Review

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Feature Review

Mitochondrial pharmacology

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Mitochondria are being recognized as key factors in many unexpected areas of biomedical science. In addition to their well-known roles in oxidative phosphorylation and metabolism, it is now clear that mitochondria are also central to cell death, neoplasia, cell differentiation, the innate immune system, oxygen and hypoxia sensing, and calcium metabolism. Disruption to these processes contributes to a range of human pathologies, making mitochondria a potentially important, but currently seemingly neglected, therapeutic target. Mitochondrial dysfunction is often associated with oxidative damage, calcium dyshomeostasis, defective ATP synthesis, or induction of the permeability transition pore. Consequently, therapies designed to prevent these types of damage are beneficial and can be used to treat many diverse and apparently unrelated indications. Here we outline the biological properties that make mitochondria important determinants of health and disease, and describe the pharmacological strategies being developed to address mitochondrial dysfunction.

The many roles of mitochondria

Mitochondria contribute to much of core human metabolism, including oxidative phosphorylation, the tricarboxylic acid (TCA) cycle, fatty acid oxidation, iron sulfur center and heme biosynthesis, and amino acid metabolism [1–4] (Box 1, Figure 1). Mitochondria are also central to apoptotic cell death and modulate calcium fluxes throughout the cell [1,5]. Superoxide is a reactive oxygen species (ROS) produced by mitochondria, and it underlies redox signaling in hypoxia sensing, cell differentiation and innate immunity [6–8].

Mitochondrial function depends on their assembly, maintenance and dynamics. Mitochondria have their own DNA (mtDNA) that encodes 37 genes necessary for assembly of the oxidative phosphorylation machinery [1]; however, most of the ~1500 other mitochondrial proteins are encoded by nuclear genes, translated in the cytoplasm and then imported into mitochondria [9] (Figure 1). This dual origin of mitochondrial proteins requires coordination of the nuclear and mitochondrial genomes, and also many other cofactors, metals and phospholipids have to be imported into mitochondria. The organelles move around the cell coordinated by the cytoskeleton, and also continually undergo fission and fusion, which is intimately linked to apoptosis, and the removal of dysfunctional mitochondria [10,11]. Damaged macromolecules within mitochondria are degraded by specialized intramitochondrial enzymes, and irreparably disrupted mitochondria are themselves degraded by generalized autophagy or more targeted mitophagy [10–12]. Mitochondrial biogenesis and turnover is regulated and coordinated by multiple transcription factors and transcriptional coactivators, notably peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), that enable mitochondria to respond to long-term alterations in metabolic demand [13]. Mitochondrial function is integrated closely into that of the rest of the cell; for example, mitochondria are preferentially located in areas of high local ATP demand. The short-term regulation of electron supply to the respiratory chain occurs by feedback pathways to upstream dehydrogenases that also respond to hormones, growth factors and neuronal stimulation via kinase cascades and changes in calcium concentration [5]. Mitochondrial oxidative phosphorylation is particularly sensitive to ATP demand through respiratory control, the rapid stimulation of respiratory chain activity in response to the lowering of the proton motive force caused by increased ATP synthesis [14] (Figure 1).

Disruption to mitochondrial assembly, turnover and function contributes to many disparate pathologies, raising the need for therapies [1,2,15,16]. Here we outline the issues and opportunities that arise in considering mitochondria as a therapeutic target for small molecule interventions. We focus on the general principles of mitochondrial pharmacology, illustrated with a few key examples that demonstrate the potential and the challenges of targeting mitochondria through pharmacological intervention.

Primary and secondary mitochondrial dysfunction

Mitochondrial disruption can be divided into two types: primary or secondary dysfunction. The primary category is characterized by a mutation to a gene encoded by mtDNA or a nuclear-encoded gene for a mitochondrial protein, or from a mitochondrial toxin. An example is Mitochondrial Encephalopathy Lactic Acidosis and Stroke-like episodes (MELAS) which is due to a mutation at np 3243 in the mitochondrial $tRNA^{leu(UUR)}$ gene that leads to the defective assembly of oxidative phosphorylation complexes and consequent defects in energy metabolism in neuromuscular systems [17]. Mutations to nuclear genes encoding mitochondrial proteins also lead to a wide range of primary

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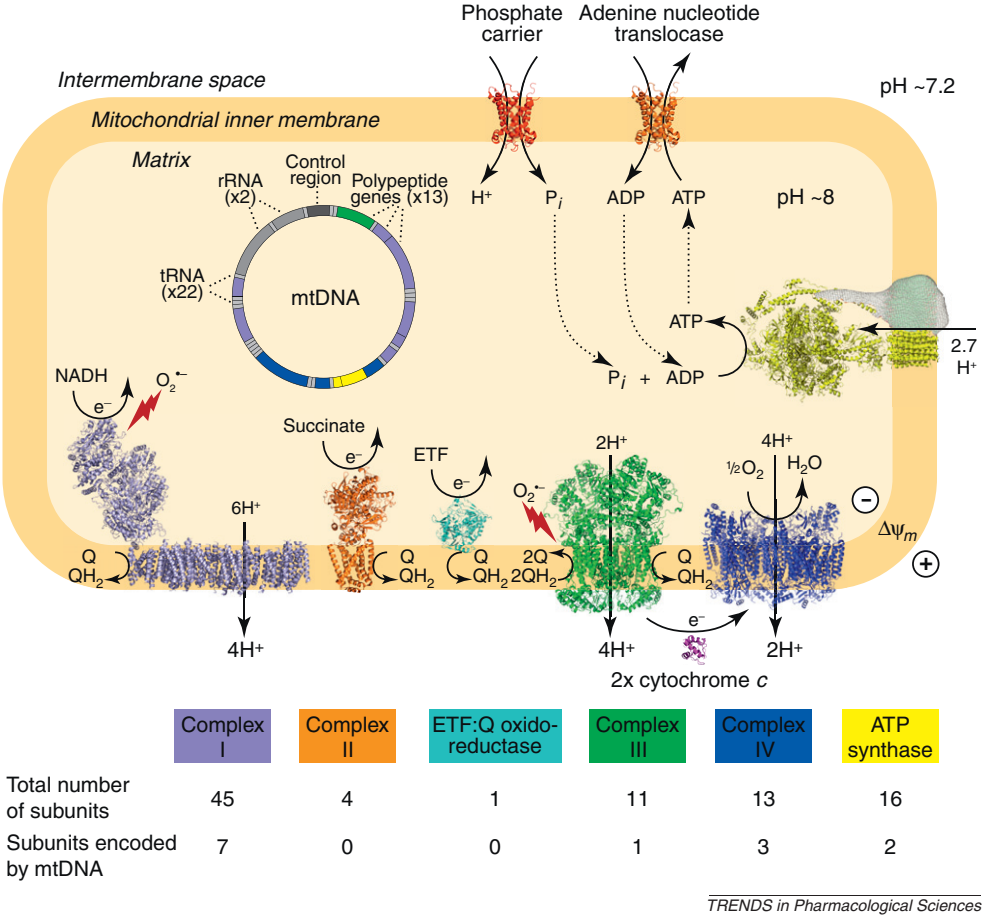
Box 1. The mitochondrial respiratory chain and oxidative phosphorylation

The central role of mitochondria is the synthesis of ATP by oxidative phosphorylation. This is carried out by a series of five large, multi-subunit complexes (I–V) illustrated in Figure I below. Oxidative Phosphorylation is made up of from 4 to 45 polypeptides that are embedded in the mitochondrial inner membrane. All these complexes except complex II are composed of subunits encoded by both the mitochondrial and nuclear genomes, the latter subsequently imported into mitochondria. The respiratory chain (complexes I–IV) channels electrons derived from food to oxygen, using the energy released to make ATP. For this various transporters exist in the mitochondrial inner membrane that transfer carbohydrates and fatty acids into the mitochondrial matrix for the first stage of their oxidation. Electrons from carbohydrates oxidized by the tricarboxylic acid (TCA) cycle and from fatty acids broken down by β-oxidation accumulate on the reduced electron carrier NADH. This NADH is oxidized to NAD^+ at complex I (NADH:ubiquinone oxidoreductase) and the electrons are passed to the CoEnzyme Q (CoQ) pool, a lipophilic electron carrier existing as oxidized ubiquinone (Q) and reduced ubiquinol (QH_2). The energy released at complex I is used to pump protons across the mitochondrial inner membrane. Electrons from the TCA cycle are also passed via succinate to the CoQ pool through complex II (succinate:ubiquinone oxidoreductase), which does not pump protons. Similarly, β-oxidation also leads to the accumulation of electrons on flavoproteins that are also shuttled to the inner membrane by electron transfer flavoprotein (ETF), which then passes the electrons to the CoQ pool by the action of electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:Q oxidoreductase). The electrons in the CoQ pool are then passed through complex III (ubiquinol:cytochrome *c* oxidoreductase) to cytochrome *c* and from cytochrome *c* the electrons are then finally used to reduce oxygen to water at complex IV (cytochrome *c* oxidase). At both complexes III and IV the redox energy is used to pump protons and charge across the inner membrane. The proton motive force generated across the inner membrane comprises a membrane potential ($\Delta\psi_m$) of up to approximately 160 mV and a pH gradient of approximately half a pH unit, equivalent to ~30 mV of proton motive force. The high proton motive force is used to make ATP from ADP and phosphate (P_i) by the flow of protons back through the F_oF_1–ATP synthase (complex V). The ATP is then exported from the mitochondrion to the cytoplasm in exchange for cytoplasmic ADP by the adenine nucleotide translocase (ANT), whereas the phosphate is replaced by transport from the cytoplasm via the phosphate carrier. The image is derived from two previous diagrams developed by Professor John E. Walker and Dr Martin King. Images of 3D structures and electron density maps are based on an image from Professor John E. Walker and were generated using PyMOL (DeLano, www.pymol.org) by Dr Martin King. Complex I is the low resolution structure of the enzyme from *Thermus thermophilus* (PDB accession code: 3M9S) to 4.5 Å [81]. Complexes III (PDB: 1BE3) and IV (PDB: 1OCC) are the high resolution structures of the bovine enzymes [82,83]; complex II (PDB: 1ZOY) is the porcine enzyme [84]. Cytochrome *c* (PDB: 1CXA) is from *Rhodobacter sphaeroides* [85]. ATP synthase (PDB: 2CLY) is a model from Professor John E. Walker's group [86]. The mitochondrial ANT (PDB: 1OKC) in complex with carboxyatractyloside is also shown [87].



	Complex I	Complex II	ETF:Q oxido-reductase	Complex III	Complex IV	ATP synthase
Total number of subunits	45	4	1	11	13	16
Subunits encoded by mtDNA	7	0	0	1	3	2

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Figure I.

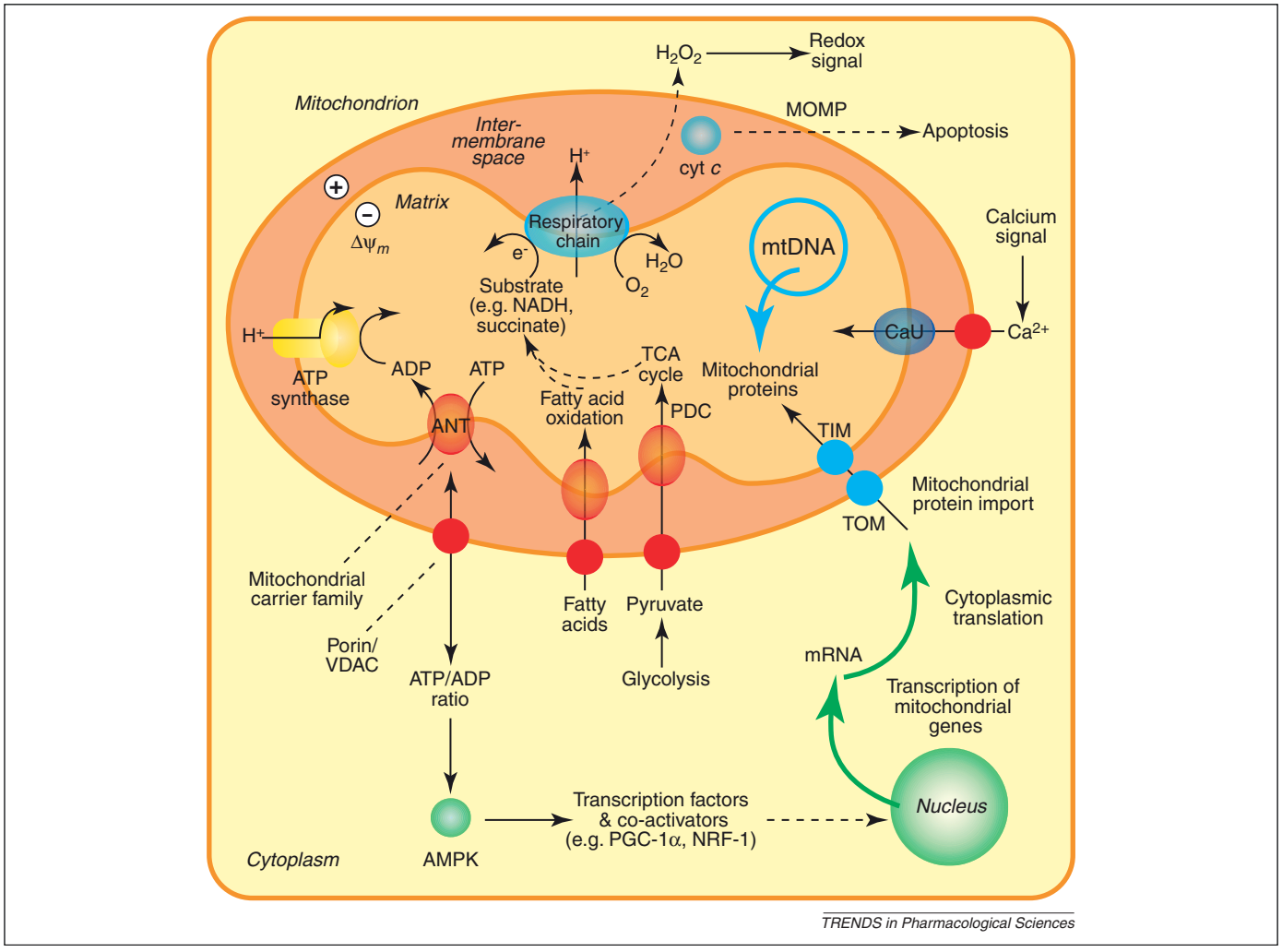

Figure 1. Mitochondrial function and biogenesis. Some of the many roles of mitochondria in cell function and aspects of mitochondrial biogenesis are illustrated. A major role for mitochondria is the production of ATP through oxidative phosphorylation. Initially, glucose is broken down to pyruvate by glycolysis in the cytosol. Porin channels, also known as voltage-dependent anion channels (VDACs), enable small molecules to pass through the outer membrane. Activation of AMP-dependent kinase (AMPK) allows the cell to respond to a low cytosolic ATP/ADP ratio through changes in AMP, and acts on various targets, such as the transcriptional coactivator PGC-1α. Mitochondrial DNA (mtDNA) encodes 37 genes that are involved in the synthesis of the respiratory chain and the ATP synthase. Additional proteins are imported through TIM and TOM, translocases of the inner and outer membranes that transport nuclear-encoded proteins into mitochondria. The adenine nucleotide translocase (ANT) enables the mitochondrion to import ADP and export ATP. Mitochondria also contribute to calcium signaling by taking up calcium into the mitochondrial matrix through the calcium uniporter (CaU) in response to changes in cytosolic calcium. In addition, mitochondria play a crucial role in apoptosis. When apoptotic signals occur, the outer membrane becomes compromised and the mitochondrion experiences mitochondrial outer membrane permeabilization (MOMP), leading to the release of cytochrome *c* (cyt *c*) and many other pro-apoptotic proteins (not shown) from the intermembrane space into the cytosol where they activate apoptotic cell death.

mitochondrial defects [18–20] such as a neonatal defect in energy metabolism due to mutation of a nuclear gene that encodes NDUFAF3, an assembly factor for respiratory complex I [21,22]. In addition, mutations to nuclear-encoded mitochondrial genes can disrupt many aspects of mitochondria other than oxidative phosphorylation including assembly, dynamics and metabolic function [18–20].

By contrast, secondary mitochondrial dysfunction is caused by pathological events that originate outside mitochondria. For example, in ischemia/reperfusion (I/R) injury, the initiating event is the interruption and subsequent reflow of blood supply to the tissue, which leads to extensive secondary mitochondrial disruption and consequent tissue damage [16,23]. Other disorders in which secondary mitochondrial damage plays a significant role include sepsis, neurodegeneration, metabolic syndrome, organ transplantation, cancer, autoimmune diseases and diabetes [1,24]. Consequently, mitochondria are an important node for therapeutic intervention, even if damage to the actual organelle is not the initial pathological event [24].

Diseases due to primary mitochondrial dysfunction are generally thought of as rare. However, with improved diagnosis it is now evident that mtDNA mutation can cause disease in as many as 1 in 5000 of the general population [25]. The cumulative prevalence of disorders due to primary mitochondrial dysfunction is not known, in part because of the diversity in their clinical presentations, and many more of these are likely to be discovered [18–20]. By contrast, many of the disorders that involve secondary mitochondrial dysfunction, including cardiac damage in I/R injury, metabolic syndrome, diabetic complications and neurodegenerative diseases, are among the most significant disorders of developed societies. Thus, there is an unmet need to treat mitochondrial dysfunction in both primary and secondary pathologies, with the treatment

of secondary mitochondrial disorders having the potential to impact on many significant and common conditions.

Correcting primary mitochondrial disorders is particularly challenging. One exception is coenzyme Q (CoQ) deficiency due to a defect in CoQ biosynthesis, in which supplying dietary CoQ ameliorates the disease [26]. However, in most cases, an effective treatment or cure is likely to require replacement or suppression of the defective gene, which may be feasible for nuclear genes, but the prospect of effective gene therapies for mtDNA diseases remains distant [27]. Thus, most pharmacological interventions in this area aim to ameliorate the consequences of the primary defect [26] rather than address the cause of the malfunction. The therapeutic situation is different for the many diseases involving secondary mitochondrial dysfunction, where treatments are not designed to affect mitochondria directly. This may give a disheartening picture, suggesting that mitochondria are an unpromising therapeutic target as distinct pharmaceuticals would be required for each disease. Fortunately this is not the case, because there are common patterns of cell disruption in both primary and secondary mitochondrial diseases, despite their disparate causes. Mitochondrial pharmacology is feasible because therapies that impact on a few common damaging pathways can treat patients with a wide range of primary and secondary mitochondrial disorders.

There are three aspects of mitochondrial damage that commonly contribute to primary and secondary mitochondrial pathologies: oxidative damage, calcium dyshomeostasis and disruption to ATP synthesis (Figure 2). The mitochondrial respiratory chain (Box 1) is a major source of superoxide that, in turn, forms hydrogen peroxide and other damaging ROS [6]. Superoxide production increases in many pathological scenarios, and mitochondria are particularly vulnerable to oxidative damage because the organelle contains several iron sulfur centers, a large expanse of inner membrane containing unsaturated fatty acids and densely packed proteins and mtDNA molecules that are essential to mitochondrial function, all of which are susceptible to reaction with ROS derived from superoxide. Oxidative damage to mitochondria disrupts the function of the organelle making cell death more probable, thereby contributing to diverse pathologies such as sepsis, organ deterioration in transplantation, I/R injury, diabetic complications and also neurodegenerative diseases [1,6]. Mitochondrial ATP synthesis is frequently disrupted by damage to the respiratory chain, the inner membrane or the ATP synthesis machinery, thereby contributing to cell

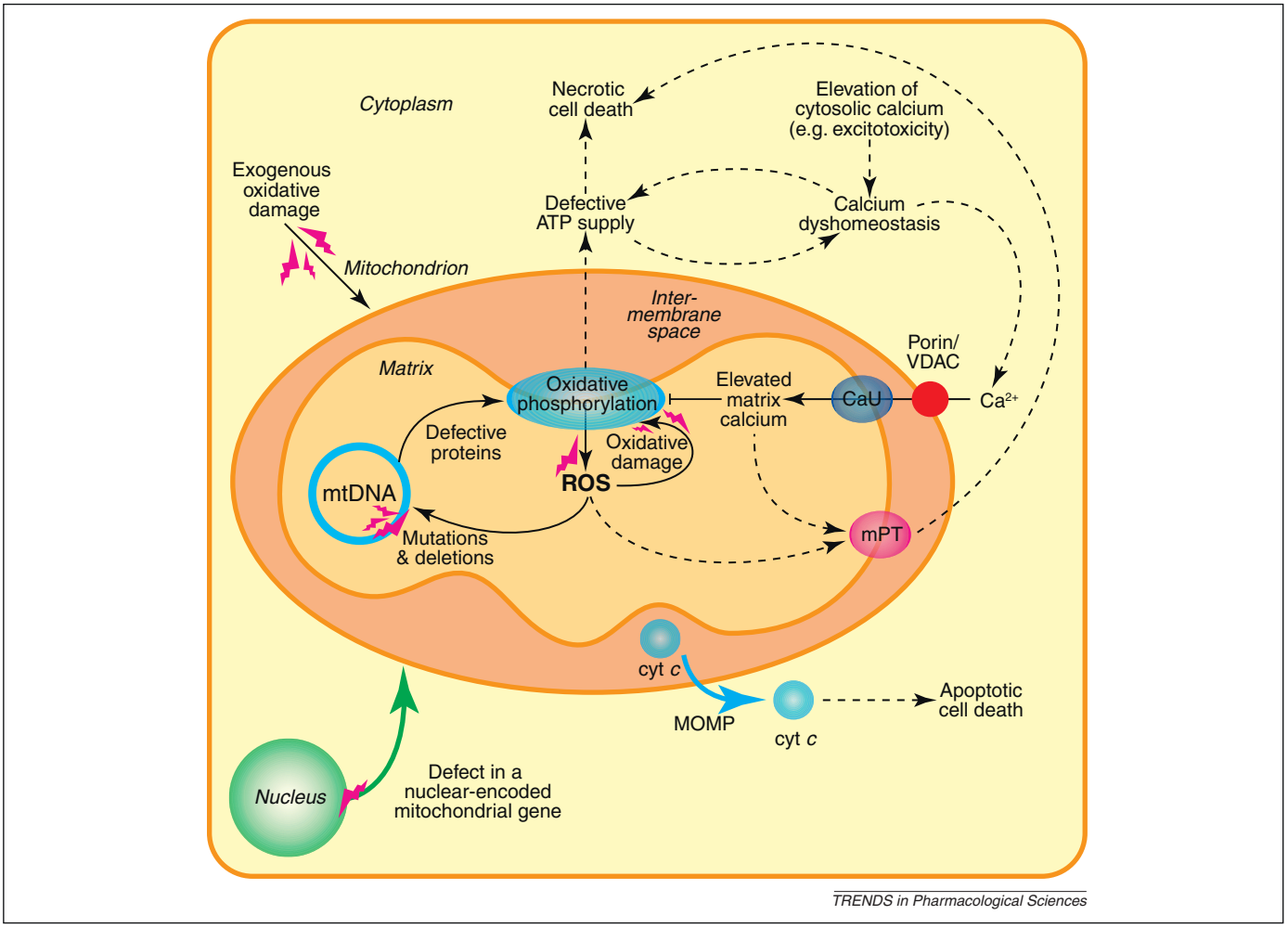

Figure 2. Mitochondrial dysfunction. Disruption to mitochondrial function can be caused by primary events, such as mutation to mitochondrial or nuclear genes. Secondary mitochondrial dysfunction arises due to causes outside the mitochondrion. There are often common factors to mitochondrial dysfunction such as increased oxidative stress, disruption to calcium homeostasis and defective mitochondrial ATP synthesis. Frequently, these occur together or lead into each other. The combination of elevated mitochondrial matrix calcium and oxidative stress leads to induction of the mitochondrial permeability transition pore (mPT), which further disrupts mitochondrial function.

death and dysfunction [1]. Defective mitochondrial ATP supply also leads to calcium dyshomeostasis by disrupting calcium ATPase activity in the endoplasmic/sarcoplasmic reticulum and in the plasma membrane, allowing cytosolic calcium levels to rise above the normal signaling range (1–2 μM) [5]. The uptake of calcium into mitochondria through the calcium uniporter is responsive to increases in cytosolic calcium, perhaps to protect against transient increase in cellular calcium, but sustained calcium elevation leads to chronic, damaging calcium accumulation within mitochondria. Oxidative damage, defective ATP synthesis and calcium dyshomeostasis frequently occur together, and as each type of damage leads to the other two, then a vicious cycle is established (Figure 2). Finally, mitochondrial oxidative damage, ATP depletion and calcium overload together induce the mitochondrial permeability transition (mPT) [28]. This phenomenon arises due to the formation of an inner membrane pore that causes swelling and disruption to mitochondrial function [28]. The physiological role of the mPT and the composition of the pore are uncertain. However, it is clear that it contributes to many pathologies such as I/R injury, and that its formation requires activity of cyclophilin D, a matrix peptidyl prolyl *cis-trans* isomerase [28].

This toxic constellation of oxidative damage, calcium dyshomeostasis, disrupted ATP synthesis and induction of the mPT occurs in many primary and secondary mitochondrial disorders. Consequently, therapeutic interventions to ameliorate these processes are applicable to many different indications. This is contrary to the usual drug development model where a well-defined protein target that is linked to a single indication can be modulated precisely by a selectively bound small molecule. Our view is that successfully intervening therapeutically in general damaging processes is essential for mitochondrial pharmacology to reach its full potential.

Strategies for mitochondrial pharmacology

There are three strategies for mitochondrial pharmacology (Figure 3) [4,15]. The first is to make molecules that selectively accumulate within mitochondria. The second is to use molecules that bind targets within mitochondria which rely on the target's location to effect specificity. The final approach is to modulate processes outside mitochondria that ultimately alter mitochondrial function.

Targeting bioactive molecules to mitochondria

Lipophilic cations and mitochondria-targeted peptides have both been developed to target drugs and bioactive molecules to mitochondria *in vivo* (Figure 3) [4,29]. These strategies lead to a dramatically higher concentration of the targeted compound within mitochondria, greatly increasing potency and enabling less of the compound to be used, thus minimizing the extramitochondrial metabolism that can lead to inactivation, excretion or toxic side effects. These delivery strategies also enable molecules that are poorly taken up by mitochondria for various reasons (e.g. hydrophobicity) to be directed to mitochondria *in vivo*. One limitation is that these procedures typically involve chemicals which tend to localize to the mitochondrial matrix and the matrix-facing surface of the inner membrane. There are many important processes that take place on the outer surface of the inner membrane, the intermembrane space and the outer membrane of mitochondria, but, as yet, there are no generic strategies to target these compartments. Another limitation is that currently these approaches are not organ-specific and the compounds generally accumulate preferentially in tissues with high mitochondrial content.

Lipophilic cations such as triphenylphosphonium (TPP) derivatives are rapidly and extensively taken up by mitochondria *in vivo* driven by the large mitochondrial membrane potential [$\Delta\psi_m$ (negative inside)] [4]. The mechanism of uptake is well understood and occurs by the movement of lipophilic cations through the plasma and mitochondrial inner membranes due to the extensive hydrophobic surface area and the large ionic radius of the cation that effectively lowers the activation energy for membrane passage. The Nernst equation adequately describes the membrane potential-dependent uptake of lipophilic cations, which increases 10-fold for every ~60 mV of $\Delta\psi_m$, leading to their several hundred-fold uptake within mitochondria *in vivo* [30,31] (Figure 3). The use of lipophilic cations to facilitate the delivery of attached 'cargo' within cells was first demonstrated with the lipophilic cation rhodamine 123 which forms a complex with the anticancer drug *cis*-platin [32]. Since then, the covalent attachment of the TPP lipophilic cation has become established as a generic and robust method to target small, bioactive and probe molecules to mitochondria *in vivo* [4].

Peptides can also be used to direct molecules to mitochondria, with the Szeto–Schiller (SS) peptides [29] and the mitochondrial-penetrating peptides (MPPs) [33] proving the most useful to date. Both classes of peptides comprise a mix of cationic and hydrophobic alkyl or aromatic amino acid residues that are taken up by mitochondria in cells and can be used to deliver attached cargoes [29,33]. The mechanism of peptide uptake by mitochondria is less clear than that for TPP species. Results obtained with MPPs suggest that charge and hydrophobicity determine accumulation in the mitochondrial matrix [34], although it is not yet established whether passage of MPPs through the phospholipid bilayer is unmediated and uptake into mitochondria is simply determined by the $\Delta\psi_m$. By contrast, the uptake of SS peptides is thought to be independent of $\Delta\psi_m$ and to rely on selective binding to the inner membrane; however, the details of how this occurs and the nature of the putative binding sites are unclear [29]. More work is required on the biophysical mechanism of uptake for the two types of peptide, although our view is that uptake is likely to be by the same general mechanism in each case.

The major therapeutic use of lipophilic cations and mitochondria-targeted peptides to date has been to deliver covalently attached, bioactive cargo to mitochondria [4,29]. This has proven to be a robust approach, and some of the molecules utilized are shown in Table 1. A variant of this approach is to design a molecule so that the targeting module is cleaved from the bioactive moiety within the mitochondria, releasing the active molecule in the mitochondrial matrix (Figure 3). Recent examples of this include the delivery of lipoic acid, temporarily attached to a

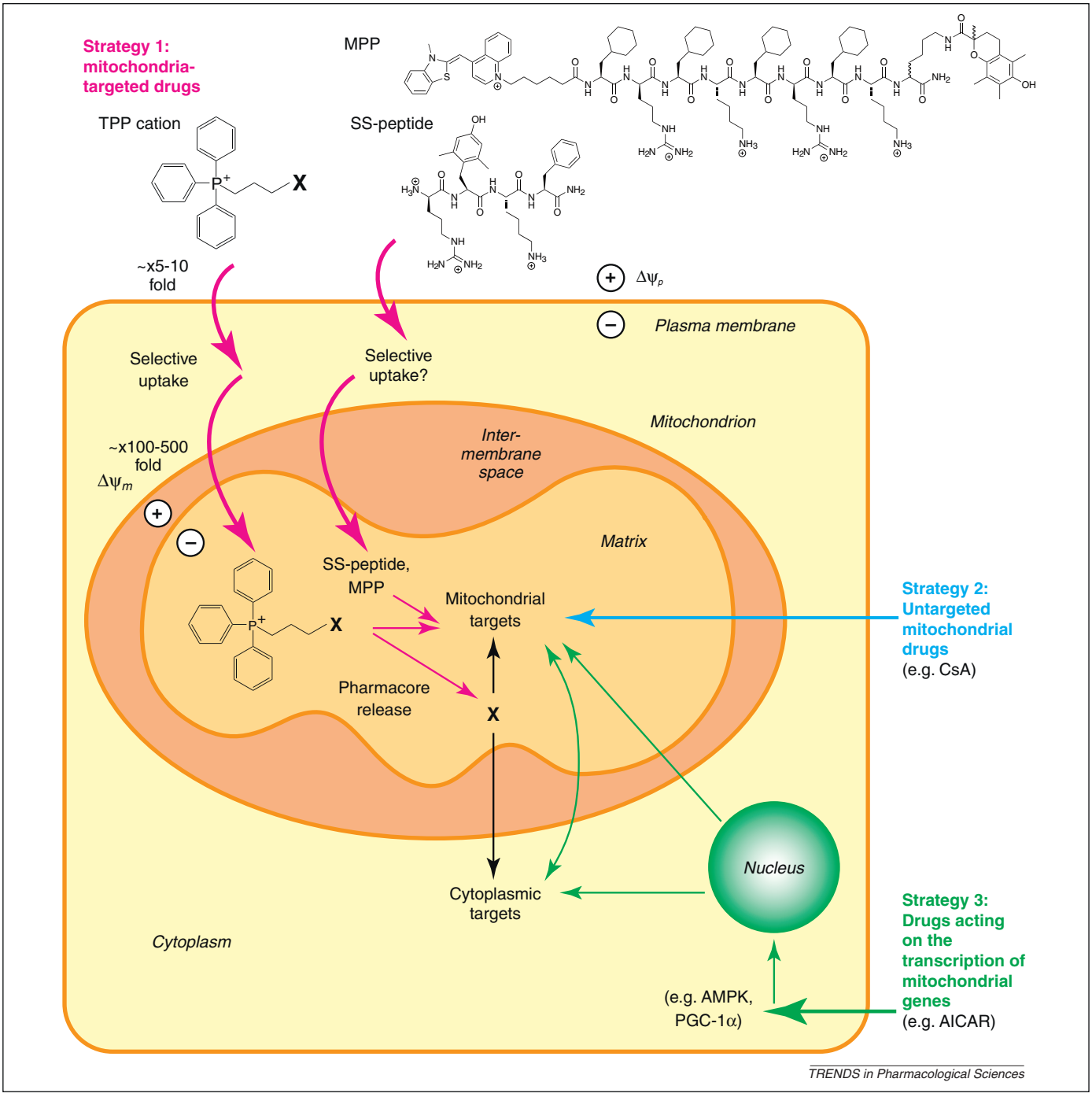

Figure 3. Three general strategies to intervene pharmacologically in mitochondrial dysfunction. The first is by targeting compounds to mitochondria. This can be done by conjugation to a lipophilic cation such as TPP leading to the selective uptake of the attached bioactive moiety or pharmacophore (X) into the mitochondrial matrix. Alternatively, peptides such as the SS or MPP peptides can also be used. These mitochondria-targeted compounds can act within the mitochondria, or the active pharmacophore can be released from the targeting moiety within the mitochondrion. Secondly, compounds which are not targeted to mitochondria but which act there by binding to specific targets can be used. Finally, many compounds can influence mitochondrial dysfunction by affecting processes outside mitochondria, such as the activity of kinases, transcription factors or transcriptional coactivators.

TPP by an enzyme-cleavable ester linkage [35], and the release of nitric oxide (NO) within mitochondria on reduction of MitoSNO [36]. A further manifestation of this approach is to use TPP connected to a bioactive moiety by a photocleavable linker that can be photolyzed *in situ*, thereby delivering the active agent within mitochondria in a particular tissue or cell. This has been demonstrated in cells for TPP connected to the uncoupler, 2,4 dinitrophenol (DNP) by a photolabile linker which releases DNP within mitochondria upon irradiation [37]. The mitochondrion can also be used as an intracellular reaction chamber, with two mitochondria-targeted compounds reacting together to form a novel product [38], or to generate a bioactive molecule within the mitochondria that can then diffuse out of the organelle to improve the efficacy or duration of its action on extramitochondrial targets [36] (Figure 3). Finally, mitochondria-targeting can also be used to enhance the therapeutic window of a known drug by sequestering active

Table 1. Selected mitochondria-targeted therapeutic compounds[a]

In vivo		
Compound	**Mode of action**	**Indications**
MitoQ	Antioxidant	Cardiac I/R injury [4,49]; toxin-induced parkinsonism [42]; endothelial nitroglycerin tolerance [4,49]; hypertension [4,49]; sepsis [4,49]; adriamycin toxicity [4,49]; kidney damage in type I diabetes [4,49]; kidney preservation *ex vivo* [44]; cocaine toxicity [4,49]; alcoholic fatty liver disease [88]; fatty liver disease [31,89]; liver inflammation in hepatitis C virus patients [41]
SS31	Antioxidant	I/R injury [29]; neuroprotection [29]; insulin resistance [29]; immobilization-induced muscle atrophy [29]; skeletal muscle burn injury [90]
MitoTempo	Antioxidant	Hypertension [91]
MitoSNO	*S*-Nitrosation	Cardiac IR injury [36,46]
TP187	Mitochondrial toxin	Cancer [50]
In vitro		
Compound	**Target**	
MitoCsA	Mitochondrial permeability transition [92]	
RevMitoLipoic acid	Cleavable delivery of lipoic acid within mitochondria [35]	
MitoPeroxidase	Catalytic peroxidase mimetic [93]	
MitoCP	Antioxidant [94]	
MitoVES	Anticancer toxin [95]	
MitoPorphyrin	Photodynamic therapy in cancer [96]	
MitoPhotoDNP	Light-activated delivery of uncoupler to mitochondria [37]	

[a]Only a representative sample of mitochondria-targeted compounds is listed here. See [4] for a more complete listing.

compound within the organelle to minimize toxicity at sites elsewhere in the cell [39].

Both of these mitochondria-targeting strategies have been shown to work *in vivo*. Extensive work on mitochondria-targeted TPP compounds has shown that they can be delivered to mitochondria *in vivo* following oral administration in drinking water [31] or by tablet [40,41], by intraperitoneal (IP) [42] or intravenous (IV) [30] injection, by eye drops [43] and into organs *ex vivo* by infusion [36,44]. TPP compounds have been given safely long-term to several rodent models [31,45]. Importantly, they have also been given orally to patients in Phase II studies for up to 1 year with no safety concerns [40,41]. TPP compounds can be delivered very rapidly to mitochondria within organs such as the heart within a few minutes following IV injection [30], enabling the rapid treatment of acute mitochondrial dysfunction [46]. The uptake of TPP compounds is not uniform across different organs, and although TPP compounds cross the blood–brain barrier (BBB) in sufficient amounts to protect the brain from degeneration in various disease models [42,45], the uptake is less than that for other organs [31]. Studies to date have shown that the metabolism of TPP compounds is mainly through reaction of the 'cargo' component, with the TPP function remaining unmodified, and the compounds being excreted in the bile and in the urine [47].

Experience with peptide delivery systems *in vivo* is less extensive, with work to date having only been carried out on the SS peptides [29]. It has been established that these can be safely delivered *in vivo* by subcutaneous, IV and IP administration [29] leading to tissue uptake. However, the uptake into mitochondria *in vivo* has been inferred indirectly from *in vitro* studies and from the observation of protection against mitochondrial damage.

The major therapeutic application of mitochondria-targeted therapies so far has been as antioxidants to block mitochondrial oxidative damage [4,29]. Conventional, untargeted antioxidants have had poor efficacy in clinical trials [48], and this is in part because they do not accumulate particularly in mitochondria – the site of much of the pathological oxidative damage. The observed several hundred-fold accumulation within mitochondria of the mitochondria-targeted antioxidants renders them far more effective in protecting against oxidative damage. The most extensively investigated mitochondria-targeted antioxidant to date has been the TPP-modified ubiquinone, MitoQ, which has shown efficacy in a wide range of animal models of disorders and has also been taken through to human studies [49] (Table 1). The unique properties of lipophilic cations mean that on oral or IV administration MitoQ is very rapidly taken up from the circulation into cells by direct movement through the plasma membrane, and this accumulation is driven by the plasma membrane potential [30]. Within the cytosol, the large $\Delta\psi_m$ leads to the further uptake of the MitoQ into the mitochondrial matrix, and the extent of this uptake can be several hundred-fold. Within the mitochondria, MitoQ is predominantly adsorbed to the matrix-facing surface of the inner membrane, facilitating its ability to protect the components of the membrane involved in oxidative phosphorylation. The ubiquinone form of MitoQ is rapidly reduced by respiratory complex II to the ubiquinol form, which is a very effective chain-breaking antioxidant that inhibits mitochondrial lipid peroxidation and directly reacts with oxidants such as peroxynitrite. In carrying out these reactions, the ubiquinol is converted to a ubisemiquinone radical that dismutates to a ubiquinol and a ubiquinone, which is then reduced back to a ubiquinol by the respiratory chain. The ubiquinone form of MitoQ can also

react directly with superoxide. It is the combination of rapid targeted and extensive uptake with antioxidant efficacy and rapid recycling back to the active form that makes MitoQ and related compounds uniquely effective antioxidants. A range of mitochondria-targeted antioxidants incorporating the TPP function have been developed including targeted versions of nitroxide, nitrones, plastoquinones and tocopherol, which have also shown efficacy in many animal models (Table 1). The SS peptide, SS31, which has an inherent antioxidant moiety, has also been shown to be effective in multiple animal models and is now being applied to human studies [29]. The broad efficacy of mitochondria-targeted antioxidants strongly supports the concept that mitochondrial oxidative damage constitutes an important therapeutic target.

Other types of mitochondria-targeted therapies have been developed. For example, the increase in $\Delta\psi_m$ in cancer cells has led to the development of various mitochondria-targeted toxins designed to accumulate in these mitochondria and thereby selectively kill cancer cells [50,51] (Table 1). The delivery strategies can also be used to target other bioactive components or pharmacophores to mitochondria (Table 1). Although several possibilities are being explored *in vitro*, only a few have established *in vivo* applications to date. A recent *in vivo* example is the mitochondria-targeted *S*-nitrosating agent MitoSNO [36], which induced the *S*-nitrosation of complex I that protects mitochondria and the heart *in vivo* from I/R injury [36,46].

Thus, the targeting to mitochondria of bioactives and pharmacophores is an established and robust procedure that can greatly expand options for designing compounds to treat mitochondrial damage in patients, and is a central platform for the future development of mitochondrial pharmacology.

Modulating druggable mitochondrial targets and processes

Mitochondria contain many targets and processes specific to the organelle that can be modulated by drugs and bioactive agents. Among these are agents that act as conventional drugs by binding to and affecting specific protein targets within mitochondria. A good example is cyclosporin A (CsA) which acts as an immunosuppressant and is also an effective inhibitor of cyclophilin D within mitochondria, thereby preventing the mPT [28]. The induction of the mPT in animal models of I/R injury is caused by elevated oxidative damage, ATP depletion and calcium dyshomeostasis, which in combination lead to the cyclophilin D-dependent induction of a pore in the mitochondrial inner membrane. Once formed, the pore causes mitochondrial swelling and disrupts ATP synthesis leading to the necrotic cell death that is a major factor in I/R injury. By binding to and inhibiting cyclophilin D, CsA can prevent the induction of the mPT. CsA is licensed as an immunosuppressant and it was utilized in a pilot human trial to test whether blocking the mPT could be therapeutic after myocardial infarction [52]. For this, CsA was administered to patients intravenously immediately before coronary angioplasty and a decrease in the area of infarcted tissue was noted in these cases [52]. CsA was also effective in two related degenerative syndromes called Bethlem myopathy and Ullrich congenital muscular dystrophy that are due to mutations in the collagen VI gene [53,54]. Surprisingly, mutations in this extracellular protein lead to increased mitochondrial dysfunction and cell death in mouse models, and these defects could be ameliorated with CsA, suggesting that induction of the mPT was central to the muscle cell death underlying the pathology [54–56]. These observations engendered an open-label pilot trial on five patients with collagen VI myopathies where oral treatment with CsA for a month significantly improved mitochondrial and cell function as assessed in muscle biopsies [57]. Inhibitors of cyclophilin D are a very promising example of how druggable targets in mitochondria can be modulated. Mitochondria-specific variants of CsA that do not bind to the cyclophilins found outside the mitochondria are being developed to avoid the immunosuppressant and toxic side effects of long-term CsA [58].

Another interesting potential mitochondrial target for pharmacological intervention is the intrinsic pathway of apoptotic cell death [51]. This can be accessed in several ways, but one promising target is the key step of mitochondrial outer membrane permeabilization (MOMP), during which rupture of the outer membrane releases proteins such as cytochrome *c* (cyt *c*) from the intermembrane space and thereby commits the cell to apoptosis [51]. Although the nature of MOMP itself is still unclear, it involves interplay between antiapoptotic proteins such as B Cell Lymphoma protein-2 (BCL-2) and related pro-apoptotic proteins such as BCL-2 homologous antagonist/killer (BAK) [51]. Activation of pro-apoptotic proteins, such as BAK, leads to the formation of a pore in the mitochondrial outer membrane and MOMP, which is counteracted through the sequestering of BAK by BCL-2. Small molecules that disrupt this interaction (e.g. ABT-737 [51]) make cells more susceptible to apoptosis and are thereby useful in killing cancer cells that have become resistant to apoptosis due to increased expression of BCL-2 [51].

Other promising mitochondrial targets for pharmacological intervention are the intimately linked processes of mitochondrial fission, fusion and autophagy that allow cells to respond to damage by either degrading damaged mitochondria or by apoptosis [10]. The accumulation of damaged and defective mitochondria decreases the pool of correctly functioning mitochondria and increases the likelihood of cell death through disrupted mitochondria releasing pro-apoptotic factors. This can occur with damaged mitochondria acting as ATP consumers by reversal of the ATP synthase and by increasing cellular oxidative stress. Enhancing the clearance of damaged mitochondria would allow the cell to replenish the pool of well-functioning mitochondria and thereby normalize ATP supply and calcium homeostasis. Therefore, the goal is to design small molecules to manipulate these processes to eliminate damaged mitochondria by upregulating autophagy. There are also compounds that interact with the mitochondrial fission and fusion machinery, such as Mitochondrial Division Inhibitor 1 (Mdivi-1) [59]. This compound inhibits dynamin related protein-1 (DRP-1) which is required for mitochondrial fission and fragmentation [59]. Inhibition of mitochondrial fragmentation by Mdivi-1 decreases MOMP and apoptotic cell death [59], as well as cell death after cardiac

Table 2. Potentially therapeutic compounds that affect mitochondria[a]

In vivo		
Compound	**Mode of action**	**Potential indications**
Cyclosporin A (CsA)	Inhibits mPT	I/R injury [52]; Bethlem myopathy and Ullrich congenital muscular dystrophy [53]
Dichloroacetate (DCA)	Activates pyruvate dehydrogenase complex	Improved cardiac function [97]
Idebenone	Bypasses blocks in complex I, antioxidant	Neurodegeneration and cardiomyopathy [98]
Methylene blue	Increases complex IV activity	Alzheimer's models [66]
2,4-Dinitrophenol (DNP)	Uncoupler	Obesity and elevated oxidative stress [61]
Bezafibrate	Pan PPAR agonist elevating PGC-1α expression	Increased mitochondrial biogenesis [70], but see [71]
AICAR	AMPK agonist to increased mitochondrial biogenesis [71]	Increases PGC-1α activity leading
CGP37157	Inhibits the mitochondrial Ca^{2+}/Na^{+} exchanger	Prevents mitochondrial calcium dyshomeostasis [99]
Mdivi-1	Inhibits dynamin related protein-1 (DRP-1) [59]	Impacts on MOMP, apoptotic cell death and cardiac I/R injury [60]

[a]Only a representative sample of compounds that interact with mitochondria in potentially therapeutic ways is listed here.

I/R injury [60], thus modulating mitochondrial fragmentation has therapeutic potential. There are many other putative drug targets within mitochondria that could be selectively modified by small molecules, and a selection of some of the most promising examples, chosen to illustrate the range of systems and approaches that can be used to affect mitochondrial function, is outlined in Table 2.

Several processes that are specific to mitochondria can be targeted in less conventional ways with potential therapeutic benefit. A good example is the proton leak through the mitochondrial inner membrane [61]. Increasing the rate of proton leak through the inner membrane has two potential benefits: mitochondrial respiration will be less efficient, thereby converting stored fat to heat [61], as occurs during thermogenesis in brown adipose tissue mitochondria through uncoupling protein 1; secondly, mild uncoupling may reduce oxidative stress by decreasing the level of the proton motive force and oxidizing electron carrier pools such as those of dihydronicotinamide adenine dinucleotide (NADH) and CoQ [61]. The mitochondrial proton leak can be increased by the use of protonophores or uncouplers that directly carry protons across the inner membrane. The best example of this is DNP (Table 2), which was found to cause weight loss in armaments workers handling nitroaromatic compounds, and was subsequently used as a slimming agent in the 1930s [61]. However, the therapeutic window was fairly narrow leading to fatalities and its use has since been banned [61]. Nevertheless, DNP was very effective at promoting weight loss so there have been several attempts to modify uncouplers so they can be used safely as treatments for chronic obesity, for example by the development of self-limiting uncouplers [62,63]. Alternatively, there has been a search for factors that can accelerate proton leak by endogenous protein pathways within mitochondria [63], but as yet none of these have been found to be effective *in vivo*.

Another potential therapeutic approach for ameliorating mitochondrial disorders is to bypass damaged sections of the respiratory chain. For example, if a particular step in the respiratory chain is defective due to an mtDNA mutation, providing a pathway for electrons to bypass this respiratory defect would enable proton pumping and ATP synthesis to continue at the undamaged steps in the chain. Conversely, facilitating the electron bypass of a proton pumping step in normally functioning mitochondria might also be beneficial, as it would lead to less efficient ATP synthesis. This would act similarly to mild uncoupling and decrease both oxidative stress and fat accumulation. The efficacy of bypassing steps in the respiratory chain *in vivo* has been demonstrated by bypassing complex I through the overexpression of a yeast nonproton pumping NADH dehydrogenase [64], and through bypassing cytochrome oxidase in flies using an alternative terminal oxidase [65]. The challenge for this approach is to achieve these effects using a redox-active small molecule to bypass a defective or normal proton pumping step within mitochondria *in vivo*. The successful compound would need to have appropriate reduction potentials and reaction kinetics to transfer electrons and thereby bypass one of the three proton pumping sites in the respiratory chain. One compound that has shown promise in this regard within cells is methylene blue [66], which can pick up electrons from various NAD(P)H dehydrogenases and then donate electrons to cyt *c*. Its mode of action is unclear but a major aspect may be to increase expression of complex IV, perhaps as a response to the more reduced cyt *c* pool [66]. The therapeutic benefit of short chain ubiquinones such as idebenone may at least in part be due to bypassing a defective or damaged complex I by picking up electrons in the cytosol from the dicoumerol-sensitive NAD(P)H:quinone oxidoreductase 1 and passing them on to complex III [67]. Finally, in a single patient study, treatment with ascorbate and menadione to bypass a defect in complex III gave evidence of both improved mitochondrial and muscle function [68]. Manipulating the pathway of electron flow through the mitochondrial respiratory chain is therefore an interesting therapeutic strategy, but the medicinal chemistry challenges are significant.

Pharmacological agents that affect mitochondria indirectly

A final strategy is to use small molecules to modulate systems outside mitochondria that control the number and activity of the organelle. Typically this involves manipulating endogenous pathways that normally enable mitochondrial content and activity to respond to environmental demands, such as increased workload or changes in nutrient supply. This can be done by altering the transcription of nuclear-encoded mitochondrial genes, and several pathways can be manipulated either directly or indirectly to do this. The most extensively studied example is PGC-1α,

the master regulator of mitochondrial biogenesis. PGC-1α upregulates the activity of transcription factors that are involved in mitochondrial biogenesis, such as Nuclear Respiratory Factor (NRF)-1, which in turn modulates the expression of other factors such as Transcription Factor A, Mitochondrial (TFAM), which are important for mtDNA replication and transcription [13]. The pharmacological upregulation of PGC-1α activity may be a way of restoring mitochondrial biogenesis to overcome a mitochondrial defect or to respond to an increase in energy demand. The level of PGC-1α expression is controlled by the activity of Peroxisome Proliferator-Activated Receptor (PPAR)γ, and in the shorter term its activity responds to AMPK which acts as a cytosolic ATP/ADP sensor that responds to an energy deficit via the phosphorylation of PGC-1α, which leads to its migration to the nucleus and activation of nuclear-encoded mitochondrial genes. PGC-1α activity can also respond to calcium/calmodulin-dependent protein kinase (CAMK) IV, enabling its activity to respond to calcium signals such as those involved in muscle contraction. PGC-1α is also affected by NO through cGMP levels and its activity can be increased via acetylation [13,69]. The activity of PGC-1α has been modified pharmacologically by upregulating its expression through the use of the pan PPAR agonist, bezafibrate, which enabled a defect in a complex IV assembly factor to be suppressed in a mouse model *in vivo* [70]. However, in a later study, bezafibrate was ineffective against a similar complex IV assembly mutation; but in this case, activating PGC-1α indirectly using the AMPK agonist AICAR prevented the complex IV defect [71]. Thus, the pharmacological manipulation of PGC-1α activity is a particularly promising strategy as a therapy for mitochondrial disorders.

Several other closely integrated and overlapping pathways can upregulate mitochondrial activity by altering transcription. Among these are the NAD^+-dependent deacetylases belonging to the sirtuin family [69]. For example, the nuclear pool of Sirtuin3 can deacetylate and thereby activate the forkhead transcription factor, FOXO3a, upregulating the expression of mitochondrial antioxidant enzymes such as MnSOD [72]. Changes in the acetylation status of histones and of other transcription factors and coactivators, such as PGC-1α, also affect the transcription of mitochondrial genes, and this complicates the interpretation of sirtuin activity on mitochondria. Nonetheless, the suggestion that small molecule sirtuin activators based on resveratrol have potential clinical applications has stimulated interest in pharmacological manipulation of mitochondria through altering sirtuin activity [73–75]. However, at this time, the knowledge of the mechanism(s) of their interactions with sirtuins is incomplete [76]. Even so, the pharmacological manipulation of mitochondrial function by small molecules acting on the endogenous pathways that control the expression of mitochondrial genes and thereby enable organelle function to match demand and respond to damage is a very appealing strategy for mitochondrial pharmacology.

Concluding remarks

In this review, we have focused on the general principles associated with the emerging field of mitochondrial pharmacology. To date, mitochondria have been a neglected drug target but show tremendous clinical potential. Of particular importance is the realization that secondary mitochondrial damage contributes to a wide range of disorders and that there are a few core aspects of mitochondrial pathology that frequently arise in many disparate diseases. This implies that therapies designed to interact with these core features can be applied to many diverse and prevalent medical disorders. One common aspect to mitochondrial pathology, elevated oxidative stress, can be treated by mitochondria-targeted antioxidants and these have proven effective across a broad spectrum of diseases. Although we have focused on therapeutic interventions to human mitochondria, the principles outlined here can be applied to many other situations, for example in veterinary medicine or in developing new ways of targeting parasites.

Several challenges will need to be recognized and overcome for mitochondrial pharmacology to achieve its full potential. One is the development of biomarkers and measurements of mitochondrial function *in vivo* to ascertain if the therapy is actually affecting mitochondria. Currently, many clinical trials are inconclusive in this regard. Often, it is unclear whether the outcome was mediated through a change in mitochondrial function or, conversely, if mitochondrial function was indeed altered but did not have a clinical impact. Biomarkers for whole body oxidative stress such as isoprostanes for lipid peroxidation have been developed, but their relationship to mitochondrial damage is not well substantiated. Methods are being developed to directly assess mitochondrial ROS production and oxidative damage in whole animals [77,78]. Mitochondrial function is also being assessed in real time *in vivo* by the development of mitochondria-targeted PET probes [79] and by the use of ^{31}P-MRI to assess ATP synthesis, although its effectiveness in clinical trials is limited [80]. It is clear that new and better ways to assess all aspects of mitochondrial function within patients is essential for the future development of mitochondrial pharmacology. To conclude, mitochondrial pharmacology is an emerging discipline of great promise and potential for new therapeutic approaches with implications for most aspects of medicine.

Conflict of interest

M.P. Murphy and R.A.J. Smith hold shares in Antipodean Pharmaceuticals Inc.

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