Cell-Penetrating Peptides for Mitochondrial Targeting

Carmine Pasquale Cerrato

Abstract

Mitochondria have simply been known as the cell’s powerhouse for a long time, with its vital function of producing ATP. However, substantially more attention was directed towards these organelles once they were recognized to perform several essential functions having an impact in cell biology, pharmaceutics and medicine. Dysfunctions of these organelles have been linked to several diseases such as diabetes, cancer, neurodegenerative diseases and cardiovascular disorders. Mitochondrial medicine emerged once the relationship of reactive oxygen species and mutations of the mitochondrial DNA linked to diseases was shown, referred to as mitochondrial dysfunction. This has led to the need to deliver therapeutic molecules in their active form not only to the target cells but more importantly into the targeted organelles.

In this thesis, cell-penetrating peptides (CPPs) used as mitochondrial drug delivery system and the pathways involved in the uptake mechanisms of a CPP are described. In particular, Paper I describes a novel cell-penetrating peptide targeting mitochondria with intrinsic antioxidant properties. Paper II expands upon this first finding and show that the same peptide can carry a glutathione analogue peptide with improved radical scavenging ability into cytoplasm and mitochondria. Paper III introduces mitochondrial targeting peptides for delivery of therapeutic biomolecules to modify mitochondrial gene expression. In Paper IV, the uptake mechanisms of the CPP delivery strategy has been investigated to gain a better understanding of the used transfection system.

Overall, this thesis summarizes our current effort regarding cell-penetrating peptides delivery system to target mitochondria and the progress made towards a potential gene therapy. It contributes to the field of CPPs and drug delivery with a set of peptides with radical scavenging ability, a strategy to deliver oligonucleotides to mitochondria as proof-of-concept for mitochondrial gene therapy, and to help understanding the pathways involved in CPPs uptake.

Keywords: Mitochondrial targeting, cell-penetrating peptides, antioxidant activity, scavenging ability, oligonucleotide delivery.
Cell-Penetrating Peptides for Mitochondrial Targeting

Carmine Pasquale Cerrato
To my parents

"Life does not end with death. What you pass on to others remains. Immortality is not the body, which will one day die. That does not matter... of importance is the message you leave to others. That is immortality."

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Mitochondria have simply been known as the cell’s powerhouse for a long time, with its vital function of producing ATP. However, substantially more attention was directed towards these organelles once they were recognized to perform several essential functions having an impact in cell biology, pharmaceuticals and medicine. Dysfunctions of these organelles have been linked to several diseases such as diabetes, cancer, neurodegenerative diseases and cardiovascular disorders. Mitochondrial medicine emerged once the relationship of reactive oxygen species and mutations of the mitochondrial DNA linked to diseases was shown, referred to as mitochondrial dysfunction. This has led to the need to deliver therapeutic molecules in its active form not only to the target cells but more importantly into the targeted organelles.

In this thesis, cell-penetrating peptides (CPPs) used as mitochondrial drug delivery system and the pathways involved in the uptake mechanisms of a CPP are described. In particular, Paper I describes a novel cell-penetrating peptide targeting mitochondria with intrinsic antioxidant properties. Paper II expands upon this first finding and show that the same peptide can carry a glutathione analogue peptide with improved radical scavenging ability into cytoplasm and mitochondria. Paper III introduces mitochondrial targeting peptides for delivery of therapeutic biomolecules to modify mitochondrial gene expression. In Paper IV, the uptake mechanisms of the CPP delivery strategy has been investigated to gain a better understanding of the used transfection system.

Overall, this thesis summarizes our current effort regarding cell-penetrating peptides delivery system to target mitochondria and the progress made towards a potential gene therapy. It contributes to the field with a set of peptides with radical scavenging ability, a strategy to deliver oligonucleotides to mitochondria as proof-of-concept for mitochondrial gene therapy, and to help understanding the pathways involved in CPPs uptake.
Populärvetenskaplig Sammanfattning


Denna avhandling beskriver utvecklingen av mitokondriella läkemedelsadministrationssystem genom användandet av cell-penetrerande peptider (CPP). Artikel I beskriver vår design och framställning av en ny CPP-kandidat med anti-oxidantegenskaper som har förmågan att ta sig genom cell-membranerna in till mitokondrier. Artikel II expanderar från den första upptäckten och visar att samma peptid tillsammans med ett glutation-derivat minska halten av re- aktiva syrearter både i mitokondrierna samt i cellerna. Artikel III introducerar kombinationen av två CPPs med resulterande förmåga att kunna leverera terapeutiska biomolekyler in i mitokondrier för att kunna modifera det mitokondriella genuttrycket. Artikel IV skildrar mekanismen för hur CPPs med eller utan terapeutiska biomolekyler tas upp och omsätts av cellerna.

Sammanfattningsvis återger denna avhandling vårt arbete inom forskningsområdet cell-penetrerande peptider med fokus mot mitokondrier. Resultaten visar de framsteg som gjorts för utvecklingen av peptider med förmågan att ta sig in i mitokondrier och leverera terapeutiska biomolekyler, vilket öppnar upp för en framtida potentiell mitokondriell genterapi.
This thesis is based on the following four publications, in the text referred to as **Paper I, II, III and IV**, respectively.


Additional Publications

Publications not included in this thesis:


**Patent application**


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Paper I in this thesis has previously been included in my licentiate thesis. ISBN 978-91-7649-312-0.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>ASO</td>
<td>antisense oligonucleotide</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>Cbz</td>
<td>benzyloxy carbonyl</td>
</tr>
<tr>
<td>CME</td>
<td>clathrin mediated endocytosis</td>
</tr>
<tr>
<td>COXII</td>
<td>cytochrome c oxidase II</td>
</tr>
<tr>
<td>CPP</td>
<td>cell-penetrating peptide</td>
</tr>
<tr>
<td>CRISPR</td>
<td>clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>Dmt</td>
<td>2,6-L-dimethyltyrosine</td>
</tr>
<tr>
<td>ESI</td>
<td>electron spray ionisation</td>
</tr>
<tr>
<td>EV</td>
<td>extracellular vesicle</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FAM</td>
<td>5(6)-carboxyfluorescein</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonylcyanide-p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenlymethylxycarbonyl</td>
</tr>
<tr>
<td>GSHPx</td>
<td>glutathione peroxidases</td>
</tr>
<tr>
<td>GSTs</td>
<td>glutathione S-transferases</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
</tr>
<tr>
<td>LHON</td>
<td>Leber hereditary optic neuropathy</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase deficiency</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>matrix-assisted laser desorption/ionization - time of flight</td>
</tr>
<tr>
<td>MAP</td>
<td>model amphipathic peptide</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mitoK&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>mitochondrial ATP-regulated K&lt;sup&gt;+&lt;/sup&gt; channel</td>
</tr>
<tr>
<td>MR</td>
<td>molar ratio</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>MVBs</td>
<td>microvesicular bodies</td>
</tr>
<tr>
<td>MTS</td>
<td>mitochondrial targeting sequence</td>
</tr>
<tr>
<td>Mtt</td>
<td>4-methyltrityl</td>
</tr>
<tr>
<td>nDNA</td>
<td>nuclear DNA</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotides</td>
</tr>
<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
</tr>
<tr>
<td>ONs</td>
<td>oligonucleotides</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation system</td>
</tr>
<tr>
<td>pDNA</td>
<td>plasmid DNA</td>
</tr>
<tr>
<td>pVEC</td>
<td>vascular endothelial-cadherin</td>
</tr>
<tr>
<td>PTDs</td>
<td>protein transduction domains</td>
</tr>
<tr>
<td>r</td>
<td>D-arginine</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNase H</td>
<td>ribonuclease H</td>
</tr>
<tr>
<td>SCARA</td>
<td>scavenger receptor class A</td>
</tr>
<tr>
<td>SCOs</td>
<td>splice correcting oligonucleotides</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TAT</td>
<td>transactivator of transcription protein</td>
</tr>
<tr>
<td>TFA</td>
<td>tri-fluoroacetic acid</td>
</tr>
<tr>
<td>TIM</td>
<td>translocase of the inner membrane</td>
</tr>
<tr>
<td>TIS</td>
<td>triisopropylsilane</td>
</tr>
<tr>
<td>TOM</td>
<td>translocase of the outmembrane</td>
</tr>
<tr>
<td>TPP</td>
<td>triphenylphosphonium cation</td>
</tr>
<tr>
<td>Y</td>
<td>L-tyrosine</td>
</tr>
<tr>
<td>Y&lt;sub&gt;Me&lt;/sub&gt;</td>
<td>O-methyl-L-tyrosine</td>
</tr>
<tr>
<td>Y&lt;sub&gt;a&lt;/sub&gt;</td>
<td>2,6-dimethyl-L-tyrosine</td>
</tr>
<tr>
<td>ΔΨ&lt;sub&gt;p&lt;/sub&gt;</td>
<td>plasma membrane potential</td>
</tr>
<tr>
<td>ΔΨ&lt;sub&gt;m&lt;/sub&gt;</td>
<td>mitochondrial membrane potential</td>
</tr>
<tr>
<td>UCP2</td>
<td>uncoupling protein 2</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage-dependent anion channel</td>
</tr>
<tr>
<td>ζ potential</td>
<td>zeta-potential</td>
</tr>
</tbody>
</table>
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1. Introduction

1.1. Mitochondria

1.1.1. Origin and Structure of Mitochondria

Researchers have made a tremendous effort to elucidate the origin of energy in life, essential for the survival of the cells. To date, two theories about the origin of mitochondria have been formulated. The first hypothesis was described in 1926 by Ivan E. Wallin suggesting that a nucleus-bearing but amitochondriate cell existed first, followed by the origin of mitochondria in a eukaryotic host (Figure 1a-d). The endosymbiotic hypothesis suggests that mitochondria originate from an ancient symbiosis that resulted when a nucleated cell engulfed an aerobic prokaryote (Figure 1e-g). The engulfed cell relied on the protective environment of the host cell and the host cell relied on the engulfed prokaryote for energy production. This engulfed prokaryote evolved over time into mitochondria. The first report of intracellular structure that could represent mitochondria dates back to the 1840s, and in 1857 the Swiss anatomist Rudolf von Koelliker described them as “sarcosomes” while studying human muscle. In 1890, Altmann called them “bioblasts” and described them as elementary organisms living inside cells and carrying out vital functions. Then, in 1898, Brenda coined the term “mitochondrion”, referring to the appearance of these organelle during spermatogenesis. The word mitochondrion was derived from the Greek words “mitos” (thread) and “chondros” (granule).

It took more than 50 years from when mitochondria were recognized to the first high-resolution electron micrographs of mitochondria (Figure 1i-l). From these micrographs and the schematic representation of a mitochondrion (Figure 2) it is possible to see the organizational structure of the organelle. Palade and Sjöstrand described the mitochondria as surrounded by a double limiting membrane, which gives form to different chambers or compartments.
Figure 1. Models for origins of mitochondria and electron micrographs. Models that propose the origin of a nucleus-bearing but amitochondriate cell first, followed by the acquisition of mitochondria in a eukaryotic host (a-d). Models that propose the origin of mitochondria in a prokaryotic host, followed by the acquisition of eukaryotic-specific features (e-g). Electron micrograph of kidney mitochondria from Palade (h) and Sjöstrand (i), 1953. Figure 1a-g and Figure 1h-I reprinted with permission.

The membranes of mitochondrion are distinguished by the outer (OMM) and inner mitochondrial membrane (IMM) and the space in between these two is referred to as intermembrane space. The space inside the IMM is referred to as the matrix, and the infold that forms ridges were named cristae mitochondriales. The OMM separates the mitochondria from the cytosol. The pas-
sage through the OMM of metabolites and nuclear-encoded proteins is regulated by the voltage-dependent anion channel (VDAC) and the translocase outer membrane (TOM)\(^9,10\). The translocase inner membrane (TIM) is instead present on the IMM and allows the passage of proteins in or out the matrix\(^11\). The *cristae* were originally described as simple invaginations of the IMM but extensive studies using electron tomography showed that they are fundamental structures for mitochondria with bag-like structures to compartmentalize and limit the diffusion of molecules that are important for the oxidative phosphorylation (OXPHOS) system\(^12\).

1.1.2. Functions and Dysfunctions of Mitochondria

Mitochondria have a central role in cell life. They are a unique organelle having their own DNA (mtDNA). mtDNA has independent origin from nuclear DNA (nDNA) and is only maternally inherited. The human mtDNA is a circular double-strand DNA of 16,569 base pairs encoding for 37 genes (22 transfer and two ribosomal DNA and 13 proteins, including enzymes involved in the OXPHOS pathway for adenosine triphosphate (ATP) production). The number of mitochondria per cell vary based on the cell type/tissue and each mitochondrion contains between two and ten copies of mtDNA. The mtDNA sequence is identical in most cells, termed homoplasmic, but mutated and wild-type mtDNA can also coexist in the same mitochondrion, termed heteroplasmic. The OXPHOS pathway occurs in the electron transport chain (ETC, also known as the respiratory chain) located on the IMM. The ETC consists of four complexes (complex I-IV) and ATP synthase that together contribute to the generation of the mitochondrial electrochemical gradient, the mitochondrial membrane potential (\(\Delta \Psi_m\)), and ATP (Figure 2). The \(\Delta \Psi_m\) is normally in the range of 80 to 140 mV, with the optimal for ATP production within 100 to 120 mV. A \(\Delta \Psi_m\) over 140 mV is usually leading to increased reactive oxygen species (ROS) production at the expense of ATP production\(^13\).

In addition to their role in cell life controlling ATP production, OXPHOS process, intracellular calcium concentration and cellular metabolism, mitochondria play an important role for cell death signaling. They can promote both necrotic and apoptotic cell death by increasing the permeability of the mitochondrial permeability transition (MPT) pore. This event leads to the dissipation of the proton electrochemical gradient with decreased ATP production, increased ROS production, calcium overload, and mitochondrial swelling\(^14\). The ATP depletion levels play a role in determining whether the necrosis or apoptosis cell death pathway is activated. Damaged mitochondria and
the regulation of their number occurs via mitophagy, an organelle-specific autophagic elimination. This process occurs via ubiquitination of mitochondrial components to facilitate mitochondrial clearance.\(^\text{15}\)

**Figure 2.** Schematic representation of a mitochondrion showing the mitochondrial permeability transition pore and the respiratory chain. Reprinted with permission.\(^\text{16}\)

The tissues with high metabolic demand have the highest number of mitochondria and are also the most susceptible to mitochondrial-driven diseases. These include brain, eye, liver, heart, and skeletal muscle and are linked to mitochondrial diseases such as mitochondrial myopathies, neuromuscular and neurodegenerative diseases (Alzheimer’s disease, Parkinson’s disease, Huntington disease and amyotrophic lateral sclerosis), diabetes, obesity and cancer. The clinical expression of mitochondrial disorders can involve different systems (Table 1). The most common mitochondrial disease associated to mtDNA mutation is the Leber hereditary optic neuropathy (LHON), due to a degeneration of retinal ganglion cells and consequent visual failure.
Table 1. Systems involved and clinical manifestations in patients with mitochondrial disorders.

<table>
<thead>
<tr>
<th>System</th>
<th>Clinical manifestations</th>
<th>System</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Heart failure</td>
<td>Musculoskeletal</td>
<td>Muscle weakness with normal creatine kinase levels and normal electromyographic and nerve-conduction studies</td>
</tr>
<tr>
<td></td>
<td>Arrhythmias</td>
<td></td>
<td>Short stature</td>
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<tr>
<td></td>
<td>Murmurs</td>
<td></td>
<td>Microcephaly</td>
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<tr>
<td></td>
<td>Sudden death</td>
<td></td>
<td>Round face</td>
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<td></td>
<td>Left ventricular myocardial noncompaction</td>
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<td>High forehead</td>
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<tr>
<td></td>
<td>Apical ballooning syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>Dyspnea</td>
<td>Respiratory acidosis</td>
<td>Skin and soft tissue</td>
</tr>
<tr>
<td></td>
<td>Orthopnea</td>
<td></td>
<td>Hypertrichosis</td>
</tr>
<tr>
<td></td>
<td>Respiratory failure</td>
<td></td>
<td>Eczema</td>
</tr>
<tr>
<td>Neurologic</td>
<td>Encephalopathy</td>
<td>Movement disorders</td>
<td>Vitiligo</td>
</tr>
<tr>
<td></td>
<td>Ataxia</td>
<td>Seizure disorders</td>
<td>Multiple lipomatosis</td>
</tr>
<tr>
<td></td>
<td>Movement disorders</td>
<td>Mental retardation</td>
<td>Reticular pigmentation</td>
</tr>
<tr>
<td></td>
<td>Seizure disorders</td>
<td>Gastrointestinal</td>
<td>Periodontosis</td>
</tr>
<tr>
<td>Renal</td>
<td>Renal failure</td>
<td></td>
<td>Anorexia</td>
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<tr>
<td></td>
<td>Benign renal cysts</td>
<td></td>
<td>Abdominal pain</td>
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<tr>
<td></td>
<td>Focal segmental glomerulosclerosis</td>
<td></td>
<td>Nausea</td>
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<tr>
<td></td>
<td>Proximal tubulopathy</td>
<td></td>
<td>Vomiting</td>
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<tr>
<td></td>
<td>Nephritic syndrome</td>
<td></td>
<td>Diarrhea</td>
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<td></td>
<td>Tubulointerstitial nephritis</td>
<td></td>
<td>Malabsorption</td>
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<tr>
<td>Hematologic</td>
<td>Anemia</td>
<td></td>
<td>Villous atrophy</td>
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<tr>
<td></td>
<td>Leukopenia</td>
<td></td>
<td>Constipation</td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia</td>
<td></td>
<td>Pseudo-obstruction</td>
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<tr>
<td></td>
<td>Eosinophilia</td>
<td></td>
<td>Pancreatitis</td>
</tr>
<tr>
<td>Endocrine</td>
<td>Diabetes mellitus</td>
<td>Ophthalmic</td>
<td>Elevated liver enzyme levels</td>
</tr>
<tr>
<td></td>
<td>Diabetes insipidus</td>
<td></td>
<td>External ophthalmoparesis</td>
</tr>
<tr>
<td></td>
<td>Hypothyroidism</td>
<td></td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td></td>
<td>Hypoparathyroidism</td>
<td>Auditory</td>
<td>Sensorineural hearing loss</td>
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<tr>
<td></td>
<td>ACTH deficiency</td>
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<td></td>
<td>Hypogonadism</td>
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<tr>
<td></td>
<td>Amenorrhea</td>
<td></td>
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<tr>
<td></td>
<td>Gynecomastia</td>
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1.1.3. Mitochondria as a Target for Drug Discovery

The potential of selective drug delivery systems in modern drug therapies has been the driving-force for developing therapeutic agents or imaging contrast formulations towards greater targeting selectivity and better delivery efficacy. One of the bottle-necks for this realization, is the inherent difficulties to penetrate mitochondrial membranes. A common approach is to increase the portion of drug accumulation in target cells versus normal cells or specific organelle in order to minimize the potential side effects and increase the therapeutic effects. The benefits of intracellular drug delivery and subcellular targeting range from significant reduction of the quantity of a therapeutic molecule for the desired effects, to decrease of the side effects.

1.1.4. Agents Targeting Mitochondria

The pivotal role of mitochondria in controlling cell life and death make them an attractive target for mitochondrial gene therapy and for the development of drugs that could treat mitochondrial related diseases. The ETC on the IMM is the major intracellular source of ROS, generated as by-products during mitochondrial electron transport. In addition, ROS are formed as necessary intermediates of metal catalysed oxidation reactions inside the cells. ROS such as anion superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion, and nitric oxide (Table 2) are highly reactive with short half-life.

Table 2. List of common reactive oxygen species. ROS are found in normal and pathological tissues.

<table>
<thead>
<tr>
<th>Reactive oxygen species</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl radical</td>
<td>OH•</td>
</tr>
<tr>
<td>Superoxide radical anion</td>
<td>O₂•</td>
</tr>
<tr>
<td>Nitric oxide radical</td>
<td>NO•</td>
</tr>
<tr>
<td>Peroxyl radical</td>
<td>RO₂•</td>
</tr>
<tr>
<td>Lipid peroxyl radical</td>
<td>LO₂•</td>
</tr>
<tr>
<td>Peroxynitrate</td>
<td>ONO₂⁻</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>O²⁻</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>HClO</td>
</tr>
</tbody>
</table>

The • designates an unpaired electron.

Mitochondria are continuously exposed to ROS and thus accumulate oxidative damage more rapidly than the rest of the cell\cite{17}. High ROS levels can cause non-specific damage to lipids, proteins, and DNA, leading to alteration
or loss of cellular functions. Many studies have associated mitochondrial dysfunction caused by ROS with both necrotic and apoptotic cell death\textsuperscript{18}. The rate of mitochondrial ROS production can be altered by several physiological or pathological conditions. Several human pathologies like LHON, dystonia, and Leigh's disease\textsuperscript{19–21} are linked to oxidative damage by mitochondrial ROS. The onset mechanisms of diseases caused by mitochondrial oxidative stress are still an object of research. Drugs like paclitaxel\textsuperscript{22}, etoposide\textsuperscript{23}, betulinic acid\textsuperscript{24}, lonidamine\textsuperscript{25}, CD-437\textsuperscript{26}, and ceramide have been clinically approved and used to initiate apoptosis in mitochondria. Different approaches have been used to successfully target the mitochondria. A schematic representation of a mitochondrion and the mode of action of representative mitochondria targeting compounds is shown in Figure 3.

Vitamin E was the first small molecule to be used for specific targeting, acting also as an antioxidant against ROS. Vitamin E and analogues have been used for different models or diseases such as pro-apoptotic and anti-cancer activity\textsuperscript{27}, breast cancer\textsuperscript{28}, cardiovascular protection\textsuperscript{29}, skin protection from UVB-irradiation\textsuperscript{30}. Vitamin E has also been covalently coupled to a triphenylphosphonium (TPP) cation to have higher targeting efficacy compared to vitamin E alone\textsuperscript{31}. The fluorescent lipophilic cation rhodamine 123 and other anticancer cyanine dyes have also been used as mitochondria-selective molecules for therapeutic purposes (anticancer or anti-apoptotic effects)\textsuperscript{32}. Another strategy used to target mitochondria was based on mitochondrial targeting signal (MTS) sequences. Proteins that are not mitochondrial-encoded need to be imported into mitochondria. Most of these proteins are encoded from nuclear DNA, translated in the cytosol and imported into mitochondria for the presence of MTS at N-terminus. The majority of MTS are composed by positively charged and hydroxylated amino acids to form amphiphilic secondary structure\textsuperscript{33}. There are also some proteins that are lacking the MTS but that paradoxically can enter into mitochondria. The MTS interact with the TIM-TOM complexes and the terminal signal sequence is proteolytically removed by proteases present in the intramembrane space or in the matrix according to the final destination of the protein\textsuperscript{10,34,35}. The MTS has been successfully used for the delivery of a variety of cargo molecules, including proteins\textsuperscript{36}, nucleic acid\textsuperscript{37}, and endonucleases\textsuperscript{38,39}.

Another molecule/nanocarrier that has been used to target mitochondria is desqualinium chloride and several other compounds have been derived from it. DQAsome, a liposome-like vesicle based on desqualinium, was reported from Weissig \textit{et al}. in 1998 as a novel potential drug and gene delivery system\textsuperscript{40}. In 2001 Weissig and Torchilin extensively reviewed the development
of mitochondrial DNA delivery systems (DNA/DQAsome complex) towards mitochondrial gene therapy. DQAsomes were able to bind plasmid DNA, form complexes between 70 and 700 nm, protect the DNA against nuclease attack, release DNA at mitochondria-like membranes, and have a cytotoxicity similar to Lipofectin and LipofectAmine.

**Figure 3.** Schematic representation of a mitochondrion and the mode of action of representative mitochondria targeting compounds. Cationic compounds (TPP-based agents, choline esters, SS peptides) are attracted by the negative potential of the IMM. Driven by their high affinity for IMM-specific phospholipids, gramicidin S (GS)-based antioxidants deliver the nitroxide ROS scavenger into the matrix. MTS can be utilized as vehicles to deliver metalloporphyrin superoxide dismutase (SOD)-mimics into the matrix. Alternatively, the mitochondrial agent can be encapsulated in a vesicle which undergoes fusion with the OMM. The filled circle represents the anti- or prooxidant payload. D-(KLAKLAK)$_2$ and analogues are cationic amphipathic α-helical peptides able to disrupt mitochondrial membranes, hence triggering apoptosis. Other chemical agents target specific mitochondrial proteins. For instance, sulfonylureas block the mitochondrial ATP-regulated K$^+$ channel (mitoK$_{ATP}$), benzothiazepines are inhibitors of the mitochondrial Na$^+-$Ca$^{2+}$ exchanger, and benzodiazepines are agonists or antagonists of the peripheral benzodiazepine receptor (PBR). ATP, adenosine triphosphate; ETC, electron transport chain; IMS, intermembrane space. Reprinted with permission.
Sulfonylureas and potassium channel openers have been shown to interact with the mitochondrial adenosine triphosphate-dependent potassium (mito-K$_{ATP}$) channels and have cardio-protective effects$^{43}$. Benzodiazepines and other peripheral benzodiazepine receptor ligands have been shown to be regulator of the mitochondrial permeability transition pore and to have potential utility as anti-apoptotic or pro-apoptotic antitumor agents, based on agonist or antagonist effects$^{44,45}$. Benzothiazepines derivatives have been reported to inhibit the mitochondrial Na$^+$/Ca$^{2+}$ exchanger and to enhance glucose-stimulated insulin secretion in pancreatic β-cells$^{46}$. D-(KLAKLAK)$_2$ is a cationic amphipathic α-helical killer peptide derived from the sequence of membrane-disrupting antimicrobial peptides (AMP). Initially it was used as mitochondria-disruption peptides to trigger apoptosis of cancer cells$^{47}$. It has been modified exchanging the leucine with cyclohexylalanine to improve mitochondrial localization and efficacy$^{48}$. Manganese metalloporphyrin conjugated to a signal oligopeptide is a class of mitochondria-targeted SOD-mimics reported for the antioxidant properties$^{49}$.

A peptide-based mitochondria-localizing antioxidant, termed SS-31, was developed and found to selectively target the inner mitochondrial membrane$^{50,51}$. This tetra peptide exerts its function through the ROS-scavenging activity of 2,6-L-dimethyltyrosine (Dmt) residue found in its sequence (D-Arg-Dmt-Lys-Phe-NH$_2$), which shares structural similarity with vitamin E. This compound was observed to display antioxidant activity and reduce cell death in two neuronal cell lines at nM concentrations, as well as decrease mitochondrial ROS production and prevent apoptosis-related events in isolated mouse liver mitochondria.$^{52}$ It was also found that this antioxidant peptide prevents myocardial stunning that is associated with reperfusion in the ischemic heart of an ex vivo guinea pig model. The chemical structures of featured mitochondria-targeting agents and clinical drug candidates discussed herein are reported in Figure 4.
Figure 4. Chemical structures of mitochondria-targeting agents and clinical drug candidates. For chimera molecules, substructures highlighted in dashed boxes represent the targeted bioactive components, and substructures highlighted in dashed circles represent the mitochondria-targeting cationic entities. Ph, phenyl; Me, methyl; Et, ethyl; Boc, tert-butoxycarbonyl; Cbz, benzyloxycarbonyl; Ac, acetyl. Reprinted with permission.\(^4^2\).
1.2. Glutathione

Glutathione (GSH) is a water-soluble tripeptide (γ-Glu-Cys-Gly). It is the most prevalent low-molecular weight (307 Da) compound containing a sulfhydryl group in eukaryotic cells, and present in mM concentration range in various mammalian cells\textsuperscript{53,54}. GSH is oxidized to glutathione disulphide (GSSG). Proteins or other molecules containing cysteine residues readily participate in thiol-disulphide exchange reactions with GSH. GSSG is usually rapidly reduced by glutathione reductase and maintained at less than 1% of the total glutathione pool\textsuperscript{55}. GSSG and other glutathione-conjugates may also be excreted from cells. GSH has roles in cellular protection against oxidants and xenobiotics, and in signal transduction. In antioxidant defence, the major reaction of GSH is reduction of hydroperoxides by glutathione peroxidases (GSHPx) and at least one peroxiredoxins, which yields to GSSG. In redox signaling, GSH participates through both the removal of H\textsubscript{2}O\textsubscript{2} and the reversal of thiolate oxidation. GSH is used for detoxification of several xenobiotics by glutathione S-transferases (GSTs), involved in the transport of nitric oxide. The glutathione system has become a drug target due to the large spectrum of bio-functionality of GSH in different pathological conditions.

Since glutathione itself cannot be administered clinically and having any effect, one strategy is to use chemically modified GSH analogues in order to mimic glutathione’s various physiologic and pharmacologic effects. Some approaches aim to enhance the antioxidant activity while others to inhibit enzymes with which GSH usually interacts. In both cases there are the conditions for a variety of molecules to be or enter clinical testing for therapeutic purposes.

1.2.1. Glutathione Analogues

GSH analogues and GSH-like compounds could support parts of the glutathione system and have an impact as an adjuvant therapeutic factor, for instance, in the case of oxidative stress when the production of the pro-oxidant GSSG is powerful. Different strategies have been used in order to maintain the functionality of GSH system. One of the main limiting factors for the \textit{de novo} synthesis of GSH is the bioavailability of cysteine. Providing commercial dosage of cysteine would not be sufficient to support rates of synthesis that are adequate to sustain normal GSH concentrations. N-acetyl-L-cysteine has been used to avoid toxicity problems\textsuperscript{56-58}. Esters derivatives of GSH were synthesized showing fast cellular uptake and subsequent de-esterification in-
inside the cells providing the native GSH. This strategy has resulted in protective effects against cerebral brain ischemia in rats\textsuperscript{59,60}, in model of stroke and spinal cord injury\textsuperscript{61}, Parkinson’s disease\textsuperscript{62}, diabetic cataract\textsuperscript{63}, LDL oxidative modification and liver perfusion injury\textsuperscript{64}.

GSH analogues have also been developed for cancer therapies, due to the reported implication of GSH in cancer progression and chemoresistance\textsuperscript{65}. One of the biochemical mechanisms reported to be responsible of drug resistance in cancer cells is the over expression of GST\textsuperscript{66}. For this reason, some GSH analogues have been designed to inhibit different GST isoenzymes, such as phosphono analogues\textsuperscript{67} and peptidomimetic analogues of GSH\textsuperscript{68}.

GSH analogues has been developed to overcome the stability problem towards peptidases and proteases. Cyclization has been one strategy; other strategies relied on the substitution of some amino acids or addition of more amino acids. The UPF1 analogue peptide followed this last strategy, adding the non-proteinogenic amino acid 4-metoxy-phenylalanine to GSH N-terminus. This modification showed to improve the antioxidant properties and to increase hydrophobicity of the GSH derivative. A series of UPF analogue peptides were designed with variations including the replacement of the native gamma-glutamyl moiety in the GSH backbone with the alpha-glutamyl moiety, using D-amino acids instead of L-isomers and amidation of the terminal carboxyl group. This modification leaded to improved hydroxyl radical scavenging properties and increased antiradical efficacy\textsuperscript{69–71}.

1.3. Cell-Penetrating Peptides

In 1988, the field of cell-penetrating peptides (CPPs) emerged from the foundational work of Frankel and Pabo\textsuperscript{72} as well as Green and Loewenstein\textsuperscript{73}. During the same time period, their laboratories discovered the transactivator of transcription (TAT) protein of human immunodeficiency virus (HIV) and described how the protein was able to cross cell membranes, efficiently internalized by cells \textit{in vitro}. In the following years, truncated versions of TAT were studied and a minimal sequence derived from TAT was identified to enable cell entry\textsuperscript{74}. A few years later, a 16 amino acid peptide derived from the amphiphilic Drosophila Antennapedia homeodomain, penetratin (pAntp), was discovered\textsuperscript{75}. Since then, several other proteins and peptides that displayed translocation activity have successfully been reported such as VP22\textsuperscript{76}, Transportan\textsuperscript{77}, model amphipathic peptide (MAP)\textsuperscript{78}, signal sequence-based peptides\textsuperscript{79}, synthetic arginine-enriched sequences\textsuperscript{80}. 

12

| | |
CPPs can be described as short positively charged peptides varying from 4 to 40 amino acids in length. They are capable of crossing cellular membranes and to be internalized into mammalian, plant, and bacterial cells. Furthermore, they can mediate the transport of a variety of biologically active molecules, cargos, and drugs with low or non-toxic effects. Numerous CPPs have been developed within the fields of biology and medicine and several have been applied for a variety of applications, showing the utility of CPPs in the basic research as well as in clinic. A variety of intracellular cargos have been used via direct conjugation, encapsulation or physical adsorption with CPPs (Figure 5).

Figure 5. Applications of cell-penetrating peptides as molecular delivery vehicles. This class of peptides has been demonstrated to successfully promote cellular internalization for a wide array of biologically active molecules. Direct conjugation, encapsulation, physical adsorption, or non-covalent complexation methods have been used to deliver imaging agents (silica nanoparticles, quantum dots, paramagnetic lanthanide ions, gold nanoparticles, dextran-coated superparamagnetic-iron oxide nanoparticles), carriers (polymeric particles, carbon nanotubes, dendrimers, micelles, solid lipid nanoparticles, liposomes), or cargoes such as peptides, proteins, drugs, plasmids, siRNA, miRNA, decoy DNA, antisense oligonucleotides into cells, nuclei or specific organelles. Reprinted with permission.

These include imaging agents such as silica nanoparticles, quantum dots, paramagnetic lanthanide ions, gold nanoparticles, dextran-coated superparamagnetic-iron oxide nanoparticles, carriers (polymeric particles, carbon
nanotubes, dendrimers, micelles, solid lipid nanoparticles, liposomes), or cargoes such as peptides, proteins, drugs, plasmids, siRNA, miRNA, decoy DNA, antisense oligonucleotides (ASO)⁸⁷. CPPs have matured as a delivery platform technology to deliver agents, providing optimism for a wide range of therapeutic applications.

1.3.1. Design of CPPs

Since the discovery and characterization of the protein transduction domain of TAT in 1988⁷²,⁷³, over 1000 individual CPPs have been reported and over 2500 papers have been published in this field to date. A statistical graphic representation of CPPs based on 1855 entries, of which 1699 are unique peptides, is shown in Figure 6 with various ways of categorizing CPPs. Most CPPs are linear peptides consisting of the natural abundant L-amino acids (Figure 6a and 6b) and about half of all CPPs are synthetically derived (Figure 6c). Examples of commonly used CPPs are included in Table 3.

![Figure 6](image_url)

**Figure 6.** Statistical representations depicting the distribution of CPPs reported in the literature based on (A) linear and cyclic conformation; (B) chirality/modifications, (C) origin and (D) type of cargoes delivered by CPPs in various in vitro and in vivo settings. Reprinted with permission⁸⁸.

One can also categorize CPPs depending on their physico-chemical properties, often into the sub-groups cationic, amphipathic and hydrophobic. Most
of the CPPs are cationic, and thus have a net positive charge at physiological pH (primarily due to arginine and lysine in the sequence). Amphipathic CPPs have the charge distribution originating primarily from lysine residues such as MAP, transportan, and Pep-1. The hydrophobic CPPs have charged and hydrophobic residues separated on the main chain, as in the vascular endothelial-cadherin (pVEC) and MPG peptides.

The number and order of amino acids in the peptide sequence is one of the factors determining the transduction properties of the CPP. Although CPPs can rapidly cross the cell membranes and be internalized, their physico-chemical properties, secondary structure, concentration, type of cargo, and cell line have all an effect on the mechanism of their internalization.

Table 3. Cell-penetrating peptides commonly used for delivery applications.

<table>
<thead>
<tr>
<th>Cell-penetrating peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRRMKWKK</td>
</tr>
<tr>
<td>Tat (48–60)</td>
<td>GRKKRRQRRRPQ</td>
</tr>
<tr>
<td>pVEC</td>
<td>LLIILRRRIRKQAHAHSH-NH₂</td>
</tr>
<tr>
<td>Transportan</td>
<td>GWTLNSAGYLLGKINLKALAALKIL-NH₂</td>
</tr>
<tr>
<td>TP10</td>
<td>AGYLLGKINLKALAALKIL-NH₂</td>
</tr>
<tr>
<td>Poly Arg</td>
<td>Rₓ</td>
</tr>
</tbody>
</table>

1.3.2. Cellular Uptake Mechanisms

Early studies of uptake mechanism suggested that CPPs could pass through the cellular membrane by direct translocation, caused by favourable electrostatic interactions and hydrogen bonding. More recently, energy-independent processes are thought to occur when the peptide has characteristics that are compatible with the plasma bilayer or if the peptide sufficiently perturb the structural integrity of the membrane. However, recent evidence suggests that direct translocation plays a less important role, especially in large cargo delivery. Instead, different energy-dependent endocytosis pathways have been proposed to be responsible for CPP-mediated intracellular delivery of large molecules and nanoparticles (Figure 7).
Figure 7. Uptake and trafficking pathways of CPPs. CPPs use predominantly different sorts of endocytosis to gain access to the interior of cells. In the context of this, all major pathways for CPP uptake have been described, including clathrin- and caveolae-mediated endocytosis, as well as macropinocytosis. Less information is available for other less-defined pathways, such as several clathrin- and caveolae-independent endocytosis mechanisms. Uptake of the cargo molecule is followed by complex intracellular trafficking events towards early/sorting endosomes, late endosomes/microvesicular bodies (MVBs), lysosomes or Golgi network. Typically, endo-/lysosomal maturation is characterized by gradual drop in the pH. Of note, recycling pathways can direct cargo also through late endosomes/MVBs for being released to extracellular milieu via extracellular vesicle (EV) release. In this case, the cargo could become incorporated into exosomes and subsequently be taken up by other cells (re-distribute) or by the same cell (reuptake). In addition to the more dominant endocytic pathways, some membrane active CPPs have also been reported to be taken up by direct translocation over the cellular membrane, which potentially allows them to avoid endocytic pathways altogether. Reprinted with permission.

These pathways are today considered as the predominant mechanisms of transmembrane delivery of CPPs. Endocytosis can be divided into four pathways: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae/lipid raft-mediated endocytosis, and clathrin/caveolae-independent endocytosis. There are often conflicting results between studies of the exact internalization pathway of a given CPP but it is now evident that various CPPs and
CPP-cargo complexes can enter cells using different (single or multiple) endocytotic mechanisms and therefore end up in different compartments into the cells\(^{100}\). This was shown for Antp, nona-arginine, and TAT where macropinocytosis, clathrin-mediated endocytosis, and caveolae/lipid raft-mediated endocytosis were used simultaneously\(^{100}\). Different results are sometimes due to different experimental setup such as choice of cell-line, CPP concentration used, type of cargo, method of link CPP to cargo molecule (covalently linked to- or complexed with the CPP).

Results from a large number of studies suggest that the mechanism of endocytic uptake for a CPP is strongly dependent on the attached cargo\(^{78,97,101-103}\). For example, Tat has been shown to use lipid raft-mediated endocytosis when conjugated to a protein and clathrin-dependent endocytosis when conjugated to a fluorophore. Macropinocytosis has been implicated in the uptake of a variety of CPP-cargo conjugates, suggesting that membrane ruffling aids the internalization of CPPs\(^{104,101}\). Additionally, the electrostatic interaction of CPPs with surface proteoglycans has been shown to be responsible for the uptake of many CPPs\(^{96,97}\).

Independent of the initial interaction leading to endocytosis, the internalized CPP and its cargo end up into endosomes or lysosomes where they can stay for extended period of time, thus reducing bioavailability and activity. If the target of the delivered molecule is not the endocytic vesicles, the peptide/cargo complex has to be further transported to the target subcellular location (cytoplasm, nucleus, mitochondria) to exert its biological effect before being either transported back to the plasma membrane for recycling by exocytosis or fused to lysosomes for degradation\(^{105}\). This process, called endosomal escape, is not completely understood but is considered a limiting factor for the success-rate of CPPs. The process of endosomal escape is in many cases not very efficient and the CPPs may deny the cargo from reaching the desired intracellular site \(^{106,107}\). For this reason, efforts have been made to improve the ability of CPPs to exit from endosomes. Different chemical endosomolytic agents have been use in vitro to induce osmotic swelling and rupture of endosomes. Examples of such chemicals are chloroquine\(^{108}\), sucrose\(^{109}\) and calcium ions\(^{110}\).

In 2012, the involvement of scavenger receptor class A (SCARA) in the uptake of non-covalent CPP/oligonucleotide (ON) complexes was shown for the first time\(^{111}\). It was previously shown that SCARA receptors bind and mediate cellular uptake of a negatively charged molecule\(^{112}\), and the involvement of SCARA was shown for the CPP PF14/splice correcting oligonucleotides (SCO), which have negative zeta-potential (\(\zeta\)-potential) and NF51/pDNA\(^{113}\).
The nature and secondary structure of the CPP, the ability to interact with cell surface and membrane lipid components, the nature, type, and active concentration of the cargo, the cell type, and the membrane composition are the parameters that play a secondary role in the cellular uptake pathway.

1.3.3. Applications in Drug Delivery and Clinical Development of CPPs

Most drugs need to cross one or more cellular membranes in order to reach their target of interest and have any therapeutic effect. Since the passage through the plasma membrane is the limiting step, optimizing cellular delivery systems of therapeutics is an important priority of today’s research. One of the major hurdles to cure a disease lies in the low potency of current available drugs, which could partially be solved by using delivery vectors for specific targeting. CPP-based drug delivery has been explored to treat various diseases, including neuronal disease, asthma, ischemia, diabetes, and cancer\textsuperscript{89,114–116}.

Despite the success reported through \textit{in vitro} studies, only a few studies have shown treatment efficacy in animal models and no CPP or CPP conjugate has passed the FDA hurdle and reached the market. The first CPP clinical trial was done using oligoarginine to transport cyclosporine into cells throughout the epidermis and dermis of human skin. It has been discontinued in 2003 after Phase II clinical trials\textsuperscript{117}. TATp has completed a Phase II clinical trial conducted by Revance Therapeutics and has been used for topical delivery of botulinum toxin across the skin. Capstone Therapeutics has evaluated AZX-100 in Phase II trials, a cell-permeant peptide mimicking heat shock protein HSP20. Their strategy relied on bypassing the signaling pathway to smoothen muscle relaxation for prevention of dermal/keloid scarring. Protein Cdelta inhibitor-TAT\textsubscript{(47-57) conjugates, developed for myocardial infarction, pain, and cytoprotection/ischemia (KIA-9803, KIA-1678, and KIA-1455) are under evaluation by KIA Pharmaceuticals in Phase I/II. 6-aminohexanoic acid-spaced oligoarginine has been tested \textit{in vivo} for splicing correction by Avi Biopharma\textsuperscript{118}. A CPP-antisense peptide-morpholino (PMO) conjugated for aortocoronary bypass therapeutic application (AVI-5126) was terminated after Phase II. Another CPP-PMO conjugate for Duchenne muscular dystrophy treatment is in preclinical development (AVI-5038). Istituto di Sanità and Novartis have a vaccine based on TAT-V2 deleted Env proteins in Phase I clinical trial. Multiple TAT peptide transduction domains (PTDs) linked to a double-strand RNA binding domain (DRBD) has been developed by Traversa Inc\textsuperscript{119,120}. Diatos has developed the agent DTS-108, an active metabolite of the
anticancer drug irinotecan and the peptide DPV1047, for cancer treatment and
in Phase I clinical trials in Europe. Data regarding clinical trials were retrieved
from ClinicalTrials.gov, a database of privately and publicly funded clinical
studies conducted around the world.

1.3.4. Targeting Intracellular Organelles

Subcellular delivery implies the delivery of a molecule or drug in its active
form to its target site of action inside the cells. Peptides have facilitated the
acellular uptake of a variety of cargoes into cells, but they have less often ex-
erted the ability to specifically target organelles. Nevertheless, several pep-
tides capable of localizing to specific subcellular compartments or organelles
have already been demonstrated. For example, quantum dots derived from
peptide JB588 and liposomes from bacteriorhodopsin helix C have been
shown to localize into the membrane\cite{121, 122}; lysosomal targeting peptide, HIV-
1 TAT, fibrinogen-derived ICAM-1 binding sequence, transportan10/siRNA
to endosomes/lysosomes\cite{123-126}; mitochondrial 3-oxoacyl-coenzyme-A, hybrid
tumor homing/proapoptotic peptide, chemoselectively ligated cytochrome-C
oxidase peptide to mitochondria\cite{122, 127, 128}; TAT, polyarginine, influenza de-
derived fusogenic peptide, nuclear localization sequence (NLS)-TAT to nu-
cleii\cite{129-131}; endoplasmic reticulum(ER)-insertion signal, ligand for ER, ER tar-
geting moiety like AAKKKAA to the ER\cite{132-134}; dentin phosphophoryn, pep-
tide derived from herpes simplex type 1 virus to the cytosol\cite{135, 136}.

Signal peptides have also been used to direct their cargo to the nucleus,
endosomes, and other organelles. The plasma membrane is the first point of
contact for peptide or complexes, thus membrane targeting using peptides as
probes to localize onto the lipid bilayer is a valuable tool to understand the
first critical interactions. The vesicles of the endosomal and lysosomal system
are another desirable target for drug delivery due to disease or disease states
associated with deficient enzymes in these pathways such as Fabry disease.
The use of CPPs can be sufficient to target these compartments since they get
entrapped and sequestrated by them as mentioned earlier. Nuclei, the site for
genetic storage and gene transcription, are one of the primary organelles for
targeting delivery. The Simian virus large T antigen nuclear localizing se-
quencing (SV40 NLS) has been commonly used for nuclear targeting\cite{137, 138}. Na-
ked oligonucleotides have been shown to not be able to freely pass through
membranes; for examples, only 0.1% of free plasmid from cytosol has been
reported to translocate into the nucleus by crossing the nuclear membrane\cite{139}.

Another effective strategy for nuclear targeting has been shown to be the
use of NLS. Macromolecules larger than 50kDa required the presence of NLS to be transported into the nucleus. The signal sequence is usually removed in the mature protein. Small peptides from viruses that show nuclear localization, such as KKKRKV peptide from SV40 have been used for nuclear delivery by non-viral strategy. Cationic lipids in complex with DNA, called lipoplex, and cationic polymers in complex with DNA, called polyplex, have also been reported to deliver DNA into the nucleus\textsuperscript{140,141}. The NLS peptides have been demonstrated to guide uptake and nuclear localization of other species, including gold nanoparticles\textsuperscript{142}, carboplatin-based anticancer therapeutics\textsuperscript{143}, and green fluorescent protein (GFP)\textsuperscript{144}.

Other organelles such as the Golgi and the endoplasmic reticulum have been targeted. Golgi was described more than a century ago by Camillo Golgi as an internal reticular apparatus. It has a central role for the cell secretory pathway and interacts with the ER. It carries out posttranslational modification of newly synthesized proteins and is involved in the synthesis of proteoglycans and carbohydrate structures. Alterations of the Golgi-associated proteins and of the Golgi apparatus give rise to a variety of neurodegenerative disorders which include, for example, Alzheimer's, Parkinson's, Niemann-Pick disease. The ER is a network of folded membrane-enclosed tubules and cisternae that extend from the nuclear membrane throughout the cytoplasm. It facilitates the folding of secretory and membrane proteins, and is involved in calcium storage and signaling, as well as regulate apoptosis against disturbances in calcium homeostasis, ischemia, hypoxia, exposure to free radical, and oxidative stress\textsuperscript{145}. For these reasons, ER-Golgi network has been considered a main target for anticancer therapy. Rapamycin, a regulator of mammalian cell growth and proliferation in response to environmental and nutritional conditions, has been delivered to target the mTOR pathway. In some cancer, mTOR is activated to enhance cell growth. Inhibition of mTOR pathway using rapamycin could decrease the anti-proliferative effect of mTOR and play a central role in fighting cancer\textsuperscript{146}.

Mitochondria are the metabolic powerhouse of the cell and the control point for regulating programmed cell death. Dysfunctional mitochondria are implicated in a variety of diseases that range from metabolic disorders, to neurodegenerative diseases, to cardiovascular disease, to cancer\textsuperscript{147–149}. It is not surprising that much attention has been dedicated to the targeting of these organelles to treat such diseases. However, the difficulties to develop vectors for mitochondrial targeting has hindered the advancement of mitochondrial medicine. Nonetheless, promising results within this area have emerged. For ex-
ample, it has been show that short peptide are also able to target mitochondria as well as mitochondrial targeting sequence fused to peptides or synthetic transporters that show promise for mito-specific delivery of bioactive cargos.

CPPs with the ability to translocate into the cells and localize into mitochondria could be highly beneficial for mitochondrial delivery applications. This require better understanding of structure and effects of CPPs on cells and organelles.

1.4. Gene Therapy

Gene therapy is still at its infancy even though the field arose in the 1960s. In 1970, Stanfield Roger proposed to use of “good DNA” to replace defective DNA in people with genetic disorders. Two years later, Theodore Friedmann and Richard Roblin published a paper in Science titled “Gene therapy for human genetic disease?” where they propose that a sustained effort should have been made to formulate a complete set of ethic-scientific criteria to guide the development and clinical application of gene therapy techniques. Thirty years after, the first patient was treated with a gene therapy for a congenital disease called adenosine deaminase (ADA) deficiency which severely affects the immune system and the ability to fight infections. The infusion was performed using a retrovirus as a vector to carry the patient’s own corrected cells. Since then, many other patients with ADA have been treated with improved gene therapy technology to achieve therapeutic benefits.

The European Medicines Agency (EMA) defines a gene therapy medicinal product (GTMP) as a “biological medicinal product that contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human to regulate, repair, replace, add or delate genetic sequence and its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence.”

The Food and Drug Administration (FDA) defines gene therapy as agents “that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administrated ad nucleic acid, viruses, or genetically engineered micro-organisms. The products may be used to modify cells in vivo or transferred to cells ex vivo prior to administration to the recipient.”

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Many hurdles have been and still need to be overcome in gene therapy such as safety, immunity and manufacturing but the logic of such treatment modality could potentially take its place as a standard of care just as the use of pharmaceutical drugs.

Gene transfer has been complemented most recently by gene editing and potentially gene correction using technologies, such as engineering endonucleases, including CRISPR/Cas, and represent part of the exciting future of gene therapy. Gene therapies are promising for a broad range of diseases with the aim to radically treat the causes of the diseases instead of only relieving the symptoms. They may be effective on a wide range of previously untreated diseases such as haematological, ocular, neurodegenerative diseases, and several cancers\(^{160}\). In Figure 8, the number of gene therapy trials per year from 1989 to 2015 is reported, with a distinct increase over time. Adenovirus, retrovirus, and naked plasmid DNA were the most used vectors in the gene therapy trials, with respectively 22.14%, 18.76%, and 18.03% of the trials. Adeno-associated virus vectors were used in 6.63% of the trials, and vaccinia virus, lentivirus, and lipofection were used as vectors in around 5% of the trials\(^{161}\).

*Figure 8. Number of gene therapy trials per year. Reprinted with permission\(^{161}\).*
To date, eleven gene therapies have been approved. These are, in the order of their approval, Gendicine®, Oncorine®, Rexin-G®, Glybera®, Neovasculagen®, Imlygic®, Strimvelis, Eteplirsen®, Zalmoxis®, Kymriah®, Yescarta®, and Luxturna®. With particular interest are: Glybera, to treat adults with lipoprotein lipase deficiency (LPL), approved in 2014 by the EMA162; Strimvelis, to treat adenosine deaminase severe combined immune deficiency, approved by the EMA in 2016163; Eteplirsen, for Duchenne muscular dystrophy (DMD), approved by the FDA in 2016164.

In 2017, researchers announced that a teenager had been cured of sickle-cell disease, an inherited blood disorder that affects 100000 people in the USA and millions around the world, after receiving an experimental gene therapy developed by Bluebird Bio. In the same year, FDA approved two pioneering treatments, Kymriah and Yescarta, that use the patient’s own immune cells to fight rare types of cancer. The first treats a bone marrow cancer that affects children and young adults, the second treats a type of lymphoma. Last December, the FDA approved the first gene therapy for an inherited disease165. Luxturna aims to correct a mutation responsible for a range of retinal diseases that make people gradually blind. More than twenty patients that were losing their sight have had their vision restored after the treatment.

There is hope also for haemophilia. In the same month, BioMartin company published early clinical trial results showing that nine patients who received its therapy saw substantial increases in the blood-clotting proteins absent in haemophilia166.

1.5. Therapeutic Oligonucleotides

Oligonucleotide pharmacology and the first attempts to silence specific genes using antisense oligonucleotides (ASOs) date back to 1970s. An ASO is a short strand of deoxyribonucleotide analogue that hybridizes with complementary mRNA in a sequence-specific manner via Watson-Crick base pairing. While conventional drugs bind directly to proteins, formation of ASO-mRNA heteroduplex either triggers RNase H activity, leading to mRNA degradation, induces translational arrest by steric hindrance of ribosomal activity, interferes with mRNA maturation by inhibiting splicing or destabilizes pre-mRNA in the nucleus, resulting in the downregulation of target protein expression.

In 1978, Paul Zamecnik et al. suggested that oligonucleotides could be used
therapeutically. In their pioneer work they reported that a 13-mer ASO containing a phosphodiester backbone complementary to the terminal repeat sequences of the Rous sarcoma virus was able to inhibit its replication and cell transformation\textsuperscript{167}. The success was not immediate and in the following years several chemical modifications were developed in order to increase stability, deliverability, potency, and improve pharmacological properties of ASO. Some of the most common modification are modification of the 2´-hydroxyl (OH) to 2´-O-methyl (O-Me), phosphorothioate (PS), 2´-fluoro (F), 2´-methoxyethyl (MOE), bicycles containing a 2´,4´-O-methylene bridge, called locked nucleic acid (LNA), and peptide nucleic acid (PNA).

Some ASOs have the ability to enter specific cell lines in naked form, a process called gymnosis\textsuperscript{168}. However, the lack of efficient cell targeting and translocation into the desired cell type, organelle, or tissue by gymnosis have motivated researchers to investigate transfection methods, such as viral and non-viral vectors as well as liposome-mediated, for their delivery\textsuperscript{169–173}. The first antisense oligonucleotide approved for marketing by the FDA, and successively by EMA, was Vitravene, also known as Formivirsen. This 21-mer phosphorothioate oligodeoxynucleotide targeting the mRNA that encode for the cytomegalovirus immediate-early-2 protein was developed by Iris Pharmaceuticals and Novartis Ophthalmics for the treatment of patients with cytomegalovirus retinitis\textsuperscript{174}. In 2013, Spinraza, an 18-mer phosphorothioate 2´-O-methoxyethoxy ASO, has been approved by FDA for the treatment of spinal muscular atrophy type 1, 2, and 3 in infants.

Additional strategies that recently are getting special attention from the pharmaceutical industry includes mRNA-based therapeutics and CRISPR-Cas9 genome editing machinery. As the subject of basic and applied research for more than five decades, mRNA has only recently come into the focus as a potentially powerful drug class able to deliver genetic information. It is based on cancer immunotherapies and infectious vaccines approaches such as \textit{in vivo} delivery of mRNA to replace or supplement proteins, or mRNA-based induction of pluripotent stem cells, or mRNA-assisted delivery of designed nucleases for genome engineering\textsuperscript{175}. CRISPR-Cas9 technology, firstly described as an adaptive immune system in bacteria and archaea, have fast developed and become a popular tool for the targeted genome modification in many organisms. The process needs two essential components: the Cas9 recombinase enzyme that cuts the DNA and a snippet of single guide RNA that guides the molecular scissors to the target sequence. These tools were shown to efficiently drive specific modification of mammalian genomes\textsuperscript{176}.

ASO is a useful tool for protein target identification and validation, but also
a highly selective strategy for diseases with dysregulated protein expression. However, the alteration of animal or patient’s genomic DNA is also associated with ethical questions. The use of gene therapy appears to be closely related to factors such as economic difficulties, particularly with regard to wealth distribution, political and cultural conflicts, as well as the scarcity of studies evaluating the impacts of the use of gene therapy on human health. In this thesis the use of oligonucleotides was limited to two types of therapeutic ONs: splice correcting oligonucleotides (SCOs) and ASO.
2. Aims

The experimental work conducted during this PhD focused on three main objectives:

- to develop cell-penetrating peptides targeting mitochondria for their primary characteristics as potential antioxidative molecules in order to protect cells from oxidative stress;
- to target mitochondria as an intracellular target for drug delivery;
- to understand uptake and transfection mechanisms of cell-penetrating peptides alone or in conjugation with oligonucleotides (ONs).

The aims for each paper are detailed below.

2.1. Paper I

The aim of Paper I was to develop a library of novel cell-penetrating peptides targeting mitochondria (mtCPPs peptides) with improved biological efficiency. The peptides were designed to have a better ability to selectively accumulate in the mitochondria and to reduce the total level of reactive oxygen species in the cells. In this project we focused on the characterization of one of the new CPPs named mtCPP1. We investigated the effects of this peptide on intramitochondrial processes such as cytotoxicity, mitochondrial membrane potential and antioxidant activity. Fluorescent microscopy using carboxyfluorescein-labelled peptides was used to determine cellular uptake and localization. The ultimate aim was to confirm the targeting ability of the peptide and for this reason localization experiments on isolated mitochondria and other cellular compartments were performed.
2.2. Paper II
The aim of Paper II was to design and synthesise a fusion peptide by covalent conjugation of the most promising mitochondrial antioxidative CPP from Paper I (mtCPP1) and a glutathione analogue peptide (UPF25), previously developed in our laboratory. The purpose was to explore the potential to synergistically increase the treatment efficacy of the novel peptide (mtgCPP). Mitochondrial membrane potential and ATP production were investigated with the aim of assessing how these antioxidant peptides influences the mitochondrial energy provision.

2.3. Paper III
In the work of Paper III, the aim was to test a new synthesised library of delivery vector complexed with nucleic acid cargo to form nanostructures able to penetrate cells and mitochondria. There are a number of diseases that originate from mitochondrial dysfunctions. These dysfunctions can be reversed or alleviated by modifying and or regulating the expression of mitochondrial genes. The main aim was to develop a delivery method to transport nucleic acids into mitochondria and alter the mitochondrial gene expression.

2.4. Paper IV
The aim of Paper IV was to examine the intracellular trafficking after transfection with an amphiphilic CPP, PF14 (used in Paper III), both alone or in complex with SCO using a transcriptomics-based approach to improve the understanding of their uptake mechanisms. Additionally, we aimed to unravel the pathways involved in fundamental cellular phenomena associated with uptake of PF14 transfection system and to use this knowledge to design more efficient CPPs.
3. Methods

The detailed description of all methods used for the work this thesis is built upon can be found in the included papers. In this section theoretical background of the methods will be provided.

3.1. Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) is the most common method to synthesise peptides for both research and therapeutic purposes. Robert Bruce Merrifield was awarded the Nobel Prize in chemistry in 1984 “for his development of methodology for chemical synthesis on a solid matrix”\textsuperscript{181}. The peptide is attached \textit{via} its C-terminus to a resin, representing the solid phase, which allows for removal of the excess soluble reagents used in each coupling step. In this work, the 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry has been used. This technique is mainly divided in two sequential steps, where cycles of addition of amino acid and removal of the Fmoc protecting group are repeated. In the coupling step, activators are used in order to convert the carboxyl group of the amino acid into a more reactive ester, susceptible for nucleophilic attack by the primary amine of the linker of the functionalized resin or the growing peptide. A schematic representation of the SPPS technique is reported in Figure 9. The activators used for the work in this thesis are 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and 6-chloro-1-hydroxybenzotriazole dihydrate (6-Cl-HOBt) or ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma Pure\textsuperscript{®}). The Fmoc group is protecting the α-amine of the amino acid in order to avoid unspecific reactions. Piperidine or piperazine are examples of weak bases used to easily remove this protecting group. The functional groups of the peptide side chains are also protected with acid labile groups. Protecting group with different characteristic can be used in order to continue the synthesis orthogonally. When the synthesis of the whole peptide is complete, then the side chain protecting groups are removed and the unprotected peptide cleaved from
the solid support. In this work, all the side-chain modifications have been performed at a lysine protected with the acid labile 4-methyltrityl (Mtt)-group. The Mtt group can be removed with a solution containing 1% of tri-fluoroacetic acid (TFA), avoiding the cleavage of other protecting groups or the peptide from the resin.

All peptides were synthesized manually, using a room temperature parallel automated peptide synthesizer (Syro II, Multisyntech GmBH) or a fully automated microwave peptide synthesizer (Alstra+, Biotage AB, Uppsala, Sweden). 5(6)-Carboxyfluorescein was coupled manually or by using the Alstra+ synthesizer after removal of Fmoc protecting group from N-terminal amino acid of peptides. Stearic acid modification was also carried out either in a manual step after automated synthesis or coupled under microwave heating conditions in the Alstra+ synthesizer. All peptides were modified at the C-terminus by using a H-Rink-Amide-ChemMatrix resin (PCAS Biomatrix, St-Jean-sur-Richelieu (province of Quebec), Canada); this resin produces amidated peptides on the C-terminal. The peptides were cleaved from the resin, using 95% TFA, 2.5% triisopropylsilane (TIS) and 2.5% H2O, precipitated in cold diethyl ether and lyophilized.

**Figure 9.** General scheme of Fmoc-solid phase peptide synthesis.
Crude peptides were purified using semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC) and analyzed using matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) mass spectrometry or by ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS). After purification, the peptides were lyophilized again and reconstituted in ultra-pure water (Milli Q, Merck Millipore) before use. Table 4 below lists the peptides used in this thesis.

Table 4. Peptide sequences used in this thesis.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>SS31</td>
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<tr>
<td>mtCPP1</td>
<td>rY'OF-NH₂</td>
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<tr>
<td>mtCPP2</td>
<td>oY'OF-NH₂</td>
</tr>
<tr>
<td>mtCPP3</td>
<td>rY'OW-NH₂</td>
</tr>
<tr>
<td>mtCPP4</td>
<td>rY'rW-NH₂</td>
</tr>
<tr>
<td>mtCPP5</td>
<td>rY'rY'-NH₂</td>
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<tr>
<td>mtCPP6</td>
<td>rY'OOF-NH₂</td>
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<tr>
<td>mtCPP7</td>
<td>oY'OF-NH₂</td>
</tr>
<tr>
<td>mtCPP8</td>
<td>rY'RF-NH₂</td>
</tr>
<tr>
<td>mtCPP9</td>
<td>rY'rF-NH₂</td>
</tr>
<tr>
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<td>rY'KF-NH₂</td>
</tr>
<tr>
<td>FAMmtCPP1</td>
<td>rY'OF-NH₂</td>
</tr>
<tr>
<td>FAMmtCPP2</td>
<td>oY'OF-NH₂</td>
</tr>
<tr>
<td>FAMmtCPP3</td>
<td>rY'OW-NH₂</td>
</tr>
<tr>
<td>FAMmtCPP4</td>
<td>rY'rW-NH₂</td>
</tr>
<tr>
<td>FAMmtCPP5</td>
<td>rY'rY'-NH₂</td>
</tr>
<tr>
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<td>rY'OOF-NH₂</td>
</tr>
<tr>
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<tr>
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<tr>
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</tr>
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<td>mtgCPP</td>
<td>rY'OF,Y'ECG-NH₂</td>
</tr>
<tr>
<td>UPF25</td>
<td>Y²ECG-NH₂</td>
</tr>
<tr>
<td>FAMmtgCPP</td>
<td>rY'OF,Y'ECG-NH₂</td>
</tr>
<tr>
<td>FAMUPF25</td>
<td>Y²ECG-NH₂</td>
</tr>
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<td>PF14</td>
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<td>rY'OFAGYLLGK(Stearyl-εN)LLOOLAAAALOOLL-NH₂</td>
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<td>Stearyl-AGYLLGK(rY'OF-εN)LLOOLAAAALOOLL-NH₂</td>
</tr>
<tr>
<td>mitFect4</td>
<td>Stearyl-rY'OFAGYLLGK(Stearyl-εN)LLOOLAAAALOOLL-NH₂</td>
</tr>
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<td>mitFect5</td>
<td>rY'OFAGYLLGK(Stearyl-εN)LLOOLAAAALOOLL-NH₂</td>
</tr>
<tr>
<td>mitFect6</td>
<td>AGYLLGK(rY'OF-εN)LLOOLAAAALOOLL-NH₂</td>
</tr>
<tr>
<td>mitFect7</td>
<td>AGYLLGK(Stearyl-rY'OF-εN)LLOOLAAAALOOLL-NH₂</td>
</tr>
<tr>
<td>mitFect8</td>
<td>Stearyl-AGYLLGK(Stearyl-εN)LLOOLAAAALOOLL-NH₂</td>
</tr>
<tr>
<td>mitFect9</td>
<td>Stearyl-AGYLLGK(Stearyl-εN)LLOOLAAAALOOLLzY'OF-NH₂</td>
</tr>
</tbody>
</table>

Y' = 2,6-dimethyl-L-tyrosine; Y² = O-methyl-L-tyrosine; FAM = 5,6 carboxy-flourescein; O = ornithine; small caps = d-amino acid, stearyl = stearic acid; NH₂ = C-terminal amidation.
3.2. Cell Cultures and Treatment

For the experiments in this thesis, a diversity of cell types has been used. HeLa cells, the first cell line that was propagated indefinitely \textit{in vivo} from biopsy of a cervical tumour taken from the patience named Henrietta Lacks, were used in Paper IV. George O. Gay was the first researcher to use this cell line in the laboratory in 1951 and from then this cell line is the mostly common used human cell line due to rapid grow and easy transfection\textsuperscript{182}.

The HeLa pLuc705 cell line is stably transfected with a luciferase-encoding gene interrupted by a mutated β-globin intron 2. These cells were used in all the papers included in this thesis. HeLa pLuc705 cells are a gift from Prof. Ryszard Kole (University of North Carolina, Chapel Hill, NC, USA). The bEnd.3 cell line is a mouse brain endothelial cell line and has been used for experiments in Paper I and II. The immortalized bEnd.3 cell line was established from an infection of the polyomavirus middle T antigen in a mice brain. The endothelial nature of these cells was confirmed by the observed expression of von Willebrand factor and uptake of fluorescently labelled low density lipoprotein (LDL)\textsuperscript{183,184}. The U87 cell line is a human primary grade IV glioblastoma cell line which was derived from a malignant glioma by J. Ponten and associates in the 1960’s and reported to produce a malignant tumor in nude mice\textsuperscript{185}. This cell line was used in Paper I and II.

The CHO cell line was derived from the Chinese hamster ovary in 1957 by T. Puck at the Boston Cancer Research Foundation. This cell line has become a staple source of cells due to their robust growth as adherent cells and usable for cell transfection\textsuperscript{186}. CHO cell line has been used in Paper II.

Cells were grown at 37°C, 5 % CO\textsubscript{2}, in Dulbecco’s modified Eagle’s medium with glutamax supplemented with 0.1 mM non-essential amino acids, 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Stockholm, Sweden). Cells were seeded 24 h prior to experiments into 96-well plates. The cells were treated with peptides alone at different concentrations or mixed with ONs at different molar ratio (MR) in MilliQ-water in 10 % of the final treatment volume (i.e., 10 µl) and incubated for the appropriate time according to the experiments.

3.3. Cell Viability Assays

The translocation of CPPs across the plasma membrane can result in toxic effects due to membrane perturbation, especially at high peptide concentrations. Exceeding the toxic threshold can result in irreversible damage to cells
which makes determination of cytotoxicity crucial. To find out if the activity of the CPPs alone or in complex with its cargo was associated with cytotoxicity, cell viability was evaluated by conventional 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) toxicity assay (Roche Diagnostics Scandinavia AB, Bromma, Sweden) according to the manufacturer’s instructions.

This assay measures cell viability as a function of mitochondrial metabolic activity. The activity of the mitochondrial dehydrogenases is measured by their conversion of tetrazolium salts to formazan, cell-viability and cell-proliferation is directly correlated to the amount of formazan dye formed, more cells means more formazan dye. The dye can be elegantly quantified spectrophotometrically, using UV-absorption at 420 nm, more intense staining means more active mitochondrial dehydrogenase. The WST-1 reagent and the formazan product are both water soluble, which means that measurements can be performed 1-4 h after addition, making it a very rapid and convenient assay compared to the commonly used MTT assay, which requires solubilisation of the formazan salt.

3.4. Isolation of Mitochondria

Mitochondria were isolated in order to detect the presence of peptide into the organelle after cell treatment. Mitochondria were extracted using the Mitochondria Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer’s instruction. This assay allows the collection of a pellet containing the isolated mitochondria as well as for the cytosolic fraction and the membranes fraction.

3.5. Mitochondrial Membrane Potential Assay

Tetramethylrhodamine ethyl ester (TMRE) fluorescent probe was used to evaluate the mitochondrial membrane potential (ΔΨm). TMRE is a cellular positively charged permeant dye that readily accumulates in active mitochondria, both in the IMM and matrix space, due to its relative negative charge. Depolarized and/or non-functional mitochondria have decreased membrane potential and fail to sequester TMRE. It was possible to evaluate the ΔΨm base on the fluorescence intensity of the dye sequestrated into mitochondria of cells treated with peptides versus untreated cells read on a fluorescence
plate reader (Flex Station II; Molecular Devices, Sunnyvale, CA, USA) with setting suitable for TMRE (Ex/Em = 549/575 nm).

3.6. Determination Assay for Reactive Oxygen Species

MitoSOX Red mitochondrial superoxide indicator (Invitrogen Detection Technologies) fluorogenic dye was used to determine reactive oxygen species (ROS) production in mitochondria of live cells. MitoSOX Red reagent is rapidly taken up and selectively targeted to mitochondria. Into mitochondria, MitoSOX Red reagent is oxidized by superoxide and exhibits red fluorescence. The selectivity of this probe is due to the oxidation by superoxide but not by other ROS- or reactive nitrogen species (RNS)-generating systems, and oxidation of the probe is prevented by superoxide dismutase. The oxidation product was read with a fluorescence reader (Flex Station II; Molecular Devices, Sunnyvale, CA, USA) with settings suitable for MitoSOX (excitation = 510 nm, emission = 580 nm).

3.7. Dynamic Light Scattering

In order to characterize the physiochemical properties of the peptides or particles that formed when mixing the peptides with ONs, dynamic light scattering (DLS) was used. This method allows the determination of the size distribution of small particles in solution. The Brownian (random) motion and the speed of the particle in solution is dependent on the size of the particles themselves. If particles are small compared to the wavelength of the laser used then the light scatters in all directions\textsuperscript{188}. Due to the Brownian motion the distance between the particles is constantly changing, therefore scattered light intensity fluctuates. The time-scale of the fluctuations is directly related to the translational diffusion coefficient of the scattering particles, which in turn is related to their size\textsuperscript{189}. From this an autocorrelation function that relates the fluctuations in intensity to the size can be used for size determination. One drawback of this method is that in order to distinguish between two intensity peaks they need to be separated by a factor of two, or they will result in a single broader peak\textsuperscript{189}. Another problem is that the method is more sensitive to larger species as they give higher intensity.
The DLS technique allows also the measurement of the electrical potential at the interface of the particle surface and the diffuse layer, known as ζ-potential. The ζ-potential is the difference in potential between the layer of ions strongly bound to the charged particle surface (Stern layer) and that of the dispersion medium (diffuse layer). It can be determined by measuring the differential migration between two electrodes using laser Doppler velocimetry. This method allows for the measurement of the charge at the interface between the particle and the solvent in which is dispersed but not the actual particle charge. From this measurement is possible to get also an indication of the stability of the particles in a solution, where potentials between -30 and 30 mV indicate low stability. In this work an instrument that can do both types of measurements was used; Zetasizer Nano ZS (Malvern Instruments, Malvern, UK).

Peptides or complexes were prepared as described in the respective papers and diluted in MQ-water or complete cell culture media DMEM. Samples were assessed in disposable low-volume cuvettes. Data were converted to relative intensity plots, from which the mean hydrodynamic diameter was derived.

3.8. Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy is a method that gives information about the overall secondary structure of peptides or proteins in solution. CD analysis is based on light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light. The light is absorbed to different extents at specific wavelength depending on the differences in extinction coefficients for the two polarized rays called circular dichroism. Circular-polarized light rays will travel through an optically active medium with different velocities due to the different indices of refraction for right- and left-circularly polarized light, called optical rotation or circular birefringence. The variation of optical rotation as a function of wavelength is called optical rotary dispersion.
It has been shown that CD spectra between 260 and 180 nm can be analyzed for the different secondary structures: alpha helix, parallel and antiparallel beta sheet, turn, and others\textsuperscript{193}. Representative CD spectra is shown in Figure 10. This method can be also used to accurately predict secondary structure using theoretically derived spectra\textsuperscript{194,195}.

3.9. Luciferase

The characteristic glow in fireflies and click beetles is due to the luciferase. Firefly luciferase is an enzyme responsible for a reaction that emits light. The enzyme catalyses the oxidation of firefly luciferin, requiring oxygen and ATP. The gene that encodes it can be used as a reporter gene in many different types of delivery assays. The light produced by the reaction is easy and convenient to measure, directly related to differences in gene expression. For example, delivering a luciferase encoding plasmid to a cell that does not normally produce the enzyme gives a measurable increase light output that is proportional to the amount of luciferase present. Conversely, delivering siRNA to a cell that already expresses luciferase will result in a decrease in the amount of enzyme and therefore a reduction in light output. The splice correcting assay
described below is another way to use luciferase as reporter gene in an ON assay.

3.10. ASO delivery

In Paper III ASOs were delivered, using non-covalent complexation strategy with peptides, to HeLa pLuc705 cells. ASO targeting the uncoupling protein 2 (ASO [UCP2]), D-arm modified ASO targeting UCP2 (D-arm ASO [UCP2]), and D-arm modified ASO targeting cytochrome c oxidase subunit II (D-arm ASO [COXII]). D-arm is a D stem-loop import signal of tRNA Tys(GUA) and has been shown to be efficiently imported into the mitochondrion of Leishmania, a kinetoplastid protozoan, as well as into mitochondrial matrix in isolated mitochondria\textsuperscript{196–198}. UCP2 is a mitochondrial anion carrier able to modulate the ROS and COXII is a mitochondrial protein that make up complex IV of the respiratory chain related to maintaining the mitochondrial membrane potential. The silencing of the mRNA coding for these two proteins yielded to significant changes of the mitochondrial membrane potential and of ROS levels.

3.11. SCO Delivery

In Papers III and IV SCOs were delivered, using non-covalent complexation with CPPs, to cells that stably express an incorrectly spliced, and non-functional, luciferase. The successful delivery results in functional luciferase. A 96 well assay was used, seeding the cells 24 h before the treatment. The cell media was changed with fresh media and the CPP/SCO complexes then added.

3.12. Splice Correction Assay

The splice correction assay that was developed by Kole \textit{et al.} provides an elegant quantitative assessment of cellular delivery efficiency of SCOs\textsuperscript{199} The assay uses HeLa pLuc705 cells, a stably transfected cell line with a plasmid containing a luciferase-encoding gene interrupted by a mutated intron from a β-thalassemic globin gene. The intronic mutations activate a cryptic splice site that produces non-functional luciferase. Masking the mutated site with an antisense ON re-orients the splicing machinery to produce functional luciferase.
Subsequent luminescence measurement by a luminometer allows for quantification of uptake.

3.13. Determination Assay for Adenosine Triphosphate

In Papers I and II adenosine triphosphate (ATP) production levels were measured after peptides treatments on HeLa pLuc705. In order to measure ATP levels, the ATP determination assay was used. The quantitative determination of ATP was assessed using recombinant firefly luciferase and its substrate D-luciferin. The assay was based on luciferase’s requirement for ATP to produce light. For the luciferase assay, a standard reaction solution was prepared. ATP was diluted serially in the standard reaction solution to generate a standard curve. Cells were treated for 24 h with peptides and thereafter lysed. Standard reaction solution was added, and immediately light emission was acquired for 30 s using a GLOMAX 96 microplate luminometer (Promega, Stockholm, Sweden).

3.14. Fluorescence and Confocal Microscopy

One of the experiments in Paper I and II uses fluorescence microscopy. Confocal microscopy was used in Paper III. These techniques are essential tools in life sciences allowing for visualization and identification of cells, submicroscopic cellular components and fluorescently labelled molecules. Fluorescence microscopy enables the study of single molecules as well as allowing for the identification of several target molecules simultaneously. Confocal microscopy offers several advantages over conventional optical microscopy, including shallow depth of field, elimination of out-of-focus glare, and the ability to collect serial optical sections from thick specimens. These techniques can be used for imaging of either fixed or living tissue/cells that have been labelled with one or more fluorescent probes. In this thesis, these techniques have been applied to study the cellular uptake of labelled CPPs and ON-cargo and to investigate the localization of fluoro-labelled peptides alone or peptides in complex with Alexa568-labelled SCO in live cells.
3.15. Transmission Electron Microscopy

The transmission electron microscope (TEM) has become a very powerful tool in science due to the increased resolution than light microscopes. This allows the observation of ultrastructure of organelles, viruses and macromolecules. The TEM operated on the same basic principles as the light microscope but electrons are used instead of light. A high energy beam of electrons is accelerated through a very thin sample, and the interactions between the electrons and the atoms can be used to observe structures, shape, size density and quality of small entities (in some cases as small as individual atoms). In Paper IV we used TEM in combination with the application of autophagy inducers and gold-tagged plasmid DNA to examine the cellular delivery and uptake of nucleic acid complexed with PF14.

3.16. Western Blot Analysis

Western blot (WB) is a common method to detect and analyse proteins. In 1979, H. Towbin et al. described the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets where the original gel pattern was accurately obtained. It is based on the separation of proteins by electrophoresis from the gel to a membrane where they can be visualized specifically.

![Western Blot Schematic](image-url)

**Figure 11.** Schematic presentation for the detection of proteins on the western blot membrane by enhanced chemiluminescence (ECL). ECL substrate is processed by the enzyme (peroxidase) tagged to the secondary antibody and generates chemiluminescence where the primary antibody reacted with the antigen on the membrane. Reprinted with permission.
The setup consists of a standard of seven steps: sample preparation, gel electrophoresis, blotting to membrane, antibody probing, detection, imaging, and analysis. After the transfer, the membrane is blocked in order to prevent unwanted membrane-protein interaction in the following steps. To visualize the protein of interest the membrane is first probed using a primary protein-specific antibody followed by a labelled secondary antibody used for detection. A schematic representation is shown in Figure 11. An image is then taken, and the result is analyzed.
4. Results and Discussion

Many human genetic, metabolic and degenerative diseases, along with normal ageing, have been linked to mitochondrial dysfunction and defective assembly of the respiratory chain, which depends on expression of genes encoded in both the nuclear and mitochondrial genomes.

This thesis describes the use of cell-penetrating peptides for their ability to cross cellular membranes and target organelles. It furthermore explores CPPs for drug development and therapeutic possibilities. The outcome of this work proposes CPPs targeting mitochondria with intrinsic antioxidant activity, complexes with ON cargoes for gene modulation, and pathway of uptake for this transfection system.

4.1. Paper I: mtCPP1, a Cell-Penetrating Peptide Targeting Mitochondria

In this study we reported a series of short peptides targeting mitochondria. The design of the peptides was taking into consideration several characteristics: positively charged amino acids, lipophilicity, and alternating aromatic and basic residues. Rearrangement of the amino acid sequence had no effect on the scavenging properties of the peptides, but substitution of Dmt with phenylalanine resulted in complete loss of antioxidant activity. The resultant antioxidant action of the mtCPPs can thus be attributed to the Dmt residue, in line with previous studies. Tyrosine is known to scavenge oxy-radicals by formation of their corresponding relatively unreactive tyrosyl-radical. The radical intermediate can either be capped by radical-radical coupling to give di-tyrosine\textsuperscript{202,203}, or scavenged by glutathione and/or ascorbate\textsuperscript{204,205}. Dmt bears many structural similarities to tyrosine, but also to vitamin E; the methylated phenolic structure\textsuperscript{50}.

Unlike other antioxidants, mtCPPs are highly water-soluble and rapidly taken up by cells. Given to their positive net charge, they might be expected...
to target the mitochondrial matrix in a potential-driven manner. By accumulating in the mitochondria, these peptides are localized to the site of ROS production. Treatment of cells with \( \text{H}_2\text{O}_2 \) causes increase of ROS levels.

![Structure of mtCPP1](image)

**Figure 12.** Structure of mtCPP1.

The most promising peptide candidate, mtCPP1 (Figure 12), demonstrated antioxidative properties that selectively targeted mitochondria, thereby enabling scavenging of ROS at the site of production. The peptide showed a 2-fold reduction of ROS levels inside the cells compared to the SS31 peptide when \( \text{H}_2\text{O}_2 \) was added to induce an oxidative stress. As a consequence, prevention of mitochondrial depolarization could be achieved as well as prevention of cell death at the optimum concentration of 5\( \mu \)M. In contrast, most antioxidants require 100 \( \mu \)M to mM concentrations to prevent oxidative cell death\(^{206–208} \).

None of the peptides showed toxic effects on cells even at as high concentration as 100 \( \mu \)M, and this is consistent with their lack of effect on mitochondrial potential and ATP production. The mitochondrial functionality was protected by maintaining a constant \( \Delta \psi \text{m} \) at physiological level. The ATP measurement confirmed that mitochondria of cells treated with mtCPP1 were not affected from the treatment with no difference in ATP production compared to untreated cells.

Fluorescence microscopy showed 70% higher uptake of mtCPP1 into the cells compared to the original peptide, based on relative quantification of the fluorescence intensity. Using isolated mitochondria, we showed the ability of mtCPP1 to pass the cellular membrane and to accumulate into mitochondria. The fluorescence intensity data from the isolated mitochondria showed a 25% increased up-take of mtCPP1 compared to the original peptide.

We designed CPPs targeting the site of ROS generation and protecting mitochondrial function with their intrinsic antioxidative properties. These antioxidant peptides may be beneficial in the treatment of ageing and diseases
associated with oxidative damage such as ischemia-reperfusion injury and neurodegeneration.

4.2. Paper II: The Scavenging Ability of mtgCPP for Reactive Oxygen Species

In this paper we took the advantage of mtCPP1 to cross the plasma membrane and to target the mitochondria to deliver a glutathione analogue peptide (UPF25) and study the antioxidant properties of the fused peptide. We hypothesized that the fused peptide, called mtgCPP, would have higher antioxidative and detoxifying effects than mtCPP1 alone.

The first finding from this work was the remarkably stronger superoxide anion scavenging ability of mtgCPP compared to mtCPP1 or UPF25 alone by 2- and 3-fold, respectively. The mitochondrial membrane potential and the ATP production were investigated to assess the influence of peptide treatment on mitochondrial energy provision. The fused peptide resulted to have similar properties to mtCPP1, rescuing the mitochondrial membrane potential to physiological levels when cells were insulted with H₂O₂ and ATP levels were similar to the one measured from untreated cells. We reasoned that mtgCPP was capable of preventing damage to cellular compartment caused by ROS acting as GSH in the GSH metabolism.

Spectrofluorometric analysis was performed on three different subcellular fractions to investigate the subcellular uptake of the fluorescein-labelled peptides. The analysis showed that FAM-mtgCPP was mainly present into mitochondria with a 10- and 33-fold higher intensity than that of FAM-mtCPP1 and FAM-UPF25, respectively. Interestingly, FAM-mtgCPP was shown to be present in the cytosolic fraction as well, with an 8- and 19-fold higher fluorescence intensity than the other two fluoro-labelled peptides. The analysis also showed that the glutathione analogue peptide was mainly present in the membrane fraction.

DLS measurements showed that mtgCPP formed particles with sizes around 150 to 400 nm in diameter when dissolved in MilliQ water or in cell culture media at 5 µM final concentration, respectively. The ζ potential of mtgCPP was mainly electropositive at all concentrations and solutions used, as expected from the nature of the peptide consisting of positively charged residues.

The synergistic antioxidative activity might be the result of the ability of mtCPP1 to transport UPF25 fragments into the cells at higher concentrations.
The presence of the peptide in the cytosol and into mitochondria resulted in increased scavenging ability.

4.3. Paper III: The Delivery of Therapeutic Biomolecules to Mitochondria

In this paper we developed a library of peptides generated from a combinatorial covalent fusion of the previously used mitochondrial-penetrating peptide, mtCPP1, and PepFect14 (PF14) in order to deliver therapeutic biomolecules to mitochondria. The first test was to elucidate the transfection ability and efficacy of the peptides complexed with SCO. Complexes were thus formed at different MRs to screen for the most optimal ratio for splice-correction activity. This activity was found to be in a MRs-dependent manner. mtFect1/SCO at MR5 and MR10 had a transfection efficacy of 20- and 30-fold increase compared to PF14 at the respective MR. mtCPP1, PF14, and SCO mixture had a remarkably 20-fold increase in luminescence over PF14/SCO treatment.

To address the main question of this study, that is to be able to deliver biomolecules to mitochondria, we evaluate the knockdown of mitochondrial mRNA using three different antisense oligonucleotides: ASO [UCP2], D-arm ASO [UCP2], and D-arm ASO [COXII]. Peptides efficiently delivered ASO [UCP2] to mitochondria affecting the mitochondrial membrane potential and ROS production. The 24 h treatment elicited higher changes in mitochondrial membrane potential and ROS production compared to the 6 h treatment. Peptides were able to efficiently deliver also D-arm ASO [UCP2] to mitochondria showing stronger effects on mitochondrial membrane potential. While there was a 1- to 4.5-fold increase compared to the treatment with ASO [UCP2], the mitochondria membrane potential was massively perturbed after 24 h treatment with D-arm ASO [UCP2], with 20-fold increase of TMRE uptake over untreated cells for mitFect4/antisense complex treatment. The delivery of D-arm ASO [COXII] complexed with peptides to mitochondria was also delivered efficiently, and the mitochondrial functionality was affected. The 6 h treatment with D-arm ASO [COXII] resulted in a strong decrease of TMRE uptake, suggesting a depolarization of the mitochondrial membrane potential. The 24 h treatment showed to be less efficient, probably due to a recovery of the mitochondrial membrane potential over time. We also investigate the effect of the ASO on the ROS production. We could observe an increase in the level of the superoxide anion.
The size and the ζ potential of the complexes were investigated. The majority of the complexes were below 100 nm. A higher compaction degree was observed at increasing MR. The electrochemical potential resulted to switch from negative values at MR3 to positive values (~ 20 mV) at MR7 and MR10.

Based on these results, we concluded that the down regulation of the UCP2 or COXII protein levels after transfection of ASO [UCP2], D-arm ASO [UCP2], or D-arm ASO [COXII] by peptides resulted in an imbalance among the subunit in the Complex I or IV of the ETC, which affected mitochondrial functions such as maintaining the mitochondrial membrane potential and the production of ROS.

4.4. Paper IV: Cellular Uptake Mechanism and Intracellular Pathway Modulation of CPP-Based Transfection System

The uptake mechanisms for transfection with CPPs alone or in complexes with cargo molecules is still need of more clarifications. In this study, we performed RNA sequencing to study the effects of PF14 and PF14/SCO complexes on the gene expression of HeLa cells. PF14 alone and PF14/SCO resulted in significant effects on the gene expression with 292 and 934 genes differentially expressed, respectively. These treatments affected multiple pathway. We validated several of the most differentially expressed genes by qPCR. The genes were selected based on their potential to regulate autophagy due to preliminary data showing the possible involvement of autophagy pathway in the regulation of the transfection process. Expression of 26 autophagy-related genes were analyzed and several genes resulted to have a high fold change in gene expression. Genes like connective tissue growth factor (CTGF), nuclear receptor coactivator 7 (NCOA7), and apolipoprotein B receptor (APOBR) resulted in 4-fold higher expression; whereas low-density lipoprotein receptor (LDLR) showed a 10-fold down-regulation in case of treatment with PF14. PF14/SCO treatment gave a completely different profile with mostly gene down-regulation. This difference can be due to the differences between the peptide alone (or nanoparticles formed by positively charged molecule) and the PF14/SCO complex (formed by positively and negatively charged molecules).

We proceeded using small molecules to modulate autophagy pathway and investigate the effect of ON transfection. The ligands were chosen based on the selected genes from the pathway analysis in order to see if particular genes
could play a role in the uptake mechanism. CTGF inhibitor and focal adhesion kinase (FAK) inhibitor reduced 75% of the splice correcting activity; HMG-CoA reductase inhibitor and Prostaglandin E2 inhibited the activity by 60%. On the other hand, other inhibitors such as toll-like receptor 4 (TLR4) inhibitor, the nuclear transport receptor importin-beta inhibitor, hydrocarbon receptor (AhR) antagonist, the heat shock protein 70 (HSP70) inhibitor, and the beta-adrenoceptor antagonist increased the splice correction activity. The induction of autophagy was detected after 8 and 24 h treatment by confocal, fluorescence and electron microscopy. The autophagy induction in cells treated with PF14 or PF14/SCO was also confirmed by Western blot analysis. PF14 was able to induce autophagy to the same extent as in cells treated with the standard autophagy inducer, rapamycin. The co-treatment with ligand molecules influenced the cellular uptake of PF14/SCOAF568; for example, co-treatment with alprenolol resulted in 50% higher level of complex uptake.

This study demonstrates the autophagy induction and cellular effects triggered by CPP transfection system in basal cell signalling. We showed that the splice correction activity could be significantly upregulated by co-transfection with small ligand molecules able to suppress the autophagy process. This knowledge, in addition to other studies, can help understanding pathways involved in uptake mechanisms to optimize non-viral delivery systems for therapeutic and clinical applications.
5. Concluding Remarks and Future Outlook

Mitochondria play an important role in controlling both life and death of cells. As a consequence, malfunctions in this organelle leads to a range of human diseases. To circumvent these malfunctions, there is a need for better understanding of the molecular mechanisms responsible for mitochondria-linked disease processes. Oxidative stress has long been reported to mediate mitochondrial dysfunctions. For this reason, strategies for targeting mitochondria and delivery of antioxidants are being developed. There is a continuing search for better and more effective organelle targeting and effective antioxidants. During the last years, research efforts developing mitochondria-targeted antioxidants have increased steadily. Within this framework, our effort has consisted of the development of cell-penetrating peptides targeting mitochondria with antioxidative properties. In Paper I we reported that mtCPP1 and other compounds studied protect mitochondria against oxidative insult. In Paper II we concluded that the fusion peptide mtgCPP, formed by covalent conjugation of mtCPP1 and a glutathione analogue peptide, resulted in synergistic antioxidative effects by scavenging H2O2 and lowering level of ROS production.

Due to the association between a range of human diseases and mutations in mitochondrial DNA, it is not surprising that mitochondria are an attractive target for gene therapy. Current therapeutic approaches are largely supportive rather than curative, and there is a lack in effective treatments for mitochondrial disorders. Alternative strategies are required to develop innovative gene therapy vectors that can be produced in large scale in order to re-establish the normal mitochondrial function in mutated mitochondria or cells. Mitochondrial gene therapy provides a new perspective to this; efficient and convenient cure due to higher targeting and prolonged duration of action. The strategy can be applied at different levels: targeting genes in the nucleus, targeting protein import into the mitochondria, or targeting genes into the mitochondria. In Paper III we reported the ability of chemically modified CPPs to efficiently deliver antisense oligonucleotides into mitochondria to affect mitochondrial functions. The results from these peptides demonstrated a fruitful avenue that
may lead to clinically useful therapeutic formulations. New technologies such as RNA sequencing, super-resolution and cryo-electron microscopy could help to optimize the design of mitochondrial targeting CPPs.

Another bottleneck to bring gene therapy into clinical use have been the difficulties to deliver cargo molecules to cell nuclei. Genetic expression profiles and intracellular signalling pathways controlling transfection efficacy can be helpful for the design of optimal delivery vectors. In order to elucidate the uptake mechanisms of CPPs for oligonucleotides transfection, we have investigated the effects of transfection by PF14 alone or in complex with oligonucleotide on gene expression. In Paper IV we reported the ability of PF14/ON complexes to induce autophagy-related genes, and consequentially the autophagy. The modulation of this process using small ligand molecules led to increased transfection efficacy. This finding opens possibilities to use autophagy modulators as combinatory strategy for future gene therapy.

Gene regulation after treatment with mitochondrial targeting CPPs is also of our interest. The finding in this thesis could reveal new insights useful for understanding the uptake mechanisms and pathways used to target mitochondria, contributing to the evolution of new suitable mitochondrial targeting peptide systems for mitochondrial gene therapy.
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