REVIEW ARTICLE

Mitochondria-Targeted Drugs

Roman A. Zinovkin[1,2,3,*] and Andrey A. Zamyatnin Jr.[1,2,*]

[1]*Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia;* [2]*Institute of Molecular Medicine, Sechenov First Moscow State Medical University, Moscow, Russia;* [3]*Institute of Mitoengineering, Moscow State University, Moscow, Russia*

Abstract: ***Background:*** Targeting of drugs to the subcellular compartments represents one of the modern trends in molecular pharmacology. The approach for targeting mitochondria was developed nearly 50 years ago, but only in the last decade has it started to become widely used for delivering drugs. A number of pathologies are associated with mitochondrial dysfunction, including cardiovascular, neurological, inflammatory and metabolic conditions.

Objective: This mini-review aims to highlight the role of mitochondria in pathophysiological conditions and diseases, to classify and summarize our knowledge about targeting mitochondria and to review the most important preclinical and clinical data relating to the antioxidant lipophilic cations MitoQ and SkQ1.

Methods: This is a review of available information in the PubMed and Clinical Trials databases (US National Library of Medicine) with no limiting period.

Results and Conclusion: Mitochondria play an important role in the pathogenesis of many diseases and possibly in aging. Both MitoQ and SkQ1 have shown many beneficial features in animal models and in a few completed clinical trials. More clinical trials and research efforts are needed to understand the signaling pathways influenced by these compounds. The antioxidant lipophilic cations have great potential for the treatment of a wide range of pathologies.

Keywords: Mitochondria, targeted drug delivery, uncouplers of oxidative phosphorylation, reactive oxygen species, antioxidants, metabolic syndrome.

1. INTRODUCTION

It has been more than a century since Paul Ehrlich postulated his famous idea of a 'magic bullet', but only recently has this concept become a reality. The main problems faced by the concept of targeted drug delivery consist of searching for a target for a particular disease, searching for a medicine and searching for an effective way of delivering drugs to the target.

Targeted drug delivery may take place on three levels: (i) first-order drug targeting to an organ or tissue, (ii) second-order drug targeting to specific cells and (iii) third-order drug targeting aimed at an intracellular compartment such as the nucleus, endoplasmic reticulum or mitochondria [1, 2].

Currently, mitochondria are recognized as an emerging pharmacological target, since this intracellular organelle has

a number of vital functions and mitochondrial damage is crucial for the development of many diseases (see section 2.4 of the current review).

There is no universally accepted definition of a Mitochondria-Targeted Compound (MTC). We suggest defining an MTC as a substance that is selectively accumulated in mitochondria in amounts of at least 90% of the total substance added to the cells. The first MTC was synthesized in 1995 by Murphy and co-workers [3]. In the last decade, a whole set of MTCs has been synthesized and tested, both *in vitro* and *in vivo*. Many experimental data have been obtained confirming the efficacy of the MTCs in the prevention and treatment of a wide range of diseases. Moreover, a few dozens of clinical trials are either in an active state or have been completed.

The purpose of this review is to consider mitochondria as a promising target for treating a wide range of human diseases and pathological conditions and to review the principles of mitochondrially targeted drug delivery and the most significant results obtained with MTCs.

*Address correspondence to these authors at the Belozersky Institute of Physico-Chemical Biology, Moscow State University, Leninskie Gory 1-40, Moscow 119991, Russia; Tel: 74956229843; Fax: 74956229808; E-mail: roman.zinovkin@gmail.com and Sechenov First Moscow State Medical University, Institute of Molecular Medicine, Trubetskaya Str., 8, Moscow, 119991, Russia; E-mail: zamyat@belozersky.msu.ru

2. STRUCTURE AND FUNCTIONS OF MITOCHONDRIA

2.1. Structural Aspects

Two membrane layers that differ in both chemical and structural composition envelop mitochondria. Between the Outer Mitochondrial Membrane (OMM) and the Inner Mitochondrial Membrane (IMM), there is an intermembrane space, and the mitochondrial matrix is located inside the IMM. The components of the Electron Transport Chain (ETC) are located in the IMM (Fig. **1**).

Mitochondria have their own self-replicating genome in the form of circular mitochondrial DNA (mtDNA). Typically, one animal cell contains from 100 to 10, 000 mtDNA copies. Human mtDNA codes for mitochondrial transport RNA, ribosomal RNA and some hydrophobic ETC proteins. Mutations in either mtDNA or in nuclear DNA genes coding for mitochondrial proteins result in the development of inherited mitochondrial diseases [4].

The outer membrane is permeable to a wide range of small molecules. The permeability of the IMM is selective; special channels regulate the transport of molecules through the IMM. Thus, the electrochemical potential and the content of the mitochondrial matrix are significantly different from those of the cytosol.

2.2. Main Functions

Mitochondria are implicated in many vital processes in animal cells, including energy production, fatty-acid oxidation and the Tricarboxylic Acid (TCA) cycle, calcium signaling, permeability transition, apoptosis and heat production.

The main function of mitochondria is to produce Adenosine Triphosphate (ATP). In the cell, the necessary energy in the form of ATP is produced in two ways: in the cytosol as a product of glycolysis, and in the mitochondria as a product of oxidative phosphorylation (OXPHOS). The substrates, in the form of fatty acids and pyruvate, are oxidized *via* fatty acid β-oxidation and the TCA cycle respectively. The Nicoti-

Nicotinamide Adenine Dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) produced by these reactions are used by the electron transport chain to generate ATP.

The mitochondrial electron transport chain consists of the enzyme complexes located in the IMM (Fig. **1**). The electrons supplied by NADH are accepted by complex I and the electrons from $FADH_2$ are transferred to complex II. Then, the electrons are transported by coenzyme Q to complex III and finally by cytochrome c through complex IV to oxygen molecules. The electron flow is accompanied by pumping of protons from the mitochondrial matrix to the intermembrane space. The resulting proton gradient generates ATP through complex V (ATP synthase).

In brown adipose tissue, mitochondria promote heat production *via* Uncoupling Protein 1 (UCP1) [5]. This protein induces mitochondrial uncoupling of oxidative phosphorylation and respiration *via* an increase in proton conductivity of the IMM. The energy of the protons is not used for ATP production but instead is dissipated in the form of thermal energy. In human adults, brown adipose tissue is found in small quantities only. However, UCP1-mediated heat production is of great importance in newborns, to avoid life-threatening hypothermia.

The main role in the storage of calcium cations in the cells is performed by endoplasmic reticulum, but mitochondria are also capable of storing calcium transiently [6]. The outer mitochondrial membrane is permeable to Ca^{2+} ions, which enter the mitochondrial matrix *via* the calcium uniporter located in the IMM. The release of calcium initiates calcium spikes or calcium waves, with a great impact on cellular signaling cascades (reviewed in [7]).

The Mitochondrial Permeability Transition (MPT) is an instant increase of the IMM permeability to molecules less than 1, 500 Da. MPT opening leads to depolarization, Ca^{2+} release and Reactive Oxygen Species (ROS) production, in response to certain stimuli, such as increased Ca^{2+}, ER stress, fatty acids and inorganic phosphate [8]. Short-term openings probably fulfil physiological functions such as Ca^{2+} regulation
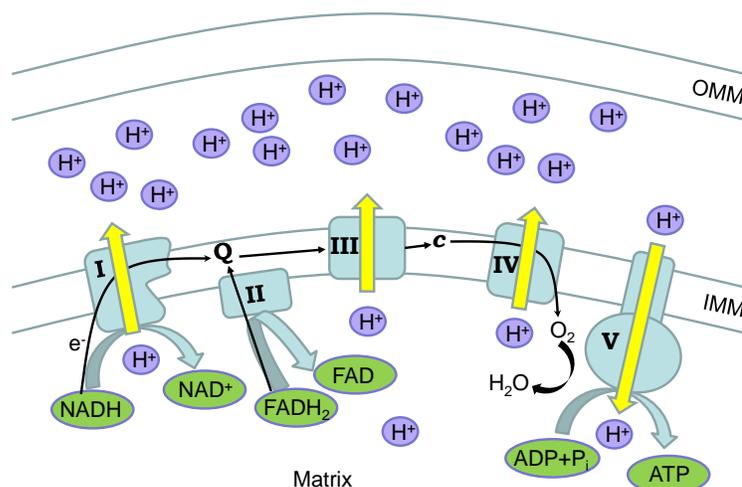
Fig. (1). Scheme of mitochondrial electron transport chain (ETC). Electron flow (black arrows) from NADH or $FADH_2$ substrates through complexes I - IV of ETC is accompanied by pumping of protons. This results in a proton gradient between the inner mitochondrial membrane (IMM) and the outer mitochondrial membrane (OMM). This proton-motive force is used by ATP synthase (complex V) to make ATP from ADP and a phosphate (Pi). Abbreviations: Q - coenzyme Q10, c - cytochrome c.

and ROS production. Long-term MPT opening may result in detrimental effects including cell death [9]. Mitochondria form a central cellular hub regulating the intrinsic pathway of apoptosis. MPT opening leads to cytochrome c exit, caspase activation and apoptosis [10]. Even without the activation of caspases, permeabilization of the outer mitochondrial membrane may induce caspase-independent cell death [11].

2.3. Mitochondria and ROS

Oxidative phosphorylation always results in ROS production. The primary ROS is a superoxide anion ($\cdot O_2^-$) produced by both complex I and complex III. Most superoxide anions are converted by superoxide dismutase (SOD) into hydrogen peroxide, which in turn is converted into water by catalase or by peroxidase enzymes. However, antioxidant enzymes are not able to inactivate all the ROS. Excessive amounts of superoxide anion may interact with other molecules, resulting in production of multiple secondary radicals including reactive nitrogen species and lipid radicals.

Mitochondrial ROS (mtROS) are also synthesized in a number of enzymatic reactions in mitochondria including those of glycerol-3-phosphate dehydrogenase, cytochrome P450, monoamine oxidase and cytochrome b5 reductase (reviewed in [12]).

It is widely accepted that excessive mtROS production is detrimental to the cells and surrounding tissues [13]. Many diseases and pathological conditions are now linked to mtROS (see the next section). However, besides their well-known destructive role, mtROS appear to be necessary for many physiological reactions, including apoptosis [14], activation of macrophages' inflammasomes [15] and possible monoamine-induced calcium signaling in astrocytes [16].

ROS are also produced from non-mitochondrial sources, including NADPH oxidase [17], myeloperoxidase [18], xanthine oxidase [19], monoamine oxidase [20] and uncoupled nitric oxide synthase [21]. NADPH oxidases are considered the major enzymatic source of ROS [22]. There are seven isoforms of NADPH oxidases, expressed in a variety of cells including phagocytes, vascular cells, smooth muscle cells and fibroblasts [23]. ROS produced by NADPH oxidases are necessary for proper functioning of innate immunity and signal transduction. However, excessive ROS production is involved in the development of many forms of the cardiovascular disease [22] and in many neurological diseases, including stroke, traumatic brain injury and neurodegenerative diseases [24]. Interestingly, though neutrophils and endothelial cells produce ROS mostly *via* NADPH oxidase and xanthine oxidase respectively, synthesis of mtROS is necessary for inflammatory signal transduction [25, 26]. In addition, secondary ROS production *via* NADPH oxidase was inhibited by quenching mtROS in an animal model of cisplatin-induced nephropathy [27]. Thus, mtROS may regulate ROS production by non-mitochondrial sources. The exact impact of mtROS in total ROS production in different cells is still not completely clear.

2.4. Mitochondria and Diseases

Mitochondrial dysfunction is typically characterized by a disturbance of the basic mitochondrial functions: Bioenergetic, antioxidant and regulatory. This dysfunction results in a decrease in ATP synthesis, dysregulated cell death processes and increased ROS production.

Proper mitochondrial functioning is crucial for every enucleated cell in a body. A number of diseases are characterized by dysfunction of muscular or neural systems or metabolic reactions. All these diseases and pathophysiological conditions are developed against a specific genetic background, together with environmental factors. Some examples of mitochondrial involvement in the development of these diseases are given below.

Our review does not focus on inherited mitochondrial diseases caused by mutations either in mtDNA or in nuclear DNA. The readers interested in this topic are directed to recent excellent reviews [4, 28, 29]. It should be noted that mitochondrial diseases affect cells in tissues with high energy demands: neural and muscular systems. These systems are also affected in all non-hereditary diseases accompanied or even caused, by dysfunctional mitochondria.

2.4.1. Metabolic Syndrome

Metabolic syndrome is a group of conditions combining hypertension, hyperglycemia, abdominal obesity and abnormal cholesterol or triglyceride levels. Metabolic syndrome greatly increases the risk of cardiovascular disease, stroke and type 2 diabetes (T2DM). There are numerous reports mentioning mitochondrial dysfunction and lower oxidative phosphorylation capacity in patients with T2DM compared with healthy individuals [30-32]. Both T2DM and obesity result in the accumulation of plasma acylcarnitines, probably due to reduction of β-oxidation of fatty acids inside mitochondria [33-35].

In skeletal muscle of both rodents and humans, a high-fat diet increases the H_2O_2-emitting potential of mitochondria, shifts the cellular redox environment to a more oxidized state and decreases the redox buffering capacity in the absence of any change in mitochondrial respiratory function. Furthermore, it was shown that attenuating mitochondrial H_2O_2 emission in mice muscles completely preserves insulin sensitivity, despite a high-fat diet [36].

2.4.2. Cardiovascular Diseases

The cardiovascular system strongly depends on mitochondrial function. Cardiomyocytes have very high mitochondrial content in order to produce the necessary ATP, and mitochondrial dysfunction inevitably leads to the development of cardiovascular diseases (for more details see [37]).

Alterations in mitochondrial calcium transport, which lead to ROS generation and MPT opening, also result in abnormalities of ATP production, thus leading to heart failure and ischemia-reperfusion injury (see the review in [38]). Mitochondrial dysfunction is also found in mice models of diabetic cardiomyopathy [39].

Peripheral arterial disease has a complex pathophysiology but is also shown to result from decreased mitochondrial respiratory capacity and oxidative stress (reviewed in [40]).

2.4.3. Neurodegenerative Disorders

There is now increasing evidence of mitochondrial dysfunction in Alzheimer's Disease (AD), Parkinson's Dis-

ease (PD), Huntington's disease, and amyotrophic lateral sclerosis (ALS).

The pathophysiology of AD remains unclear and the amyloid cascade hypothesis has dominated others to date. However, none of the drug candidates preventing accumulation of amyloid β-peptide has succeeded even in the delay of this disease. Thus, mitochondrial dysfunction was proposed as the primary event in the sporadic form of AD that causes amyloid β -peptide (Aβ) deposition, synaptic degeneration and formation of neurofibrillary tangles [41]. This hypothesis is supported by the evidence of mitochondrial dysfunction in AD patients [42] and in animal models [43, 44]. This approach also identifies new alternative therapeutic targets located in the mitochondria [45].

PD is characterized by resting tremor, bradykinesia and rigidity. These symptoms are developed due to loss of dopaminergic neurons in the *substantia nigra*. Patients with PD have a deficiency in the activity of mitochondrial electron transport chain complex I in a number of tissues, including the *substantia nigra* (reviewed in [46]). The primary role of mitochondrial dysfunction in PD was proved using transgenic mice lacking the mitochondrial transcription factor A gene. These animals slowly developed a typical Parkinsonian phenotype [47, 48].

Huntington's disease is caused by an abnormal accumulation of CAG repeats in the huntingtin gene. Multiple mitochondrial abnormalities are found in the neurons of Huntington's disease patients (reviewed in [49]). The main neuropathological features of Huntington's disease are reproduced in animals treated with the mitochondrial toxin 3-nitropropionic acid [50].

Amyotrophic lateral sclerosis is a progressive neurodegenerative disease affecting motor neurons in the brain and spinal cord. ALS etiology is still largely unknown, but in some familiar forms of ALS it occurs due to a mutation in the cytoplasmic Superoxide Dismutase (SOD1) gene. This mutated enzyme is also located in the mitochondria, causing alterations in axonal mitochondrial morphology and distribution [51]. Expressing mutant SOD1 targeted to mitochondria is sufficient to produce loss of motor neurons and an ALS phenotype [52].

2.4.4. Muscular Disorders

Duchenne muscular dystrophy is an X-linked recessive disease with a deleterious mutation in the dystrophin protein [53]. Duchenne dystrophy also has some features of mitochondrial dysfunction, including decreased ATP synthesis and MPT pore opening leading to the death of the myofibers [54-56].

Inclusion body myositis is a type of inflammatory myopathy that, like AD, is an amyloid disease characterized by inflammation and accumulation of Aβ oligomers and other protein aggregates in muscle fibers. Mitochondrial homeostasis is greatly affected in this disease (reviewed in [57]).

2.4.5. Inflammatory Diseases

Indeed, nearly all the diseases mentioned above are characterized by systemic chronic inflammatory reactions. In addition, mitochondrial dysfunction plays a significant role in the inflammatory response in acute human pathologies. Systemic Inflammatory Response Syndrome (SIRS) is a pathological state with a systemic immune reaction to severe damage, including ischemia, acute pancreatitis, trauma and sepsis [58]. SIRS is elicited by different factors including Damage-Associated Molecular Patterns (DAMPs), Pathogen-Associated Molecular Patterns (PAMPs) and cytokines [59]. Excessive inflammatory reactions may result in the development of fatal multiple organ failure. The important role of mitochondrial ROS in SIRS was reviewed recently [60].

Production of the pro-inflammatory cytokine IL-1β is regulated by NLRP3 inflammasome, which has been linked with various human auto-inflammatory and autoimmune diseases [61]. ROS-generating mitochondria greatly enhance the activity of the NLRP3 inflammasome complex [15].

Since mitochondria have a bacterial ancestry, it is not surprising that many mitochondrial components may function as DAMPs, promoting acute inflammatory reactions. Mitochondrial DAMPs elicit a strong immune response through interaction with the corresponding pattern-recognition receptors. It was recently shown that newly synthesized mtDNA enhances activation of the NLRP3 inflammasome [62]. Extracellular mtDNA also triggers inflammatory reactions in many cell types, including neutrophils [63, 64]. Cardiolipin, a phospholipid of the inner mitochondrial membrane, also functions as a DAMP [65]. Like bacteria, mitochondria start protein synthesis with the N-formylmethionine residue. These proteins also function as DAMPs, activating neutrophils and driving their chemotaxis [66].

2.4.6. Other Diseases

Many other diseases are interconnected with mitochondrial dysfunction. Due to space limitation, we direct readers to the recent review on this topic covering liver diseases, cancer, kidney diseases, eye diseases, pulmonary diseases (including chronic obstructive pulmonary disease) and drug-induced mitochondrial toxicity [67].

2.5. Mitochondria and Aging

A number of age-related processes are associated with mitochondrial dysfunction (reviewed in [68]) and most popular aging theories take this into account. The mitochondrial theory of aging suggests that the accumulation of damage to mitochondria and mtDNA promotes aging of humans and animals [69]. The theory supposes that there is a so-called vicious cycle: the accumulation of damage in mtDNA leads to the synthesis of nonfunctional proteins of the respiratory chain, which in turn increases the production of mitochondrial ROS, again damaging mtDNA. A number of facts confirm this theory: transgenic 'mutator' mice with defective mitochondrial DNA polymerase γ had accelerated accumulation of mutations in mtDNA, decreased lifespan and a senescent phenotype [70]. Later, Schrinner and colleagues showed that the overexpression of the mitochondria-targeted catalase increased lifespan in these mice [71]. Nowadays, however, this theory is seriously challenged by data showing that: (i) oxidative mtDNA damage during the lifetime does not result in accumulation of the mutations [72] and (ii) heterozygous 'mutator' mice have a higher mutational load in mtDNA than old wild-type mice, although their life spans are identical.

Another theory describes aging as a chronic low-grade inflammation causing cumulative damage to organic compounds *via* ROS [73]. Aging is accompanied by increased mitochondrial ROS production in the heart [74] and in vessels [75]. The ROS are able to activate redox-sensitive pathways, including transcription factor NF-κB, and can also stimulate secretion of pro-inflammatory cytokines, leading to the development of vascular inflammation and/or promoting atherosclerosis [76]. Both mitochondrial dysfunction and mitochondrial DAMPs are suspected sources of chronic inflammation in aging [77].

Apart from these theories, there are many experimental data confirming the key role of mitochondria in aging. For instance, two recent papers published in the journal *Cell* have shown that mitochondrial stress early in nematode development causes chromatin rearrangement and a subsequent increase in the life span of *Caenorhabditis elegans* [78, 79]. Thus, mitochondria also appear to be an attractive target for aging retardation.

3. HOW TO TARGET MITOCHONDRIA

3.1. Targeting Principles

It is likely that mitochondria originated *via* endosymbiosis with ancient prokaryotic organisms such as proteobacteria [80]. Thus, among all other organelles, mitochondria still have some unique features that can be used for targeting (Fig. **2**): high transmembrane potential (ΔΨm) across the inner mitochondrial membrane, unique phospholipid composition in the IMM and a special protein import machinery.

Firstly, mitochondria are the only intercellular organelles with a negative charge inside. The ΔΨm between the matrix and the intermembrane space is around 180 mV. Mitochondria use this potential as a proton-motive force to drive ATP synthesis, and ΔΨm is a key parameter that indicates the bioenergetic competence of mitochondria [81]. The ΔΨm is exploited for mitochondrial targeting by the use of positively charged ions (cations) that are attracted to the mitochondria *via* an electrostatic force.

Secondly, the IMM has a unique structure and lipid composition, which can also be used for the targeted delivery of drugs to the mitochondria. The phospholipid cardiolipin is found almost exclusively in the IMM, where it provides structural support for respiratory chain complexes and also plays a key role in apoptosis [82].

While establishing endosymbiotic relationships between the primary eukaryotic cell and mitochondria, the latter lost most of their genetic material and were forced to use proteins encoded in the nuclear genome. Thus, mitochondria have their own protein import machinery that recognizes proteins with a special amino acid sequence [83] (Fig. **2**).

Thus, drug delivery to mammalian mitochondria is carried out using one of these approaches, or a combination thereof. The main groups of MTCs exploiting various targeting principles are listed below.

3.2. Lipophilic Cation Moieties

Mitochondria-penetrating cations were discovered about half a century ago [84]. These cations should possess the following properties, allowing them to penetrate through the cellular and mitochondrial membranes of the animal cell. Firstly, they must be to an optimal degree lipophilic. If lipophilicity is less than a certain limit, then the molecules
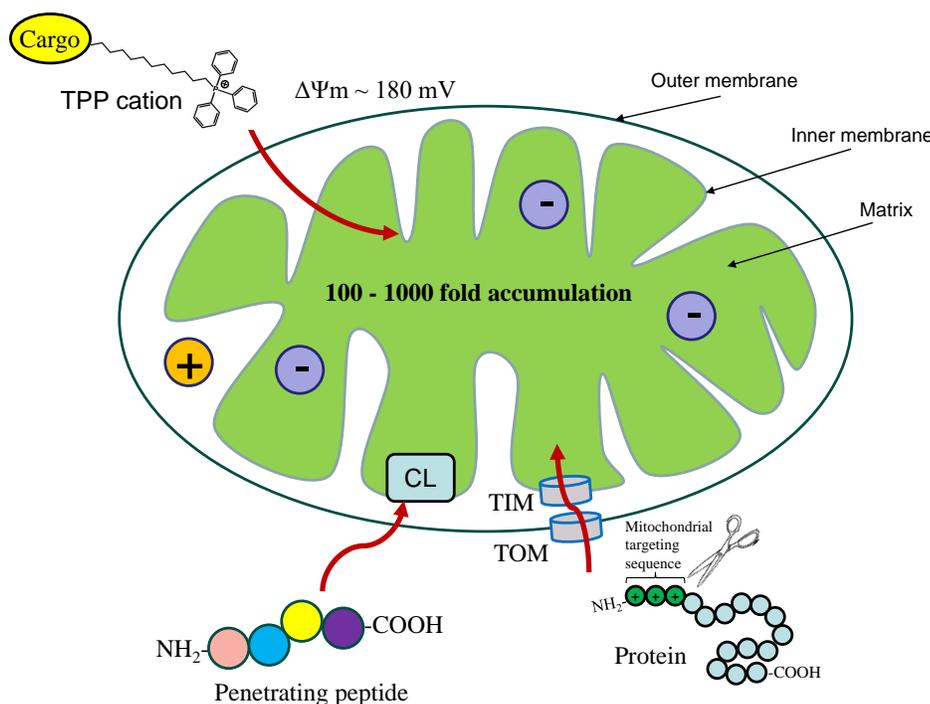
Fig. (2). Three main types of mitochondrial targeting. Mitochondrial uptake of lipophilic cations such as triphenylphosphonium (TPP) occurs due to the transmembrane potential (ΔΨm), and the cations are accumulated at the inner mitochondrial membrane. Peptides penetrating mitochondria and binding to the mitochondrial phospholipid cardiolipin (CL). Cellular proteins with N-terminal mitochondrial targeting sequences are imported into mitochondria *via* TOM and TIM channels.

cannot penetrate biological membranes. If lipophilicity is excessively high, then the compounds will accumulate mainly in the cell membrane. Secondly, the positive charge of lipophilic cations should be delocalized, *i.e.*, the charge should be distributed between at least three atoms but not within the single internuclear region between two adjacent atoms.

The most widely used lipophilic cation is triphenylphosphonium (TPP) (Fig. **3**). Normally, the drug of interest is linked to the TPP cation *via* a carbon aliphatic linker. The idea of using lipophilic cations as the carriers to mitochondria was first implemented by Murphy and co-workers [3] to synthesize thiobutyltriphenylphosphonium bromide. Later, the thiobutyl group was replaced by the natural antioxidant α-tocopherol (MitoVitE) [85]. Nowadays, many dozens of TPP-based MTCs have been synthesized [86] and characterized in various *in vitro* and *in vivo* studies.

There are also less common lipophilic systems besides TPP, such as rhodamine, quaternary ammonium salts, pyridinium, cyanine and berberine derivatives. Dequalinium (DQA) is another lipophilic molecule with two cations. DQA has been used for a long time as a topical antimicrobial agent. DQA can form liposome-like aggregates called DQA-somes that are able to deliver different molecules to mitochondria [87]. Unfortunately, the usefulness of this promising delivery system is limited by low transfection efficiency [88].

3.3. Cardiolipin Targeting (Penetrating Peptides)

Penetrating peptides are small, cell-permeable, mitochondria-targeted, antioxidant peptides that can protect mitochondria from oxidative damage [89]. The exact mechanism of the peptides' uptake into mitochondria is still unclear, but most probably it does not rely on mitochondrial potential [89] but depends on direct interaction with cardiolipin in the IMM [90].

These peptides (also sometimes called Szeto-Schiller peptides) carry aromatic residues providing necessary hydrophobicity and basic amino acids providing positive charge, at physiological pH values [89]. The peptides have antioxidant properties attributed to the tyrosine or dimethyltyrosine residue. Tyrosine reacts with ROS forming relatively inert tyrosyl radicals, which either create dityrosine or react with superoxide to form tyrosine hydroperoxide [91]. Dimethyltyrosine acts in the same way but appears to be more effective than tyrosine. These peptides efficiently neutralize ROS in mitochondria and prevent the oxidation of cardiolipin, thus inhibiting mitochondrial apoptosis associated with oxidative stress [89].

The first of the Szeto-Schiller peptides, SS-31, is currently in multiple clinical trials, mostly targeted at cardiovascular, age-related and mitochondrial diseases (please refer to the informative reviews in [92, 93]).

3.4. Mitochondrial Targeting Signal Peptides

More than 1, 000 mitochondria proteins are encoded in the nuclear genome. Mitochondrial protein import machinery is naturally used by cells for the delivery of nuclear-encoded mitochondrial proteins. These proteins normally have a cleavable 20-40-amino acid N-terminal targeting sequence. Though not conserved, this sequence is typically charged positively and contains amphiphilic α-helices [94]. TOM importing complex is located on the OMM and is responsible for the translocation of the imported proteins to the TIM complex located at the IMM. After passing through the IMM, the targeting sequence is cleaved by mitochondrial peptidases and the imported protein is folded into mature conformation by the mitochondrial chaperones (reviewed in [95]).

Most of the researchers' efforts were aimed at using a mitochondrial targeting sequence for DNA or gene delivery into the mitochondria as a possible way to cure mitochondrial diseases [96, 97]. This approach is also used to study the specific role of mitochondria in the development of some diseases, *e.g.*, the role of mitochondrial accumulation of Aβ peptide in the alteration of mitochondrial function and cell death [98].

However, no potential drug with mitochondrial targeting signal peptides has yet been tested, since this approach has many practical limitations: (i) large molecules can hardly be transported, (ii) efficient targeting peptides are quite long, (iii) the peptides have low hydrophilicity and cellular permeability and (iv) the price for chemical synthesis is very high. These drawbacks severely limit the use of MTCs based on the mitochondrial targeting signal peptides.

4. MTCs IN PRECLINICAL AND CLINICAL TRIALS

4.1. Main Types of MTCs and New Drug Discovery

The vast majority of synthesized MTCs fall into one of the following categories:

antioxidants - lower mitochondrial ROS production

uncouplers of oxidative phosphorylation and respiration - lower $\Delta\Psi m$ and ATP production

poisons - mitotoxic and cytotoxic compounds inducing cell death, mainly apoptosis

probes and sensors - for detection of reactive oxygen, nitrogen and sulfur species.

It should be mentioned that applying any of the MTCs nearly always has multiple effects, such as alterations in the redox status, ETC activity, ATP synthesis, etc. This occurs due to the complex interconnected processes existing within mitochondria. For example, at certain concentrations, uncouplers of oxidative phosphorylation and respiration also lower ROS production in mitochondria [99]. High doses of the antioxidants may exert pro-oxidant effects [100] and inhibit ETC [101].

Mitochondria-targeted antioxidants represent the major part of all tested MTCs because of the important role of mtROS in many pathophysiological processes (see above). Most of the *in vivo* results and all the clinical trial results are obtained using either the lipophilic TPP antioxidants MitoQ and SkQ1 (Fig. **3**) or the cell-penetrating tetrapepride SS-31
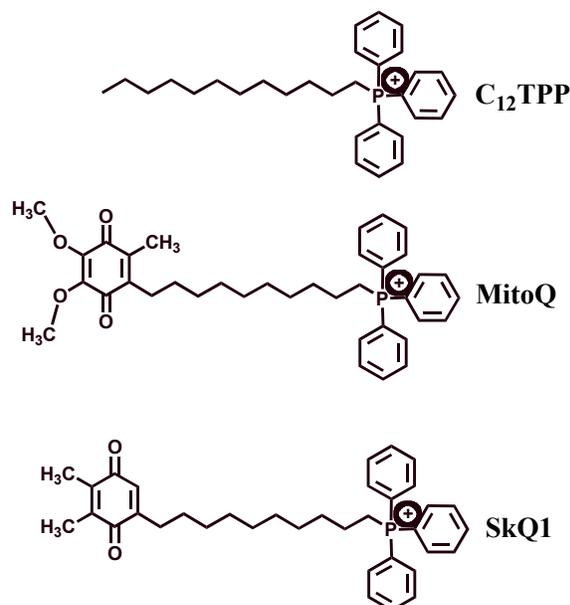
Fig. (3). Chemical structures of the lipophilic cations triphenyl-phosphonium (TPP), MitoQ and SkQ1. MitoQ and SkQ1 are shown in the oxidized (quinone) forms.

(also known as elamipretide, MTP-131). Below, we review the most important preclinical data and clinical trials with MitoQ and SkQ1 (Table **1**).

Development of new mitochondria-targeted antioxidants is an urgent task in modern pharmacology. Antioxidant capacities of 'classical' antioxidants like glutathione, ascorbate or N-acetylcysteine may easily be evaluated using established assays to determine total antioxidant capacities both *in vitro* and *in vivo* [106]. Other types of antioxidant, (*e.g.*, MitoQ and SkQ1) have the ability to quench radical reactions inside mitochondria and to be subsequently recharged by ETC. There is no established method of quantifying the potency or potential of a new drug candidate of this type. We can suggest the following steps. The pro-oxidant and anti-oxidant activities of the drug candidate are determined in aqueous solutions, planar bilayer phospholipid membranes, isolated mitochondria and cell cultures. Measurement of lipid peroxidation is of particular importance in these systems [107]. The main assays on the isolated mitochondria include measurements of transmembrane mitochondrial potential, oxygen consumption, ATP synthesis, the ability of the drug to be reduced by ETC and overall OXPHOS efficiency. The *in vitro* assays include cell survival assays under oxidative stress, (*e.g.*, treatment with hydrogen peroxide). The MTCs with good characteristics proceed to further *in vivo* tests.

4.2. MitoQ and SkQ1

MitoQ and SkQ1 are the derivatives of ubiquinone and plastoquinone respectively (Fig. **3**) attached *via* the C_{10} hydrophobic linker to the TPP cation. Importantly, both compounds may not only be oxidized by ROS but also reduced afterwards by the mitochondrial ETC. This ability makes these antioxidants 'rechargeable' in contrast to many other mitochondria-targeted antioxidants [108].

SkQ1 has lower pro-oxidant activity than MitoQ [100]. Thus, SkQ1 exerts antioxidant effects at very low concentrations. The range of working 'antioxidant' activity of SkQ1 is also larger than that of MitoQ, providing a potential advantage for clinical SkQ1 applications [100].

Interestingly, SkQ1 also possesses the ability to bind to mitochondrial cardiolipin and prevent its oxidation, just as the penetrating peptide SS-31 does [109]. MitoQ also binds to the cardiolipin, though with lower affinity than SkQ1 [109]. Nevertheless, unlike SS-31, MitoQ and SkQ1 do rely on ΔΨm to penetrate mitochondria.

Another important feature of both MitoQ and SkQ1 is the uncoupling activity. It was shown that TPP-based cations may be paired with fatty acids and these pairs cycle across the IMM increasing the transmembrane proton-conducting activity [110]. This feature may be used to treat a variety of conditions associated with mitochondrial hyperpolarization.

There are many other data concerning the *in vitro* properties of MitoQ and SkQ1 (see a recent review [111]). The main properties are decreased ROS levels, prevention of lipid peroxidation, decreased protein oxidation and prevention of cell apoptosis and necrosis. It should be mentioned that these compounds are active at nanomolar concentrations [111].

Mitochondria-targeted TPP-based compounds are inevitably lipophilic. SkQ1 and MitoQ have octanol-PBS partition coefficients of 13, 000:1 and 3, 000:1 respectively [100, 112]. It should be noted that TPP-based compounds are 'membranophilic' rather than lipophilic, since they tend to accumulate at the layer between water and octanol and at the lipid bilayer membranes. This may produce biased results in different experimental settings. The water solubility of these compounds was determined as ~ 1.1 μM. At higher concentrations they start to form micelles [100]. Orally consumed SkQ1 and MitoQ are subjected to first-pass metabolism in the liver and intestinal wall [113], thus reducing their quantities. The oral bioavailability of MitoQ was determined as ~ 10%, and the major metabolites in urine are glucuronides and sulfates of the reduced hydroquinone form, along with demethylated compounds [114]. Nevertheless, there is a possibility that cardiac, hepatic and/or renal failure may strongly affect the pharmacokinetics and the *in vivo* effectiveness of mitochondria-targeted lipophilic drugs.

Significant doses of TPP-based compounds could be fed safely to mice over long periods, coming to steady-state distributions within the heart, brain, liver and muscle [115]. Animal studies with tritiated compounds indicate that the half-life of TPP-based antioxidants is about 1.5 days [115]. In phase I trials, MitoQ showed satisfactory pharmacokinetic behavior with oral dosing at 80 mg (1 mg/kg), resulting in a plasma Cmax of ~ 33 ng/ml and Tmax of ~ 1 hour (h) [114].

MitoQ and SkQ1 have both shown many beneficial features in animal models, including neuroprotection and protection against ischemia-reperfusion injury, kidney diseases, metabolic syndrome and obesity, wound healing, SIRS, cardiovascular pathologies, eye diseases, arthritis and many age-related pathologies (reviewed in [111, 116-118]). Importantly, these compounds also exhibited anti-aging effects.

Table 1.   **Mitochondria-targeted compounds used in clinical studies.**

Molecule Name (Type)	Targeted Disease	Study Title	Clinical Trial Identifier[1]	Status	Main Outcome(s) / References
MitoQ	Multiple Sclerosis Fatigue	MitoQ for Fatigue in Multiple Sclerosis	NCT03166800	Recruiting	ND
MitoQ	Aging	The Efficacy of Oral Mitoquinone (MitoQ) Supplementation for Improving Physiological in Middle-aged and Older Adults	NCT02597023	Completed	Improved vascular function [102]
MitoQ	Parkinson's Disease	A Trial of MitoQ for the Treatment of People with Parkinson's Disease	NCT00329056	Completed	No effect on the PD progression [103]
MitoQ	Diastolic Dysfunction	MitoQ Supplementation and Cardiovascular Function in Healthy Men and Women	NCT03586414	Recruiting	ND
MitoQ	Chronic Hepatitis C	Trial of MitoQ for Raised Liver Enzymes Due to Hepatitis C	NCT00433108	Completed	Significantly decreased plasma alanine and aspartate aminotrans-ferase [104]
MitoQ	Peripheral Arterial Disease	Impacts of Mitochondrial-Targeted Antioxidant on Peripheral Artery Disease Patients	NCT03506633	Not yet recruiting	ND
MitoQ	Non-Alcoholic Fatty Liver Disease	A Study to Compare MitoQ and Placebo to Treat Non-Alcoholic Fatty Liver Disease (NAFLD)	NCT01167088	Terminated	Terminated due to poor participant recruitment
MitoQ	Alzheimer's Disease, Early Onset	Effects of Mitochondrial-Targeted Antioxidant on Alzheimer's Disease	NCT03514875	Not yet recruiting	ND
MitoQ	Chronic Kidney Disease	Mitochondrial Oxidative Stress and Vascular Health in Chronic Kidney Disease	NCT02364648	Recruiting	ND
MitoQ	Chronic Obstructive Pulmonary Disease, Pulmonary Artery Hypertension, Heart Failure, Hypertension	Vascular Function in Health and Disease	NCT02966665	Recruiting	ND
MitoQ	Hypogonadism	Cardiovascular Outcomes of Low Testosterone	NCT02758431	Recruiting	ND
SkQ1	Keratoconjunctivitis Sicca	A Clinical Study to Assess the Safety and Efficacy of an Ophthalmic Solution (SkQ1) in the Treatment of Dry Eye Syndrome	NCT02121301	Completed	Significant improvements in dry eye signs and symptoms [105]

[1] - as indicated at www.clinicaltrials.gov.

For example, SkQ1 greatly improved the health and life span of the PolG 'mutator' mice [119] and MitoQ extended the life span of a transgenic *C. elegans* model of Alzheimer's disease [120].

Based on impressive results, clinical trials began a decade ago (Table **1**). Below, we review the results of clinical trials and the possible molecular mechanisms underlying the observed effects.

MitoQ is now available as a dietary supplement that is well tolerated and safety-approved when administered orally for three weeks to healthy young adults without adverse effects [121].

Another double-blinded and placebo-controlled study tested the efficacy of MitoQ against impaired vascular function in healthy older adults aged 60-79. After six weeks of

administration of MitoQ, these individuals had improved endothelial function at ~ 42%, measured as brachial artery flow-mediated dilation. They also had reduced levels of plasma oxidized low-density lipoproteins, a marker of oxidative stress [102]. These results confirmed previously obtained preclinical data showing that four weeks of oral supplementation of MitoQ in old mice completely restored endothelium-dependent dilation by improving nitric oxide (NO) bioavailability [122]. Endothelial dysfunction is thought to be caused by reduced NO bioavailability due to increased oxidative stress [123, 124]. The mitochondria-derived superoxide anion reacts with NO to form peroxynitrite, which in turn can irreversibly damage tetrahydrobiopterin (a cofactor for endothelial NO synthase). Thus, NO synthase produces more additional superoxide, rather than NO [123]. SkQ1 also showed angioprotective and anti-

inflammatory activity, lowering expression of NO synthase and interleukin-6 in lipopolysaccharide-treated mice [125].

SkQ1 decreased age-related heart hypertrophy in mice [126] and also prevented age-related inflammatory activation of endothelium in the aortas of old mice *via* NF-κB-dependent reduced expression of cell adhesion molecules (ICAM-1) [26, 127]. Probably, the complex interplay between mtROS and endothelium influences many signaling pathways that require further investigation.

MitoQ demonstrated promising results in a cell model of Parkinson's disease [128]. However, in human clinical trials, MitoQ had no effect on the PD progression [103]. Probably, lack of MitoQ efficacy could be due to insufficient brain penetration or the severity of neuronal damage and dopaminergic deficiency at the time the treatment was applied [103].

Hepatitis C virus (HCV) infection induces mitochondrial oxidative stress leading to cell death and tissue fibrosis in liver [129]. Randomized placebo-controlled clinical trials of MitoQ in patients with chronic HCV infection did not result in a decrease of viral load but significantly decreased plasma alanine transaminase and aspartate aminotransferase levels, thus indicating reduced liver damage [104]. In agreement with these results, both MitoQ and SkQ1 also protected against liver damage in various animal models [130, 131].

Early animal studies of SkQ1 revealed its high efficacy against a wide range of eye diseases, such as dry eye syndrome, retinopathy, glaucoma, uveitis and conjunctivitis [100, 132-134]. The first human clinical trials of the SkQ1-based Visomitin eye drops for treatment of dry eye syndrome were successfully carried out in 2011. Visomitin greatly reduced hyperemia and edema of the conjunctiva and healed corneal microerosions [135]. In another international, multicenter, randomized, double-masked, placebo-controlled clinical study of Visomitin, the eye drops increased tear film stability and reduced corneal damage in patients with dry eye syndrome [136]. Phase 2 US clinical trials of Visomitin eye drops for safety and efficacy confirmed significant improvements of the signs and symptoms of dry eye [105]. The underlying mechanism of SkQ1 therapeutic action is likely to involve protection of the cardiolipin against oxidation [100]. In a rabbit model of dry eye disease, SkQ1 also stimulated the activity of the anti-oxidant enzymes (glutathione peroxidase and glutathione reductase) and lowered pro-inflammatory cytokine production in tears [137]. Nevertheless, most of the involved signaling pathways affected by SkQ1 probably remain unstudied.

CONCLUSION

Mitochondria are crucial cellular organelles, and mitochondrial dysfunction, accompanied by excessive mtROS production, decreased ATP synthesis and increased cell death, plays an important role in the pathogenesis of many diseases. Most of the prominent results in the treatment of these diseases were obtained with the lipophilic antioxidant cations MitoQ and SkQ1, or the penetrating peptide SS-31. These antioxidants were also investigated in multiple clinical trials completed up to phase 2. Further studies are needed to elucidate the signaling pathways influenced by these compounds, including their long-term effects on gene expression,

proteomics, metabolomics and epigenetics. Such studies will also help in understanding the pathophysiology of mitochondria-related diseases.

The prospects for new drug discoveries for treating mitochondrial dysfunction will mostly depend on increasing the bioavailability of the membranophilic drugs. Unfortunately, current pharmaceutical technologies do not offer a ready solution, though the use of microemulsions seems to be very promising [138]. Another problem is that existing MTCs are not targeted to certain organs or tissues and their distribution in the body relies only on their physico-chemical properties. There is a need for the combination of both first- and second-order drug targeting with MTCs.

LIST OF ABBREVIATIONS

AD	=	Alzheimer's Disease
ALS	=	Amyotrophic Lateral Sclerosis
Aβ	=	Amyloid β Peptide
ATP	=	Adenosine Triphosphate
DAMPs	=	Damage-associated Molecular Patterns
DQA	=	Dequalinium
FADH$_2$	=	Flavin Adenine Dinucleotide
HCV	=	Hepatitis C Virus
ETC	=	Electron Transport Chain
ICAM-1	=	Intercellular Adhesion Molecule 1
IMM	=	Inner Mitochondrial Membrane
MPT	=	Mitochondrial Permeability Transition
MTC	=	Mitochondria-targeted Compound
mtDNA	=	Mitochondrial DNA
mtROS	=	Mitochondrial Reactive Oxygen Species
NADH	=	Nicotinamide Adenine Dinucleotide
NO	=	Nitric Oxide
OMM	=	Outer Mitochondrial Membrane
OXPHOS	=	Oxidative Phosphorylation
PAMPs	=	Pathogen-associated Molecular Patterns
PD	=	Parkinson's Disease
ROS	=	Reactive Oxygen Species
SIRS	=	Systemic Inflammatory Response Syndrome
SOD	=	Superoxide Dismutase
T2DM	=	Type 2 Diabetes Mellitus
TCA	=	Tricarboxylic Acid Cycle
TPP	=	Triphenylphosphonium Cation
UCP1	=	Uncoupling Protein 1
ΔΨm	=	Transmembrane Potential

CONSENT FOR PUBLICATION

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The authors declare no conflict of interest, financial or otherwise.

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