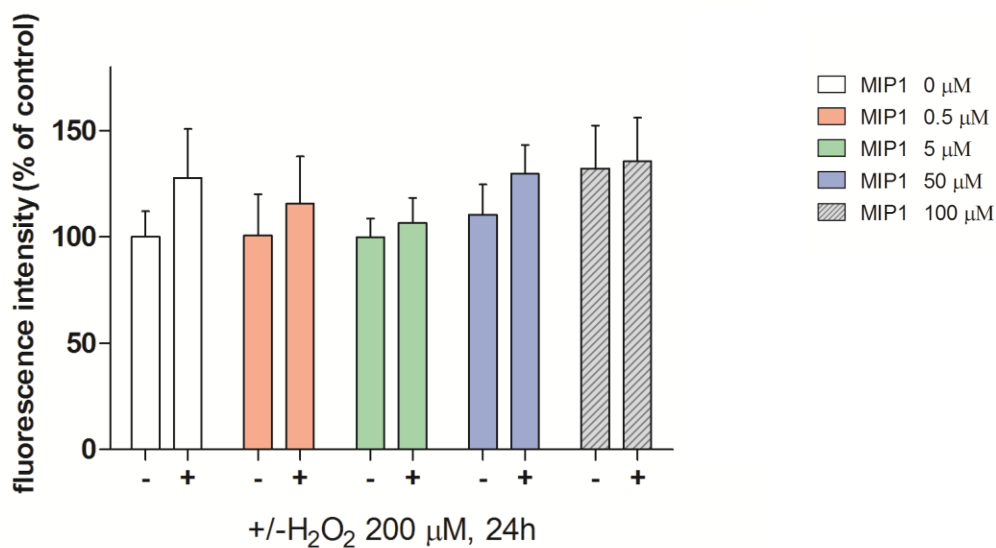


Fig. 9. Effect of MIP1 peptide (0.5-5-50-100 μM) in ROS production on HeLa 705 cells in absence or presence of hydrogen peroxide. ROS production was quantified by fluorescent probe MitoSOX Red mitochondrial superoxide indicator

MIP1 treatments 24 h did not increase amount of ROS on HeLa 705 cells ***(first bar of each group)***. In the second bar of each group H_2O_2 200 μM treatment was added 2h after MIP1 treatments. Pretreatment with MIP1 (0.5-5 μM) preserved aa amount of ROS near to value on non treated cells. Maximum protective action was seen in cells pretreated with MIP1 at 5 μM 2h before H_2O_2 200 μM

Data shown represent means of a minimum of three trials, standard error for each data point is shown.

MIP1 cellular uptake and localization

To demonstrate that MIP1 is cell-penetrating peptide, it was conjugated to 5,6-carboxyfluorescein (FAM). HeLa 705 cells were treated with FAM-MIP1 (5 µM) and incubated for 4 h at 37°C, then imaging was performed using fluorescence microscopy. MIP1 was taken up into HeLa 705 cells after 4 h of treatment. In order to verify the ability of the peptide to target the mitochondria a red fluorescence dye, tetramethylrhodamine, ethyl ester (TMRE) that specifically localizes into active mitochondria was used. In order to compare the cellular localization between the original peptide and the new one Hela 705 cells were treated also with FAM-SS31. As shown in *Figure 10*, Leica DM/IRBE 2 epi fluorescence microscope captured identical patterns in HeLa 705 cells when TMRE, FAM-MIP1 were co-incubated, as a proof that MP1 is targeting mitochondria. In order to compare the cellular localization between the original peptide and the new one, HeLa 705 cells were treated also with FAM-SS-31. Afterwards, the cellular localization of FAM-MIP1 was compared with FAM-SS31 one, the original peptide. The SS31 peptide was used as model of CPPs targeting mitochondria. The evaluation of relative fluorescence of both MitPep and SS31 showed similar intracellular distribution. Moreover HeLa 705 cells taken up more FAM-MIP1 than FAM-SS31 as shown in the microscopy picture ***(Fig. 10)*** and confirmed from the relative quantification based on the fluorescence intensity ***(Fig. 11)***.

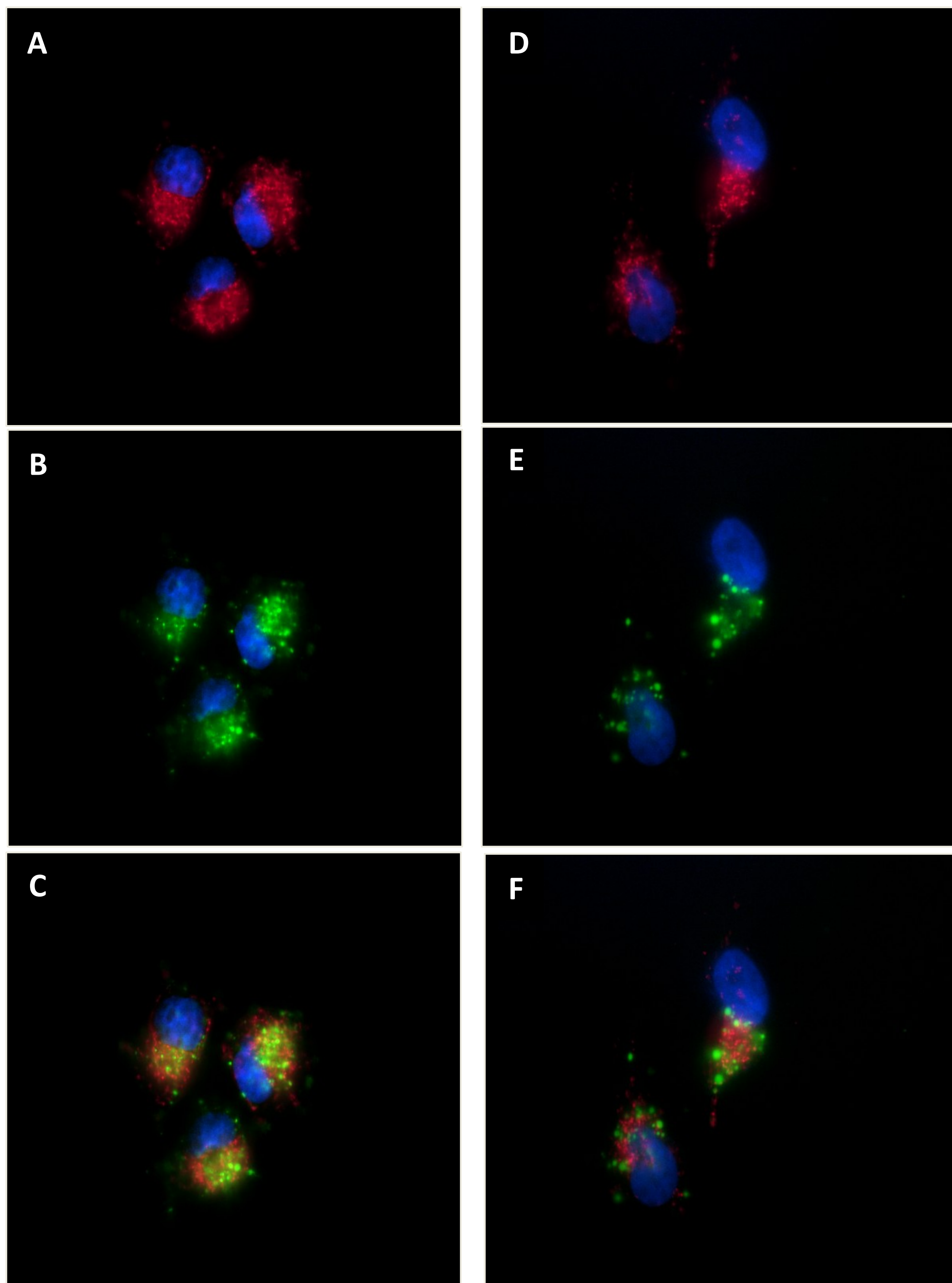


Fig.10 . Fluorescence image of cellular uptake and localization of MIP1 and SS-31 on HeLa cells

HeLa cells were treated with FAM-MIP1 (A, B, C) or FAM-SS-31 (D; E; F) at 5μM, (green fluorescence). After 4 H cells were counterstained with mitochondrial dye, TMRE (red fluorescence) and nucleus dye, Hoechst 33258 (blue fluorescence). Merged images (C) shows co-localization of TMRE and FAM-MIP1 fluorescence, proving that MIP1 is targeting mitochondria. The evaluation of relative fluorescence of both MIP1 and SS31 showed similar intracellular distribution (C; F)

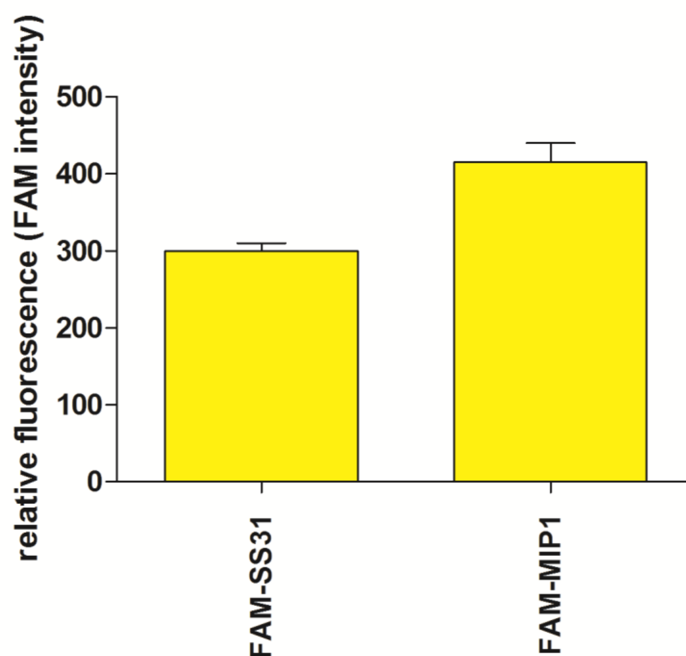


Fig. 11. FAM relative fluorescence intensity

Graphs show relative fluorescence of FAM-MIP1 and FAM-SS31 in HeLa cells. After 4 h treatments intensity was more on FAM-MIP1 treatment than on reference control FAM-SS31 treated cells.

Mitochondrial uptake of MIP1

Mitochondrial uptake of MIP1 was examined using isolated mitochondria. In this study, isolated mitochondria were treated with MIP1 conjugated with FAM for 4 hours at 5 µM. FAM alone was used as a positive control at the same concentration. After 4 h of treatment the fluorescence was read with a spectrofluorometer. 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-MIP1 showed higher fluorescence intensity, statistically significant in comparison to FAM mitochondria treated. In mitochondria FAM-MIP1 treated group the intensity of fluorescence was eight times higher than FAM treated group. ***(Fig. 12)***

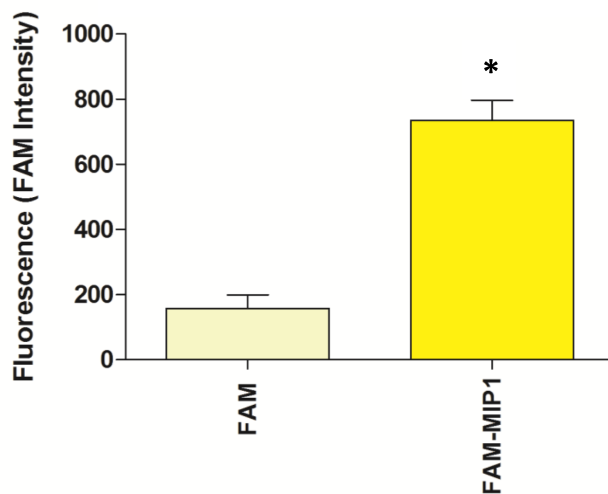


Fig. 12. FAM Fuorescence intensity on isolated mitochondria.

Black bar shows FAM relative fluorescence in isolated mitochondria, Blue bar relative fluorescence in FAM-MIP1 conjugated treated mitochondria.*$p < 0.05$ compared to FAM fluorescence intensity.

Data shown represent means of a minimum of three trials, standard error for each data point is shown

DISCUSSION AND CONCLUSION

Nowadays is known that mitochondrial impairment results in over ROS productions and this gave rise to onset or to exasperation of diseases characterized by different clinical symptoms. Sometimes antioxidants may be harmful because need really high dose supplementation to get specific target resulting in prooxidant effects ***(Steinhubl*. *2008)***. Mitochondrial ROS are generated as normal byproducts of oxidative metabolism. Approximately 3% of mitochondrial oxygen consumed is incompletely reduced ***(Gauuan et al., 2002)***; those “leaky” electrons can easily interact with molecular oxygen to generate ROS such as superoxide anion ***(Tieu et al., 2003)***. This knowledge increased exponentially the number of studies on mitochondrial functions and resulting in a wide range of strategies to develop mitochondrial targeted antioxidants and drugs delivery strategies into this compartment. To this day a range of natural and synthetic molecules showed great antioxidant activity in *in vitro* studies, but do not proved to be particularly effective in clinical trials, of these VitE ***(Miller et al., 2005)***, turmeric ***(Kaminaga et al., 2003)*** This limit could be due to physical and chemical characteristics that obstruct antioxidants to reach the relevant sites of free radical generation. CPPs are one of the most widely used and effective drug delivery strategies applicable on basic research and medical trials. Nowadays this strategy is drawing more and more attention and many studies have been come out characterizing and developing new classes of CPPs enlarging their applicability window as in medicine as in biological tools. This thesis is focused on mitochondrial target antioxidants peptides. The main characteristics of CPPs targeting mitochondria has been spread in 2006 ***(Sheu et al., 2005)*** then increasingly new CPPs went out. In 2000 a new class of cell penetrating antioxidant peptides targeting mitochondria was synthesized by Szeto and coworkers ***(Schiller et al., 2000)***. At today, a number of publications showed and confirmed SS-peptides as the most promising tool applicable in the treatment of all diseases and impairments associated with oxidative injury such as

neurodegenerative diseases. In this dissertation a new short carrier antioxidant peptide target mitochondria, denominated as MIP1, was designed, manually synthesized taking SS-31 peptide as reference model. At today there are not CPPs sequence less than four amino acids long and MIP1 is a tetrapeptide, water soluble and showing positive net charge. Sequence of MIP1 was designed following rules and experiences found in literature and displaying two important properties for crossing both the plasma and mitochondrial membranes: positive charge and lipophilic character. To evaluate toxic effects of MIP1 peptide, HeLa 705 human cervical cancer cells were chosen as cellular model. Interestingly, MIP1 did not exhibit toxicity even at 100µM. In order to evaluate eventual antioxidant activity an oxidative condition was generated using hydrogen peroxide. In absence of efficient antioxidant systems, H_2O_2 can react with transition metals such as ferrous iron, which is disproportionately increased during neurodegenerative diseases ***(Dusek et al., 2014)***. That reaction, named Fentom's reaction, leading to the formation of the highly reactive hydroxyl radical. Large amount of hydroxyl radical has been implicated in several pathological conditions ***(Antus et al., 2014)***. This radical can react and oxidize all types of cellular macromolecules: carbohydrates, nucleic acids, lipids and amino acids ***(Reiteher et al., 1995)****.* At to date, antioxidant systems protecting hydroxyl radical has not been known yet; hence, the prevention of its formation is a critical anti oxidant process ***(Sheu et al., 2006)***. MIP1 showed great antioxidants properties at 0.5 and 5µM, more activity was appreciated on 2h pre treated cells, suggesting a time dependent mechanism of uptake. The scavenger ability was further confirmed by studies on mitochondrial membrane potential and ROS production. Antioxidant peptide MIP1 was able to decrease mitochondrial depolarization and overload of ROS production in exposed cells to H_2O_2. On the other hand, the higher concentrations of MIP1 totally lost antioxidant capacity. An explanation could be that the uptake of MIP1 is self-limiting and it is reduced at greater concentration than 50µM. Moreover, MIP1 get net positive charge then, limited uptake is may be a consequence of inevitable mitochondrial depolarization due to excessive amount of cations accumulated into matrix ***(Smith et al., 1999)***. As

shown in literature ***(Roy et al., 2008)***, loss of mitochondrial membrane potential and oxidative stress are part of vicious circle that give rise to increased mitochondrial permeability transition. In view of the above, MP1 results showed gradually increased of ROS at the same time decreased mitochondrial membrane potential. The sequence of MIP1 is focused on alternating aromatic residues and basic amino acids (aromatic cationic peptides) and the antioxidant activity can be assigned to Dmt in second positions. Amino acid Tyrosine could 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 ***(Pichorner et al., 1995)***. Zhao and coworkers demonstrated that, due to methylation of the phenolic ring, no natural amino acid Dmt holds more antioxidant properties than Tyrosine ***(Zhao et al., 2004)***. MP1 is easily water soluble and quickly goes through cellular membrane. The uptake of MIP1 has not been studied yet, but showing net charge 3^+ could make use of the potential gradient across the mitochondrial inner membrane to get into mitochondrial organelle. As showed by Zhao and co-workers ***(Zhao et al., 2004)***, the uptake of SS-peptides were decreased by only 10% to 15% in mitochondria that were depolarized by FCCP (carbonyl cyanide 4-(trifluorometoxy) phenylhydrazone), then being MIP1 based on SS-31 sequence could be fairly supposed a similar way to get inside cell. Fluorescence microscopy confirmed rapid uptake of a fluorescent MIP1 analog in living cells. The intracellular distribution pattern of the fluorescent MIP1 analog was identical to Mitotracker TMRE, a fluorescent dye that is taken up into active mitochondria in a potential-driven manner, suggesting that MIP1 peptide is targeted to mitochondria. In this study SS-31- Szeto-Schiller peptide was taken as reference model. Literature is plenty of publications about SS-31 ability to target mitochondria and is concentration in mitochondria is 1000 fold more than cytosol compartment ***(Zhao et al., 2004)***. As shown in the fluorescence microscopy and the relative quantification based on the fluorescence intensity, the amount of MIP1 into the cells was more than reference model SS-31 underlining its great potentialities as antioxidant targeting mitochondria. The ability of MIP1 to overpass cellular

membrane and accumulate into mitochondria was confirmed by uptake study on isolated mitochondria. In order to evaluate mitochondrial uptake, MIP1 was conjugated to membrane impermeant dye FAM. MIP1 peptide was totally taken up within 4 hours by isolated mitochondria confirming this organelle as one of potential targets of MIP1. Moreover, the higher relative fluorescence FAM-MIP1 on isolate mitochondria underlines the ability of MIP1 to make easier delivering of molecules inside specific compartments. Potentially, MIP1 peptide could be conjugated with different cargoes to be used as a mitochondrial delivery machine for mitochondrial diseases treatment. In summary, MIP1 is cell-permeable peptide antioxidants that accumulate into the major site of ROS production and prevents mitochondrial impairments. MIP1 was great in reducing intracellular ROS and preventing cellular death with less than 5µM concentration, in contrast most antioxidant require 100µM to millimolar concentrations to prevent oxidative cell death ***(Pias et al., 2003)***. MIP1 is a small peptide; fast and easy to synthesize, it is completely soluble in water and able targeting mitochondria without toxicity, moreover showed antioxidant property and is potentially conjugable to different molecules. In view of all these characteristics MIP1 should be taken as hopeful candidate against oxidative stress and it could be beneficial as mitochondrial delivery drugs machine for making easier treatment of diseases characterized by mitochondrial impairment. The ability to target mitochondrial organelles creates new opportunities to study physiological and pathological processes at the subcellular level and to deliver therapeutics to targets within mitochondrial compartments.

SUMMARY IN ITALIAN

L'insorgenza e il peggioramento di un'ampia schiera di malattie, tra cui malattie cardiocircolatorie, neurodegenerative, diabete e neoplasie sono associati all'eccessiva produzione di radicali liberi dell'ossigeno (ROS) ***(Hroudová 2014)***. I ROS sono molecole altamente instabili che si formano all'interno dei compartimenti cellulari in seguito a reazioni endogene enzimatiche e non enzimatiche. Numerosi studi hanno dimostrato che la fisiologica presenza intracellulare di ROS non è di per se una condizione dannosa, infatti sono implicati in fondamentali processi cellulari come autofagia, comunicazione e difesa immunitaria ***(Chen, 2007. Niess, 1999)***. L'inizio della compromissione cellulare è legata ad uno squilibrio spaziotemporale tra produzione di ROS e difese antiossidanti, generando una condizione nota come stress ossidativo ***(Zamzami et al., 1997)***. In assenza di difese antiossidanti, i ROS possono potenzialmente reagire con diversi substrati cellulari come proteine, lipidi, carboidrati e DNA, provocandone alterazione strutturale, danneggiamento e perdita delle normali funzionalità. La letteratura scientifica presenta numerose pubblicazioni riguardanti il ruolo principale dei mitocondri nella produzione di ROS. Circa il 90% dell'ossigeno richiesto da una cellula, è utilizzato per sostenere i fisiologici processi metabolici mitocondriali ***(Chance, 1979)***. L'O_2 molecolare rappresenta l'accettore finale degli elettroni con la sua conseguente riduzione ad acqua, tuttavia non tutto l'ossigeno è completamente ridotto, infatti circa il 2% dell'anione superossido origina da un'incompleta riduzione dell'ossigeno ***(Chance, 1979)***. Sviluppare antiossidanti o molecole con specifico target mitocondriale è la strategia di scelta per contrastare lo stress ossidativo, e allo stesso tempo crea nuove opportunità per studiare i numerosi processi biologici che hanno luogo a livello mitocondriale sia in condizioni fisiologiche sia patologiche. Nel 2000 è stata sintetizzata una serie di sequenze tetrapeptidiche, SS-peptide, dotate di attività antiossidante in grado di accumularsi specificamente a livello mitocondriale ***(Schiller et al., 2000)***. I risultati ottenuti da

esperimenti *in vivo* ed *in vitro* candidano SS-peptide come futuro promettente approccio per il trattamento delle disfunzioni mitocondriali causate da stress ossidativo. In questa tesi, prendendo come modello di riferimento SS-31 peptide, il più promettente tra le sequenze SS-peptide, un nuovo tetrapeptide, MIP1, è stato disegnato e sintetizzato. Gli effetti biologici e la localizzazione di MIP1 sono stati valutati su linea cellulare HeLa 705. In seguito ad un ampio screening, MP1 non ha mostrato alcuna tossicità anche ad alte concentrazioni, non ha perturbato il potenziale di membrana mitocondriale e non ha aumentato la produzione di ROS. In seguito ai promettenti risultati precedenti è stato deciso di valutare l'eventuale attività antiossidante di MIP1. Le cellule sono state pretrattate con MIP1, dopo 30 minuti o 2 h sono state trattate con perossido d'idrogeno. I risultati hanno evidenziato nei pretrattamenti di 2 h a 0.5 e soprattutto a 5µM un'interessante attività antiossidante in grado di proteggere le cellule dal danno indotto dal perossido d'idrogeno. In seguito ai risultati precedenti, si è deciso di utilizzare per gli studi di microscopia la concentrazione 5µM. Grazie all'utilizzo di un analogo di MIP1 coniugato con il fluorocromo 5(6)-carboxyfluoresceina (FAM) sono stati condotti studi di miscroscopia in fluorescenza con lo scopo di identificare la localizzazione intracellulare. Utilizzando Tetramethylrhodamine methyl ester (TMRE) mitochondrial dye e SS-31 peptide come indicatori mitocondriali, è stato possibile identificare i mitocondri come potenziale target di MIP1. Grazie alla quantificazione della fluorescenza relativa di FAM, è stato possibile dedurre che MP1 possiede una maggior capacità di localizzarsi all'interno dei mitocondri rispetto al modello di riferimento SS-31 peptide. Al fine di valutare le potenzialità di MP1 come carrier, sono stati condotti esperimenti su mitocondri isolati. La quantificazione della fluorescenza di FAM ha dimostrato una maggior facilità di attraversamento delle membrane mitocondriali quando coniugato a MIP1. In conclusione, MIP1 è un nuovo tetrapeptide con target mitocondriale. Le sue eventuali proprietà antiossidanti potrebbero assicurargli un suo futuro ruolo nella protezione da

danno ossidativo, inoltre potrebbe essere impiegato come carrier per facilitare, a cargo di diversa natura, l'ingresso a livello mitocondriale.

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