Chapter 15
Development of Treatments and Therapies to Target Mitochondrial Dysfunction

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Abstract Mitochondrial diseases affect 1:10,000 people worldwide, with a further 1:6,000 at risk of developing symptoms, and can be caused by mutations in the nuclear genome or by mutations within/deletion of mitochondrial DNA (mtDNA). There is currently no therapy for patients with these diseases. In addition, mitochondrial dysfunction can be considered contributory or causal for other neurological diseases, such that therapies that improve mitochondrial function may have wide-reaching benefits. This chapter summarizes cell- and animal-based attempts to find a therapy by modulating major mitochondrial pathways including mitochondrial biogenesis, fission, fusion and altering metabolic intermediates, mitophagy and the mammalian target of rapamycin (mTOR) pathway. Clinical trials currently in progress include drugs that are direct or indirect antioxidants, a mitogenesis activator, an antiapoptotic compound and a compound that improves electron transport chain efficiency.

Keywords Leber's hereditary optic neuropathy (LHON) • Mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) • Mitochondrial biogenesis • Mitochondrial fission and fusion • Mitophagy • Antioxidant • PGC1-alpha • AICAR • Resveratrol • NAD+ • Rapamycin • Bezafibrate • Clinical trial

15.1 Introduction

Mitochondria are known as the powerhouse of the eukaryotic cell, utilizing nutrients and oxygen to generate ATP, an absolute requirement for life. They are considered endosymbionts, derived originally from proteobacteria and still carrying the remnants of their own genome, the mitochondrial DNA (mtDNA). Human

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mtDNA exists as 1,000s of copies per cell and encodes 13 proteins, 22 tRNAs, and 2 rRNAs. Together they encode 13 proteins that are critical components of the ATP-generating electron transport chain (ETC), with the remaining 77 proteins of the ETC being encoded by the nuclear genome [1, 2] (see Chap. 1). An additional 1,000–1,500 nuclear-encoded genes encode the remaining proteins that make up mammalian mitochondria, representing circa 5 % of the protein encoding genome [3–5]. This complexity of mitochondria highlights the fact that they are not just ATP-generating organelles, but perform many other functions including metabolism, iron storage and iron-sulfur cluster formation, calcium homeostasis, apoptosis, hormone signaling/synthesis and thermogenesis, among others [6]. It is therefore not surprising that mitochondrial dysfunction, via inborn or accumulated genetic lesions or via environmental factors, causes a wide variety of diseases affecting many different organs in the body including the central and peripheral nervous system [2, 7–12].

This chapter will briefly describe the causes of mitochondrial defects and then summarize attempts to find therapeutic agents that ameliorate or cure diseases of mitochondrial dysfunction. It will describe molecular pathways and targets most likely to yield therapies when intersected with pharmacological approaches, including a brief summary of those therapeutic agents that have shown promise in cellular or animal models for a given pathway, finishing with a summary of current clinical advances and activities for mitochondrial diseases. The chapter focuses mainly on mitochondrial dysfunction in neurological disorders that represent a clear unmet medical need, with some reference to other disorders. The great hope of everyone in this challenging field is that once we have successfully ameliorated cellular mitochondrial dysfunction with therapeutic agents, those agents will also have broad utility in all mitochondrial disorders as well as in diseases that have been linked to mitochondrial dysfunction, including neurodegenerative diseases such as Parkinson's disease (PD).

15.2 Mitochondrial Disease

Mutations in mtDNA and nuclear DNA (nDNA) can cause mitochondrial disease, generally understood as diseases caused by defective oxidative phosphorylation. Over 300 different point mutations or deletions in mtDNA have been attributed to human disease [13]. Mutations of nuclear mtDNA maintenance genes can also cause mtDNA deletions and depletion (reduction in copy number). mtDNA mutations are primarily linked to a loss of function of the ETC, and the existence of significant copies of mtDNA leads to an additional complication of mtDNA-related disease, the coexistence of mutant and wild-type mtDNA – known as heteroplasmy [14]. One hundred seventy-four nDNA genes have been functionally linked to mitochondrial disease to date [15], and with the advent of inexpensive whole exome/genome sequencing disease, genes are being uncovered at an ever-increasing rate [16–19].

Together the elucidated and as yet unknown mutations account for mitochondrial disease that has an overall clinical prevalence estimated to be 1:10,000 [20]. This would suggest that there are significant numbers of patients such that clinical trial recruitment should be easy – but the major challenge of mitochondrial diseases is the genetic range and clinical complexity, depending on the mutation(s), heteroplasmy (for mtDNA diseases), age, and environment. For this reason there is much interest in targeting the major pathways regulating mitochondrial biogenesis, fission and fusion, and mitochondrial autophagy (mitophagy), in addition to attempts to directly shift heteroplasmy or to modulate metabolism related directly to the electron transport chain.

15.3 Mitochondrial Biogenesis for Improved Metabolic Function

Perhaps the most widely studied *in vitro* and *in vivo* approach, increasing the total mass of mitochondria is an obvious approach to increasing overall function of a partially defective electron transport chain [21–23]. This will only be useful if a negative aspect of mitochondrial (dys)function is not also amplified, e.g., a defective respiratory chain that increases reactive oxygen species (ROS) to damaging levels.

15.3.1 PGC1-α

This approach pivots primarily around the activation of PGC1-alpha(α), the key transcriptional co-activator protein that regulates nuclear mitochondrial genes [23–26]. PGC1-α interacts with multiple transcription factors to bring about the transcriptional program that produces nuclear-encoded mitochondrial genes [27]. There are multiple routes to PGC1-α activation using the low-molecular-weight compounds (LMWs) resveratrol, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), and bezafibrate that target sirtuin (SIRT1) and/or AMP-activated kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR), respectively (Fig. 15.1). These three routes to mitochondrial biogenesis exemplify the use of both genetic and pharmacological *in vivo* experiments in relevant mouse models [21, 23].

Muscle-specific overexpression of muscle PGC1α increased mitogenesis in *cox10*, *Surf1-/-*, and *Polg* "mutator" mice [28–30] (for more detail of these models, see Chap. 13), improved muscle function and lifespan in the *cox10* mouse and improved motor function and cardiac performance in the mutator mouse [28, 30]. In addition, PGC1α has been overexpressed in mouse models of Duchenne muscular dystrophy (DMD), amyotrophic lateral sclerosis (ALS), and a 1-methyl-4-phenyl-

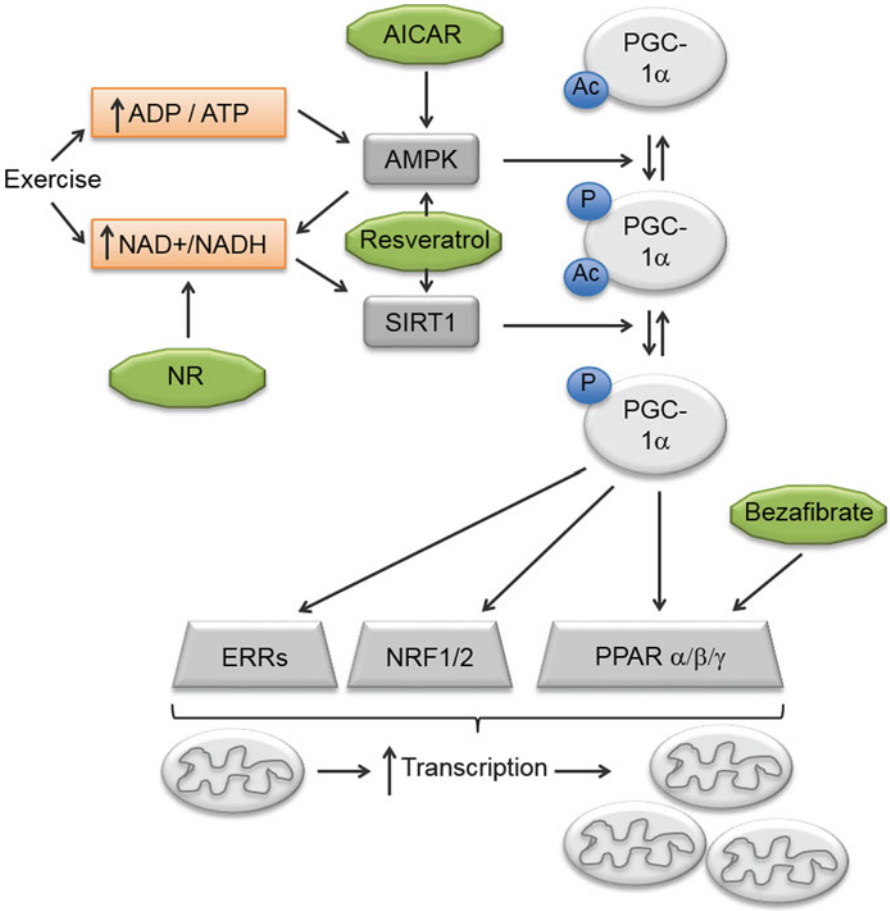

Fig. 15.1 ATP and NADH are depleted during energy expenditure. The subsequent increase in the ADP/ATP ratio is sensed by AMPK that activates PGC-1a. In addition, via NAMPT (not shown), AMPK affects the NAD+/NADH ratio that regulates the deacetylase SIRT1 that fully activates PGC-1a. PGC-1a interacts with groups of transcription factors to increase the transcription of nuclear-encoded and subsequently mitochondrially encoded mitochondrial genes; *ERR* (estrogen-related receptor), *NRF* (nuclear respiratory factor), and *PPAR* (peroxisome proliferator-activating receptor). Compounds that stimulate this pathway are noted in green and are discussed in depth in the text (*NR* nicotinamide riboside)

1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's model. Increased mitochondrial biogenesis linked to an improvement in phenotype was shown in some, but not all, cases [31–33]. In particular, in mice treated with MPTP to induce Parkinson's-like phenotypes, PGC1α overexpression through the neuronal-specific thy-1 promoter significantly protected dopaminergic neurons from MPTP-induced degeneration, demonstrating PGC1α's potential utility in ameliorating neuronal phenotypes of mitochondrial disease [33]. Overall there is strong evidence that

increased mitochondrial biogenesis via increasing PGC1α activity could have therapeutic utility in mitochondrial disease.

15.3.2 Resveratrol

PGC1α is regulated by AMPK and the deacetylase Sirt1, a member of the sirtuin family (Fig. 15.1). Resveratrol, a natural stilbenoid found in the skin of grapes, was initially proposed as an activator of Sirt1, thereby regulating yeast and metazoan lifespan [34–37]. Sirt1 is a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, which is regulated by AMPK activity via altered NAD+/NADH levels. Importantly, NAD+ is also required by the Krebs cycle at multiple steps during oxidative phosphorylation. While resveratrol effects are not necessarily disputed, there is still controversy about the molecular mechanism of action. One view is that resveratrol regulates Sirt1 indirectly via AMPK, regulating NAD+ levels that in turn control Sirt1 activity [38–42]. At the same time resveratrol can act directly on Sirt1 homologues *in vitro* to catalyze a deacetylation reaction on acetyl lysine [34, 43]. Interestingly, it has been proposed that resveratrol may act via tyrosine tRNA synthetase, to activate poly(ADP-ribose) polymerase (PARP-1), a major consumer of NAD+. This appears to contradict the concept that an increase in NAD+ improves mitochondrial function but there is a transient increase in NAD+ levels in mice treated with low doses of resveratrol, before a longer term reduction [44]. This result will require further investigation before the molecular mechanism is truly understood. Nevertheless, resveratrol does increase mitochondrial biogenesis and oxidative respiration in mouse models of obesity and in doing so reduces the development of obesity and retains insulin sensitivity [45, 46]. Based upon this, structurally unrelated compounds have been sought that activate Sirt1 (SRT1720, SRT2183, SRT1460, SRT2104, SRT501) and have been tested in many *in vitro* and *in vivo* situations alongside or independently of resveratrol. In general their effects are consistent with the activation of Sirt1, showing improvement of insulin sensitivity in mouse obesity models [47, 48].

For neurological indications and mitochondrial disease, resveratrol has been tested in mouse models of Friedreich's ataxia, the *mdx* model for DMD, SOD1 ALS models, and models for Alzheimer's disease (AD), Huntington's disease (HD), and PD [21, 49]. Resveratrol was not beneficial in every case. The beneficial mechanisms may or may not be caused solely or partly because of increased mitochondrial function because mitochondrial measurements were not carried out in most of these studies. One exception is the MPTP PD model where PGC1α is a likely efficacy target [33]. Resveratrol has not been tested in any mouse models of direct oxidative phosphorylation defects, but the Friedreich's ataxia study mentioned above does represent an indirect model of mitochondrial function, given that ETC complexes I/II and III contain iron-sulfur clusters and frataxin is required for iron-sulfur cluster synthesis. However in this study there was no data to suggest that resveratrol was having a beneficial effect in terms of the amelioration of disease symptoms [50], but

only upon the levels of frataxin. In sum, there is good evidence to suggest that resveratrol could be useful for patients with mitochondrial disorders or neurological dysfunction, although significant analysis of mitochondrial function in the animal models mentioned here is still lacking.

15.3.3 AICAR

AICAR is an adenosine monophosphate (AMP) analogue that stimulates AMP-dependent kinase (AMPK) following intracellular conversion into an intermediate known as 5-amino-4-imidazolecarboxamide ribonucleotide (ZMP) [51, 52]. AMPK acts as the sensor of metabolic status of the cell – the ATP/ADP and ADP/AMP ratios – and has already been implicated in mitochondrial regulation via the use of resveratrol. Thus AICAR is simply another activator of AMPK, but it does so without affecting the ATP/ADP ratios, i.e., it does not affect the energy status of the cell. In *in vitro* experiments, AICAR was shown to improve growth and increase ATP concentration/production better than nine other potential mitochondrial compounds in multiple mitochondrial disease patient fibroblast lines [53].

AICAR is orally effective in mouse models and improved muscle performance in sedentary mice by increasing nuclear mitochondrial gene expression. It also increased COX enzyme expression and function and other mitochondrial gene expressions in multiple muscle-specific COX-deficient mouse models (ACTA-Cox15-/-, Surf1-/-, and Sco2KO/KI). Cox15 is a key enzyme in the heme biosynthesis pathway, and Sco2 is a metallochaperone that regulates cytochrome *c* oxidase (COX); mutations in either of these genes lead to encephalocardiomyopathy in children. Surf1 is a COX assembly factor that when mutated causes severe Leigh syndrome in children [29]. AICAR fully rescued the motor defects of the Sco2 mouse by unknown mechanism(s) but had no effect on the Cox15-/- model, likely reflecting the severity of this model. So, AICAR clearly induces mitochondrial function *in vivo*, but it exhibits poor brain penetrance [54], preventing its obvious utility in neurological situations. Many other direct and indirect LMWs have been proposed to act upon AMPK, mainly indirectly [55], but there are reports of improved direct AMPK activators [56–58]. The driving force behind AMPK activator research is the concept that AMPK is a relevant target for type II diabetes and the metabolic syndrome, as well as certain cancers [59], which may result in better molecules in terms of activity but not necessarily in terms of distribution (i.e., blood-brain barrier penetration).

15.3.4 Bezafibrate

Bezafibrate is a pan-peroxisome proliferator-activated receptor (PPAR) agonist developed initially to treat hyperlipidemia [60]. More recently PPARs were shown to regulate mitochondrial biogenesis via PGC1α [61] and were shown to increase respiratory function in patient-derived cells [62–64]. Parallel to these cell-based activities, bezafibrates were tested in multiple mitochondrial disease model mice with partial success. *ΔCox10* mice receiving 0.5 % w/w dietary bezafibrate exhibited increased muscle mitogenesis, a delay in the onset of myopathy, and an extended lifespan [28]. However, similar dosing in *Surf1*-/- and *ACTA-Cox15*-/- mice resulted in loss of body weight with hepatic toxicity or death, respectively [29]. Further studies in the *Twinkle* mutant "deletor" mouse [65] and the *Polg* "mutator" mouse [66] had varying degrees of success. The "deletor" mouse did exhibit reduced mtDNA deletion load and had fewer COX-negative muscle fibers, but at the expense of body weight and hepatic complications (treatment in this model started significantly later than the other models mentioned here) [67]. The *Polg* "mutator" mouse, despite not displaying an increase in mitochondrial biogenesis during bezafibrate treatment, did show multiple beneficial phenotypes, including a delay in hair loss, reversion of an abnormal spleen seen in this model, and reduced weight loss [68]. Overall bezafibrate has a reasonable success rate in cellular and animal models in terms of increasing mitochondrial function and ameliorating mouse model phenotypes.

15.3.5 NAD+ Precursors

Finally, it is important to consider recent promising developments utilizing NAD+ precursors directly as therapeutic agents, in particular nicotinamide riboside (see Fig. 15.1) [69, 70]. In mitochondrial disease respiratory chain deficiency leads to a decrease in the NAD+/NADH ratio, which sends a "high nutrient" signal to the cell – confounding the original mitochondrial metabolic defect. It makes sense to increase the levels of NAD+ using precursors of NAD+ that can reach the correct cellular compartments. Nicotinamide riboside (NR) is such a precursor and has been shown to increase muscle mitochondrial performance in wild-type and high-fat diet mice [71]. Subsequently, NR was used to treat the "deletor" adult-onset mitochondrial disease model mouse in two dosing schedules representing pre- and post-disease-onset situations (400 mg/kg/day) [69]. NR was beneficial in both dosing situations, increasing intracellular NAD+, increasing mitochondrial biogenesis, and reducing mtDNA deletion accumulation, adding to the hypothesis that increasing NAD+ is likely to be of very significant potential in the treatment of mitochondrial disease.

15.4 Mitochondrial Dynamics and Quality Control: Fission, Fusion, and Mitophagy

Mitochondria are highly dynamic and undergo fission and fusion, as well as being able to move with varying degrees of speed within cells – something of particular relevance in neurons with long cell processes [10, 72–74] (see Chap. 7). Fission and fusion are regulated by GTPases, with Drp1 driving fission and the collaboration of mitofusins 1 and 2 (Mfn1 and Mfn2) with Opa1 and Opa3 facilitating fusion. Fusion allows content mixing, including the mixing of mitochondrial genomes or mitochondrial-encoded proteins, perhaps allowing the dilution of damaged mtDNA or other damaged factors to buffer against stresses [25, 73, 75–77]. Fission allows the regulation of individual mitochondrial size, important for their transport [78] but also for their degradation (see below).

15.4.1 Fusion

Mutations in fusion factors lead to neurological disease. Opa1 defects lead to autosomal dominant optic atrophy (DOA) [79, 80], and Mfn2 mutations cause a peripheral neuropathy, Charcot-Marie-Tooth disease type 2A (CMT2A) [81]. Dominant mutations in Drp1 have been linked to a lethal neurodegenerative condition presenting with multiple phenotypes including microcephaly, optic atrophy, and lactic academia [82]. An extensive review of mouse models generated so far suggests that there are multiple tractable models to study the consequences of these mutations [83]. However, for example, in the case of Mfn2, multiple models have been generated with differing outcomes; some models proved too severe (lethal) [84], while others were too mild in relation to human Charcot-Marie-Tooth neuropathy type 2A [85], This variability in mouse models highlights the challenge of drug discovery for fission-fusion-related diseases.

15.4.2 Fission-Fusion Compounds

There are a limited number of compounds that specifically regulate mitochondrial fission and fusion: Mdivi-1 that inhibits Drp1 and 15-oxospiramilactone (S3) that inhibits USP30, a mitochondrially localized deubiquitinase and a hydrazine (M1) with an unknown target [86–89]. These molecules may represent useful starting points for drugs that are pro-fusion, i.e., they either block fission (mdivi-1) or enhance fusion (M1, S3). While M1 and S3 have never been tested in animal models, mdivi-1 has been tested in multiple studies, mainly concerned with reduction of infarct size and cell death in ischemic-reperfusion models [90–93].

In C57BL/6 mice, mdivi-1 reduced retinal ganglion cell death following pressure-induced ischemia, suggesting potential use for the treatment of glaucoma [91]. More recently inhibition of Drp1 has been tested in two Parkinson's disease models, a PINK1-/- mouse and a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model [94]. This study is important because it was demonstrated that mdivi-1 crosses the blood-brain barrier and in doing so was able to restore a striatal dopamine release deficit and reduce neurotoxicity. This suggests that pushing the balance of fission-fusion toward fusion is beneficial in PD models with altered mitochondrial function. However it should be noted that in this case, the exact relationship between loss of PINK1 function and the pro-fusion function of mdivi-1 is not completely clear, especially given the proposed roles of PINK1 in mitophagy (see below) [94, 95].

15.5 Mitophagy

Mitophagy is the process whereby dysfunctional mitochondria are destroyed by the autophagic machinery (see Chap. 11). This occurs as a developmental process, for example, during the creation of mature erythrocytes [96], or it can occur as a result of loss of function or damage to mitochondria in many different cell types, i.e., a form of mitochondrial quality control [97, 98]. Mutations in *PARK2* (Parkin) and *PARK6* (PINK1) cause juvenile-onset autosomal recessive PD [99–101], and PINK1 and Parkin are now known to be key regulators of mitophagy [95, 102–104]. Briefly, PINK1 accumulates on the surface of damaged mitochondria (those with reduced membrane potential), binds Parkin, and the E3 ubiquitin ligase activity of Parkin converts multiple mitochondrial outer membrane proteins to an ubiquitinated form. These ubiquitinated proteins are recognized by specific receptors of the pre-autophagosome, causing engulfment of whole mitochondria for ultimate destruction in the lysosome. Mitophagy is closely linked to fusion because mitofusin is regulated by PINK1, acts as a receptor for Parkin and is ubiquitinated by Parkin to inactivate it. Presumably mitochondria above a certain size cannot fit within the pre-autophagosomal membrane so linking loss of fusion and mitophagy improves the efficiency of mitophagy. In addition, PINK1 and Parkin have been shown to regulate mitochondrial fission and fusion [105–107].

Thus, one hypothesis for PD is that the poorest functioning mitochondria are normally removed from the cellular pool through mitophagy, but failure of mitophagy due to PINK1 and Parkin mutations permits the accumulation of damaged mitochondria, leading to neuronal death in the *substantia nigra* [97, 98, 108]. It is likely that the *substantia nigra* neurons are more susceptible to loss of mitophagy than other neurons because there is a more dynamic redox environment, due to dopamine metabolism, that increases mtDNA mutations (see Chap. 6) [109]. Indeed, high levels of somatic mtDNA deletions are detected in *substantia nigra* neurons of healthy subjects and PD patients [110–112].

Conceptually then, it makes sense to activate the mitophagy pathway in PINK1/Parkin PD patients – as long as the fidelity and selectivity of damage-dependent mitophagy is maintained – potentially not possible with all alleles of PINK1 and Parkin responsible for disease [97]. In addition, activation of this pathway could preferentially remove mutant mtDNA from heteroplasmic tissue in mitochondrial disease patients. This concept has been genetically validated in several ways. Overexpression of wild-type Parkin in a cybrid cellular model of mtDNA disease (see later for details) was able to improve the heteroplasmic ratio of the wild-type/mutant mtDNA and improve respiratory function [113]. Furthermore, USP30, a de-ubiquitinating enzyme, has been shown to oppose the action of Parkin on mitochondrial outer membrane proteins (MIRO, TOM20) and to inhibit mitophagy. Knockdown of USP30 restores mitophagy in pathogenic Parkin mutant cells and improves mitochondrial function in paraquat-treated flies [97, 114, 115]. In addition, USP8, USP15, and USP35 have also been implicated in the regulation of mitophagy [116–118], These are interesting targets, mainly because the USP proteases are considered "druggable" [119, 120], and an inhibitor of USP30 has already been reported to be involved in fission-fusion regulation of mitochondria via the regulation of mitofusin ubiquitination [88].

15.5.1 Compounds Increasing PINK/Parkin Function

To find compounds that increase Parkin function, a chemical screen was performed using a cell line carrying multiple reporters linked to the PARK2 promoter. This approach yielded several classes of compounds that appear to increase Parkin function *in vitro*, including epigenetic agents, cholesterol synthesis modulators, and Jun kinase (JNK) inhibitors. These all represent potential compounds for further studies in PD and mtDNA disorders [121], given that one of the JNK inhibitors (SR-3066) is known to have neuroprotective effects in an MPTP rodent model [122]. A novel-directed approach to activating PINK1 yielded kinetin triphosphate, a PINK1 neo-substrate, which was able to increase the activity of both wild-type and disease-mutant $PINK1^{G309D}$ in cellular models, resulting in increased Parkin recruitment to depolarized mitochondria [123]. Thus, there are promising compounds that likely influence damage-dependent mitophagy, and future experiments in relevant animal models will tell us more about their therapeutic potential.

15.5.2 mTOR Inhibition

Rapamycin inhibits mammalian target of rapamycin (mTOR), which regulates cell growth via multiple downstream effectors. mTOR is also a negative regulator of autophagy and mitophagy, and as such rapamycin could be beneficial based upon the arguments above [124–126]. However, mTOR positively regulates

mitochondrial function in skeletal muscle but does the opposite in adipose tissue, confounding the issue when one thinks of the whole organism [126]. Perhaps more importantly, mTOR regulates aging, with mTOR inhibition increasing the lifespan of eukaryotes across evolution [127, 128].

Mouse models of mitochondrial disorders are often considered useful models of aging, with reduced survival times being common [129, 130]. Based upon this, *Ndufs4-/-* mice that have a lifespan median of 50 days have been treated with different dosing schedules of rapamycin at relatively high doses, such that daily treatment significantly reduced multiple neurological parameters and extended lifespan dramatically [131]. Interestingly mitochondrial mass and function were not altered, but a shift in central metabolism observed in the mutant compared to wild-type mouse was reversed upon rapamycin treatment of the mutant mouse. Importantly, low doses of a rapamycin derivative, RAD001, improve age-dependent immune function in healthy aged subjects, with few side effects [132]. Thus, there is a rationale for testing rapamycin in mitochondrial disease patients, but achieving therapeutic benefit while limiting side effects may be challenging.

15.6 Unbiased Screening in Cellular Systems

One can consider three conceptual approaches to obtaining novel bioactives using large libraries of compounds: First, biochemical screening of a known (protein) target such that modulation of that target should rescue a disease phenotype. Second, screening a cellular model of a known disease [133]. For this phenotypic approach, there must be a detectable phenotype, related to the genotype of interest, and the detection method must be amenable to miniaturization to facilitate large-scale screens. A third approach, a chemical biology approach, is to perform chemical screens on otherwise "normal" cells, organs, or organisms in culture to identify compounds that alter phenotype of choice, yielding tool or probe molecules that can perturb different biological functions [134]. These tools can then be assessed as potential leads for drugs based upon the knowledge surrounding their target.

Several labs have performed medium-throughput phenotypic screens or chemical biology or to identify compounds that alter mitochondrial function in cell lines in culture, using assays that measure cellular viability, cellular dehydrogenase activity, mitochondrial membrane potential, intracellular ATP, NAD+/NADH ratio, reactive oxygen species, and oxygen consumption as primary approaches [135–139]. Additional approaches for LMW altering some or all of these readouts included transcriptomics or assessing compound effects on *C. elegans*. Mining of these datasets for therapeutic opportunities yielded several compounds of interest: first, microtubule inhibitors that drive PGC1α transcription [135]; second, meclizine, an FDA-approved antiemetic, which protects from ischemic-reperfusion injury in animal models if used as a pretreatment and Huntington's disease models [137, 140]; third, a group of FDA-approved drugs that increased oxygen consumption (acarbose, metaraminol, gallamine triethiodide, and acamprosate) [139]; and fourth, a

compound (BRD6897) that increases mitochondrial mass and function in endothelial cells [138]. However, given the inherent toxicity issues of microtubule inhibitors [141], it is unlikely that they can be relevant for mitochondrial-dependent neurodegenerative diseases. Previously, FDA-approved drugs that have a positive effect on mitochondrial function in normal and diseased cells and/or fly and rodent models may represent a worthwhile approach, but there is to date no further literature supporting treatment of patients with mitochondrial dysfunction with these drugs.

15.7 Screening in More Relevant Cellular Systems

15.7.1 Patient-Derived Cybrids

By definition, the nature of heteroplasmic mutations makes their study in derived cellular systems challenging. Initial attempts to recreate heteroplasmy *in vitro* led to the production of a cytoplasmic hybrid – known as a cybrid, created by fusion of a rho-zero (lacking mtDNA) transformed cell line with an enucleated cell line carrying donor mtDNA(s) [142]. Creation of cybrids carrying varying point mutations, deletions, and haplotypes have since been created to address a range of questions surrounding mitochondrial disease and the role of mtDNA in other diseases such as Parkinson's or diabetes [143].

Since cybrids can be heteroplasmic, they represent cellular models to test for nutrients or compounds that alter heteroplasmy, increasing the ratio of wild-type to mutant mtDNA. Indeed, several approaches have been described to alter cybrid heteroplasmy, reducing the proportion of mutant mtDNA, including altered nutrient regimes and treatment with compounds. For instance, coenzyme Q [144], glucose/arginine [145], and rapamycin [146] have all been shown to shift the wild-type/mutant mtDNA ratio positively. However, cybrid lines are by definition transformed and may thus reflect abnormal metabolism, and these cells are exclusively mitotic, preventing any examination of the effects of mtDNA mutations in postmitotic cells, which are most often affected in mtDNA disease.

15.7.2 Induced Pluripotent Stem Cells (iPSCs) and Differentiated Neurons

Some of the limitations of using cybrids are now being overcome with the advent of induced pluripotent stem cell (iPSC) technology, enabling the creation of many different mitotic and postmitotic cell types following specific differentiation processes [147]. Since their initial discovery several labs have produced iPSCs derived from patients with mitochondrial defects [148]. In two studies, fibroblasts from patients carrying the MELAS mutation m.3243A>G have been reprogrammed into stem

cells where the level of heteroplasmy has a bimodal distribution that remains stable during serial outgrowth of a given clone [149, 150]. In a third study, fibroblasts carrying the MELAS-associated mtDNA mutation, m.13513G>A, were made into iPSCs in which the heteroplasmy remained stable or was significantly reduced [151]. iPSCs carrying mtDNA deletions have also been produced [152] from three different patients with Kearns-Sayre syndrome (KSS)/Pearson's syndrome (PS). During both reprogramming and further outgrowth of clones, a variability of heteroplasmy was observed. Overall it appears that iPSCs can be derived with stable mtDNA mutations and can be differentiated into heteroplasmic neuron-like and hematopoietic cells that demonstrate significant mitochondrial dysfunction [149, 150], and this will aid research and screening for novel therapeutics for these diseases.

To date there have been no reports demonstrating effective rescue of mitochondrial defects in postmitotic-derived mitochondrial disease cells (nuclear or mtDNA). However, several groups have derived neural-like cells from iPSCs carrying PD-causative PINK1 or LRRK2 (leucine-rich repeat kinase 2) mutations [153, 154] and shown pharmacological [153] or genetic rescue [154] of the associated mitochondria dysfunction. Although the overall effects are small, coenzyme Q10 and rapamycin could both rescue valinomycin-induced lactate dehydrogenase (LDH) leakage in LRRK2 G2019S patient-derived neurons, and Q10 could also rescue LRRK2 R1441C and PINK1 Q456X neurons [153]. These initial results using single doses of compound are promising, but the molecular mechanisms involved are unclear. In a genetic approach, neurons carrying LRRK2 G2019S were corrected to wild type using Zn-finger nucleases to demonstrate that the mutant cells accumulate a higher rate of mtDNA mutation than the corrected cells [154]. Whether these mtDNA mutation rates affect mitochondrial function was not assessed in this study.

15.8 Clinical Approaches

Three recent exhaustive reviews assessed all prior reports of clinical trials in mitochondrial disease patients to assess trial design quality and to determine beneficial outcomes [155–157]. The key message is that there is no attempted treatment for mitochondrial disorders that has a clear statistically relevant positive outcome. There are several reasons for this, but a major reason is the trial design itself, because there is a clear bias toward positive reporting when the trials are less well designed – e.g., open label instead of double blind. This sets a poor precedent for all concerned, because patients will receive drugs that are likely to be ineffective, damaging patient's confidence in the medical and research community [155].

Current clinical trials for pharmacological agents in mitochondrial disease space are shown in Table 15.1 (www.clinicaltrials.gov). For mitochondrial disease patients, mercaptamine is proposed to increase levels of glutathione – which acts an antioxidant to combat reactive oxygen species produced by damaged mitochondria. Vatiquinone is also an antioxidant that has been shown to be beneficial in open-label

Table 15.1 Current pharmacological agents in clinical trials for mitochondrial disease

Chemical entity	Phase (mitochondrial disease), trial ID at clinicaltrials.gov	Notes, trial completion dates
Mercaptamine (RP103, transglutaminase inhibitor)	Ph II/III (Leigh syndrome) [launched for nephropathic cystinosis 2013], NCT02023866	Prevents abnormal lysosomal cysteine crystal accumulation. Expected to complete June 2016
Vatiquinone (EPI-743, NADH, or NADPH oxidoreductase modulator)	Ph II (Pearson syndrome (PS), mitochondrial respiratory chain disease, Leigh syndrome). NCT01721733	Antioxidant. PS completion Apr 2016, respiratory chain disease completed, Leigh syndrome completion Apr 2016
RTA-408 (nuclear erythroid 2-related factor 2 stimulator)	Ph II (mitochondrial myopathy). NCT02255422	Antioxidant, anti-inflammatory agent. Completion Jun 2016
Cyclosporine (calcineurin inhibitor)	Ph II (LHON). NCT02176733	Limits apoptosis in acute phase of disease. Completion expected Oct 2015
Bendavia (MTP-131)	Ph I/II (LHON, mitochondrial myopathy). NCT02367014	Targets cardiolipin in mitochondrial membranes. LHON study expected to complete in 2016. Myopathy study expected to complete Dec 2015
Benzafibrate (lipase stimulator; PPAR agonist)	Ph II (mitochondrial myopathy, mt.3243A>G). NCT02398201	Mitochondrial biogenesis stimulator. Completion 2016
KH176	Ph I (mitochondrial disease, MELAS, LHON, Leigh, mt.3243A>G). NCT02544217	Redox modulator. Completion 2015

trials [158] and is now in a randomized double-blind trial for pediatric Leigh syndrome patients. RTA-308 is also an antioxidant and anti-inflammatory agent that acts by increasing Nrf2 activity that in turn enhances the expression of prooxidant genes and represses inflammatory genes in animal models [159]. It is currently in a safety/efficacy trial to assess workload during exercise training in mitochondrial myopathy patients. Cyclosporine A, an immunosuppressive drug that protects cells from cell death caused by opening of the mitochondrial permeability transition pore, is being open-label-trialed in Leber's hereditary optic neuropathy, a mitochondrial disease that affects the basal retinal ganglia – hopefully reducing cell death in the retinal ganglion cell layer of the eye. Bendavia is a peptide that prevents cardiolipin from converting cytochrome c into a peroxidase but leaves its electron chain function intact [160] and is also proposed to improve electron chain efficiency by improving coupling by reducing proton leak. Also, based upon the preclinical data presented earlier and the fact that it has been used extensively in humans already, a trial using bezafibrate in mitochondrial myopathy caused by mutation in

mt.3243A>G is under way, with planned completion in mid 2016. Finally, KH176, an antioxidant trolox derivative, is being trialed in phase I rising dose trials in patients with either MELAS, LHON, or mt.3243G<A or Leigh syndrome patients.

It is interesting to note that six of the seven entities being tested are essentially repurposing or expanded indication approaches. Many of these are also general antioxidant therapies that aim to redress the proposed redox defects seen in mitochondrial patients. This reflects the fact that historically there has not been sufficient interest in this class of patients or sufficient knowledge of the pathways in the cell regulating mitochondrial function. This is changing and the hope is that in the immediate future more therapies will be developed with mitochondrial disease patients as the primary focus. Dedicated approaches to processes such as biogenesis and mitophagy, for example, coupled with better clinical trial design, will be key to improving the chance of success for mitochondrial disease patients.

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