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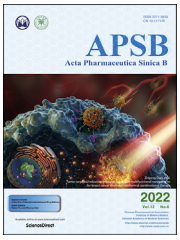

REVIEW

Mitochondria targeting drugs for neurodegenerative diseases—Design, mechanism and application

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Evaluation

Abstract Neurodegenerative diseases (NDDs) such as Alzheimer's disease (AD) and Parkinson's disease (PD) are a heterogeneous group of disorders characterized by progressive degeneration of neurons. NDDs threaten the lives of millions of people worldwide and regretfully remain incurable. It is well accepted that dysfunction of mitochondria underlies the pathogenesis of NDDs. Dysfunction of mitochondria results in energy depletion, oxidative stress, calcium overloading, caspases activation, which dominates the neuronal death of NDDs. Therefore, mitochondria are the preferred target for intervention of NDDs. So far various mitochondria-targeting drugs have been developed and delightfully some of them demonstrate promising outcome, though there are still some obstacles such as targeting specificity, delivery capacity hindering the drugs development. In present review, we will elaborately address 1) the strategy to design mitochondria targeting drugs, 2) the rescue mechanism of respective mitochondria targeting drugs, 3) how to evaluate the therapeutic effect. Hopefully this review will provide comprehensive knowledge for understanding how to develop more effective drugs for the treatment of NDDs.

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1. Introduction

Neurodegenerative diseases (NDDs) are a heterogeneous group of disorders characterized by progressive degeneration of neurons especially in central nervous system including highly prevalent Alzheimer's disease (AD) and Parkinson's disease (PD)[1]. Despite impressive efforts taken, NDDs remain incurable and no effective medication could delay or stop its progression. Ascending evidences suggest that dysfunction of mitochondria plays crucial role in the pathogenesis of NDDs[2]. Clinical trials based on mitochondria cascade related hypothesis account for 17% of all AD trials[3]. Mitochondria are organelles that generate basic energy, adenosine triphosphate (ATP), for most types of cells in our body. Besides maintaining energy homeostasis, mitochondria are also the central mediator of cellular signaling, cell survival[4]. Neurons are particularly energy demanding cells due to its unique structure and function[5]. Dysfunction of mitochondria leads to energy depletion, oxidative stress, calcium overloading, caspases activation, which confers the neuronal death of NDDs *via* apoptosis, pyroptosis or ferroptosis[6]. Therefore, to combat NDDs, mitochondria are the preferred target for intervention. So far various mitochondria-targeting drugs have been developed and applied and delightfully some of them demonstrate promising outcome. In present review, we will summarize and address 1) how to design mitochondria-targeted drugs, 2) what is the respective rescue mechanism of the drugs, 3) how to assess the therapeutic effect.

2. Strategy for mitochondria targeting

Mitochondria are the pivotal organelle of cells with unique structure formed by two lipid-bilayer membranes. Drugs with mitochondrial targeting ability could not only enhance the efficiency but also minimize side effects induced by distribution in the cytoplasm. Efficient and specific mitochondrial targeting is the precondition to develop drugs for mitochondrial related diseases such as PD, AD and cancer. To achieve mitochondria targeting, many strategies have been proposed and verified to be feasible (Fig. 1 and Table 1[7−30]).

2.1. Small molecule facilitated mitochondria targeting

2.1.1. Triphenyl phosphonium cation (TPP^+)

There is a large transmembrane potential of 120−140 mV (negative inside) in mitochondria, which could be utilized to drive the positively charged molecules to mitochondria. TPP^+, one kind of lipophilic cations, could easily pass-through lipid bilayers duo to the existing of the potential gradient. The uptake of lipophilic cations into mitochondria increases 10-fold for every 61.5 mV of membrane potential at 37 °C leading to 100−500-fold accumulation[7]. Many mitochondrial targeted drugs are developed with TPP^+ assistance, including Mito-Q, Mito-Vit-E, 2,2,6,6-tetramethyl-4-[5-(triphenylphosphonio)pentoxy]piperidin-1-oxy bromide (Mito-TEMPOL). Mito-Q was prepared by conjugating ubiquinone to TPP^+ moiety, which could be taken up rapidly by mitochondria due to the existence of $\Delta\psi$[8]. Mito-Q has been widely used as antioxidants[7] to preventing cell death. Mito-Vit-E was constructed by conjugating vitamin E and TPP^+, and the latter drove the compound to mitochondria. Mito-Vit-E as antioxidant was also proved to invigorate mitochondrial function and prevented cell death[9] Mito-Q and MitoVit-E, at low concentrations, showed higher efficiency in protecting cells against peroxide-induced oxidative damage and apoptosis, compared to the untargeted antioxidants (*i.e.*, Vit-E and ubiquinone-10). In addition, the antioxidation ability of Mito-Q was affected by membrane potential, while MitoVit-E was not affected[10]. Mito-TEMPOL was a well-known mitochondria-targeted superoxide scavenger. Mito-TEMPOL treatment could attenuate ATP depletion mediated necrosis and apoptosis by preserving mitochondrial integrity and decreasing BAX translocation to mitochondria[11].

Besides utilized in preparing compounds, TPP^+ was also applied in the nanoparticles (NPs) construction, including functionalized liposomes, dendritic polymers, cilia, silica, which endowed the NPs with mitochondria targeting capacity. Following liposomal formation with conventional method, TPP^+ was decorated on the surface of liposomes to get functionalized TPP^+-liposomes. To reduce the cytotoxicity of liposomes, dimyristoyl triphenylphosphonium (DM-TPP) was admitted, which did not compromise its ability to target mitochondria[12]. Dendritic polymers were widely used in drug delivery due to its capacity of encapsulating drugs. Presence of TPP^+ moiety on the surface of dendrimetric core enabled mitochondria targeting and rendered better drug delivery effectiveness[13]. Hyeon's group synthesized small and positively charged TPP^+-ceria NPs capable of localizing to mitochondria in various cell lines. TPP^+-ceria NPs were biocompatible and scavenged mitochondrial reactive oxygen species (ROS) efficiently to reduce oxidative stress *in vitro* and *in vivo*[14]. This kind NPs was single function and could just remove ROS, which greatly limited its application. TPP^+ was further incorporated into universal and biodegradable silica nanoparticles (BS-NPs) to deliver functional proteins into the mitochondria, which showed higher efficiency in drug packaging, protection of protein activity and controlled release in mitochondria[15].

2.1.2. Pyridinium salt

Pyridinium salts are another type of commonly utilized mitochondrial targeting groups with positive charge and lipophilicity. Unlike TPP^+, which is modified with long alkyl chain links, pyridine salts are often modified with ethylenic bonds to form large conjugated systems. The conjugated systems and electron-withdrawing ability of pyridine salt can produce or regulate molecular luminescence, which is applicable for the optical detection of mitochondria. However, lipophilicity of pyridine salts hinder its accessibility to tissues *in vivo*. To cope with this issue, Cheng's group developed human serum albumin facilitated pyridine salt complex which easily accessed to tumor tissue with mitochondria targeting ability and induced tumor cell apoptosis[16], which achieved a breakthrough in pyridinium salt *in vivo* application. The cytotoxicity of the pyridine salt molecule was a defect of its mitochondrial detection, but it displayed advantages in tumor treatment.

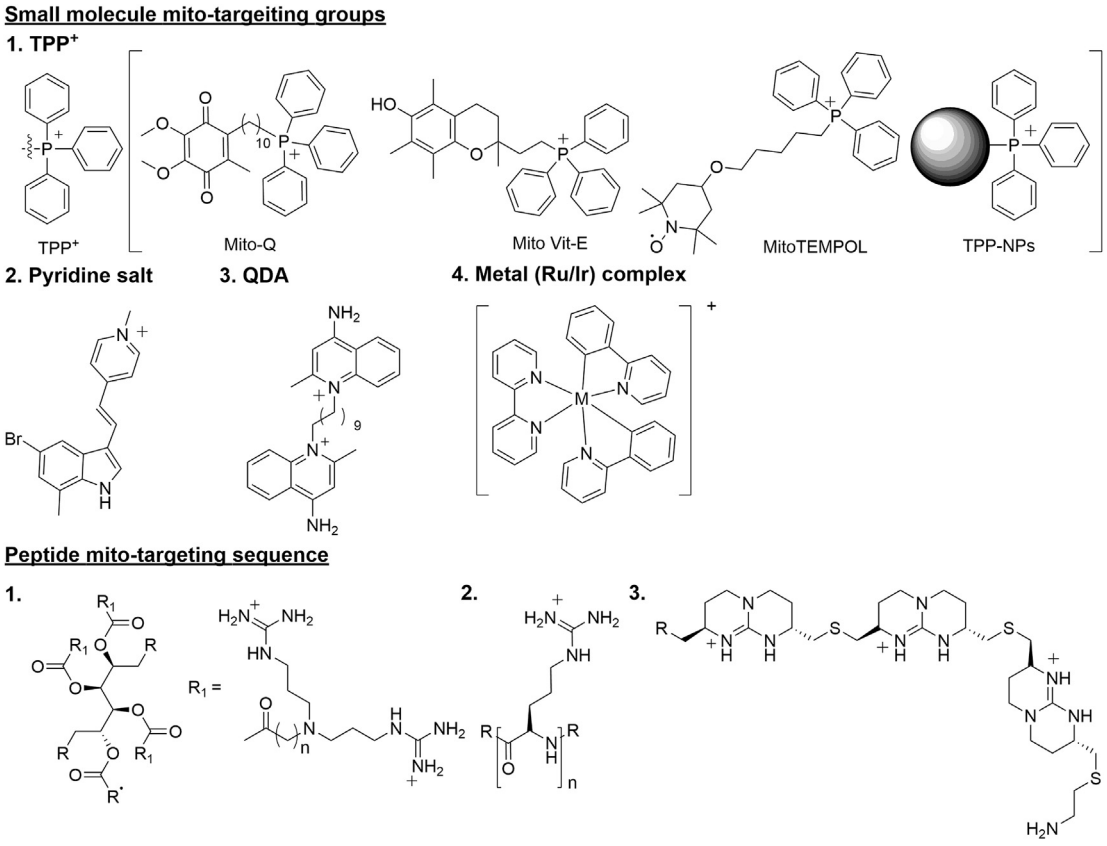

Figure 1 The structures of small molecules and peptides with mitochondria targeting ability.

2.1.3. *DQA/DOA somes*

Dequalinium (DQA) is a cationic bola amphiphilic composed of two quinaldinium rings linked by ten methylene groups which can self-assemble into liposome-like vesicles DQA-based liposomes (DQAsomes). DQA exhibited mitochondria-targeted ability and could induce ROS production by inhibiting ATP synthesis. DQA has been demonstrated to synergize the antitumor effects of tumor necrosis factor (TNF) against the growth of rat colon tumor isografts[17]. DQAsomes exhibited mitochondria-targeting ability and selectively delivered biologically drugs (*e.g.*, DNA) to mitochondria of mammalian cells[18]. However, the low endosomal escape ability and transfection efficiency of DQAsomes limit its application in mitochondria-targeted delivery.

2.2. *Metal complexes*

Metal complexes were reported to be able to quickly penetrate the membrane and highly accumulate in mitochondria due to its high positive charge and good lipophilicity[19]. So far most of the metal complexes were prepared for anti-tumor. Hetero-binuclear iridium-platinum (Ir-Pt) and iridium-ruthenium (Ir-Ru) complex were found to induce cell death *via* mitochondrial DNA damage, metabolism alteration and mitochondrial superoxide accumulation[19,20], which served as a dual function, mitochondria-targeting, photoactivated chemotherapy (PACT) and photodynamic therapy (PDT) agent. Noteworthy, these mitochondria-targeted complexes contain heavy metals, which makes it difficult to be used *in vivo* or in clinical trials duo to the toxicity of heave metals.

2.3. *Mitochondria targeting peptides*

Peptide is another tool used for facilitating mitochondria targeting. Peptide-based delivery scaffolds offer attractive features such as ease of synthesis, tunability, biocompatibility, and high uptake both in cell and *in vivo*. Owing to its advantages, peptides are highly adaptable for delivering chemically diverse cargo. Mitochondria targeting peptides normally are cationic charged and lipophilic, which allows them to harness the negative membrane potential of mitochondria, and favorably interact with hydrophobic membranes of mitochondria. Compared with TPP^+ and pyridinium salt, peptides did not cause mitochondrial depolarization at millimole concentrations, whereas TPP^+/pyridinium salt caused cytotoxicity at concentrations higher than 10 μmol/L. And peptides were more suitable for disease treatment and drug delivery in the living body for their biocompatibility. The structures of common peptides targeting mitochondria are showed in Fig. 1.

2.3.1. *Szeto-Schiller (SS) peptides*

The SS tetra peptides represent a series of mitochondria targeting peptides that feature a common structural motif of alternating aromatic and basic residues. The antioxidant properties of SS-02 and SS-31 likely originate from their dimethyl tyrosine (Dmt) residues. More specifically, tetra peptides SS-31 and SS-02 were found to be equally effective in scavenging H_2O_2 and inhibiting linoleic acid oxidation *in vitro*. The basic residues served for localization in the inner mitochondrial membrane, and the dimethyl tyrosine phenol moieties of SS-02 and SS-31 were likely

Table 1 The list of mitochondria targeting molecules and its targeting mechanism.

Chemical nature	Group	Molecule	Targeting mechanism	Advantage/disadvantage	Ref.
Small molecule	TPP^+	Mito-Q; Mito-Vit-E; Mito-TEMPOL; TPP^+ functionalized liposomes; Ceria nanoparticle; Silica nanoparticles	Positive charge and lipid solubility	Easy to synthesis/cytotoxicity for drug delivery	7−15
Small molecule	Pyridinium salt	5BMF@HSA complex	Positive charge and lipophilic, HSA increasing water solubility	Easy to synthesis, tunable luminescence/ cytotoxicity for drug delivery	16
Small molecule	DQA	QDAsomes	Amphipathic	Easy to synthesis, cytotoxicity for drug delivery/low endosomal escape ability and transfection efficiency	17,18
Small molecule	Ru/Ir	Ru/Ir complexes	Positive charge and lipophilic	Cytotoxicity for cancer therapy/ multimodal therapy	19,20
Peptide	S-S peptides	SS-02, SS-31	Positive charge and lipophilic	Hypotoxicity, antioxidant/complex	21
Peptide	MPP	MPP	Positive charge and lipophilic	Hypotoxicity, tunability, desirable pharmacokinetic profiles/complex synthesis	22,23
CPMs	CPM	CPM1, CPM2, CPM3	Amphipathic	Efficient and universal delivery of cargos/complex synthesis	24
Mito-Porter	Octa arginine	(DF)-Mito-Porter, ASO-Mito-Porer, DOX-Mito-Porter	Lipid compositions promote its fusion with the mitochondrial membrane	Efficient and universal delivery/ complex synthesis	25 −29
Gramicidin S	Gramicidin S	XJB-5-131	High affinity for the membrane	Antioxidant/complex synthesis	30

responsible for chemically reducing reactive oxygen species and peroxide bonds. SS tetra peptides displayed anti-oxidation property. Peptides SS-02 and SS-31 were demonstrated to ameliorate apoptosis of t-BHP-treated cells. In an *ex vivo* reperfusion study of guinea pig heart, both SS-02 and SS-31 were able to prevent myocardial stunning and significantly improved contractile force[21].

2.3.2. *Mitochondria-penetrating peptides (MPPs)*

The MPPs are cationic and lipophilic which facilitate its permeation into the hydrophobic mitochondrial membrane. Mitochondria targeting capacity of MPPs could be finely tuned by altering lipophilicity and charge[22]. MMPs did not show obvious cytotoxicity even at high concentrations. But MMPs could efficiently target mitochondria and facilitate robust delivery of bioactive compounds, such as drugs, antioxidants, and photosensitizers, to achieve the treatment of disease[22,23]. Numata's group reported that low concentrations of peptides were sufficient to deliver DNA into the mitochondria and rendered protein expression in a short incubation period[23]. The advantages of MPPs facilitated delivery included tunability, ease of synthesis, desirable pharmacokinetic profiles *in vitro* and *in vivo*.

2.4. *Cell-penetrating motifs (CPMs)*

CPMs are consisted with four guanidinium groups and one or two aromatic hydrophobic groups (naphthalene) assembled through a central scaffold (a benzene ring). CPMs showed high efficiency of mitochondria delivery and with minimal effect on the viability or the mitochondrial membrane potential of mammalian cells[24]. They efficiently and specifically delivered small molecules, peptides, and other cargoes into the mitochondrial matrix of mammalian cells with greatly enhanced anticancer activity.

2.5. *Mito-Porter*

Liposome-based carrier (Mito-Porters) was first reported in 2008 by Harashima's group. Mito-Porters carried with octa arginine modifications to facilitate entering cells *via* micropinocytosis and the lipid compositions promoted both its fusion with the mitochondrial membrane and the release of its cargo to the intramitochondrial compartment in living cells[25]. In 2012, Dual Function (DF)-Mito-Porter encapsulating DNase I was constructed, which effectively delivered the DNase I into the mitochondria and achieved selective mitochondrial genome edition[26]. Mito-Porters displayed delivery ability for different kinds of molecules, and was utilized for drug delivery in mitochondrial disease. In 2019, Harashima's group reported the efficient packaging of ASO in the Mito-Porter *via* a nanoparticle packaging method, which showed a 10-fold higher delivery efficiency than the conventional method[27]. In 2020, Harashima's group encapsulated DOX in the Mito-Porter, which led to enhanced DOX accumulate in tumor mitochondria[28]. Mito-Porter facilitated wild-type mitochondrial pre-tRNAPhe (pre-WT-$tRNA^{Phe}$) transfection into disease associated mitochondrial cells. The mutation rate of $tRNA^{Phe}$ was decreased, and therapeutic effect sustained for more than one week[29].

2.6. *XJB gramicidin S (GS) analogs*

XJB peptides are derived from membrane-active GS antibiotics which had a high affinity for the mitochondria membrane. The mitochondrial targeting portion of XJB-5-131 consists of the

Leu−DPhe−Pro−Val−Orn fragment of GS and antioxidant 4-amino-TEMPO (4-AT). The nitrogen-oxide radicals in XJB-5-131 accepted an electron and converted them into hydroxylamine, which scavenged ROS. McMurray's group reported that XJB-5-131 eliminated mitochondria ROS[30].

3. Rescue effect and mechanism of mitochondria targeting drugs for NDDs

Given the crucial role of mitochondria dysfunction in pathogenesis of NDDS, mitochondria targeting drugs especially attract the interest of clinician and researchers. So far, abundant kinds of drugs have been designed and prepared which displayed gratified effects. To better understand the rescue effect and mechanism of mitochondria targeting drugs, we classify them into four categories based on the biological nature of the drug targets: mitochondrial nucleic acid, mitochondrial protein, small molecule, and metal ions (Table 2[27,31−55]). The pharmacological mechanisms of drugs are elaborately described.

3.1. Drugs targeting mitochondria nucleic acid

Mitochondria, different from other organelles, have its own DNA, mitochondria DNA (mtDNA). mtDNA encodes 22 tRNAs, 2 rRNAs and 13 mRNAs which dominate the translation of electron transport chain proteins, mitochondrial inner and outer membrane proteins[56]. Mutation of mtDNA is implicated in the pathogenesis of NDDs. Therefore, drugs targeting mtDNA repairing or editing are developed and applied for the treatment of NDDs.

Gammage et al.[31] used the zinc finger nuclei (ZFNs) structure to knockout mtDNA with disease associated mutation. Transcription activator-like factor nucleases (TALENs) were also reported to edit mtDNA and effectively ameliorated phenotypes of disease[32]. Both ZFNs and TALENs can recognize and eliminate the mutated sequence of mtDNA by nuclease. Regretfully it can only delete aberrant mtDNA, but it cannot correct or insert deficient mtDNA sequence. In 2012, one novel gene editing technique, CRISPR/Cas9 was developed, which provided alternative way for mtDNA editing especially when combined with mitochondria targeting strategy. Jo et al.[33] constructed mitochondrial targeting sequence (MTS) conjugated Cas9 (MTS-Cas9) which realized mtDNA edition and interrupted mitochondria protein expression. In 2019, Bian et al.[34] constructed a mito-CRISPR/Cas9 system which inserted a single stranded homologous DNA into mtDNA, which for the first time achieved mutated mtDNA reparation by homologous recombination strategy. In 2020, Mok et al.[35] constructed DddA-derived cytosine-based editor (DdCBE) by introducing a mitochondrion targeting internal toxin DddA. DdCBE penetrated into inner membrane of mitochondria, and catalyzed the mtDNA CG to AT conversion. Kawamura et al.[27] constructed a Mito-Porter to silence the target mtRNA which rendered knockdown of complex II RNA. To date, a number of systems for gene editing have been developed and achieved therapeutic effect in hereditary NDDs. For example, ISoldner et al.[36] used ZFNs to repair A53T mutation of *SNCA* gene in patient-specific induced pluripotent stem cells (iPSC) in familial Parkinson's disease with *SNCA* gene mutation. An et al.[37] used TALENs technology to replace pathogenic CAG repeats with normal repeats gene sequences through homologous recombination in iPSC. Merienne et al.[38] used KamiCas9 self-inactivating CRISPR/Cas9 system to inactivate mutant huntingtin in Huntington's Disease (HD) mice model. Regretfully, those gene editing strategy had not been endowed with mitochondria targeting ability.

Though mtDNA editing techniques have been proved to be feasible for intervention of mitochondria genes. There were still gaps in applying them into NDDs treatment, indicating more efforts are needed to broaden it in the future. The mitochondrial genome-targeted edition represents one promising therapeutic candidate for treatment of mitochondrial related diseases including NDDs.

3.2. Drugs targeting mitochondria proteins

Mitochondria proteins, including respiratory chain complexes I to IV, ATP synthase, mitochondria membrane protein, are the executors of mitochondria function. Dysfunction of those protein is closely related to the pathogenesis of NDDs. Some proteins, such as PARKIN, PINK1 though not mitochondria derived ones, dysfunction of which are also implicated in NDDs. Drugs targeting those proteins exhibit promising therapeutic effects for NDDs.

3.2.1. Drugs targeting mitochondria membrane proteins (MMPs)

MMPs played crucial roles in maintaining the integrity and permeability of mitochondria and disruption of which often leads to neurons apoptosis and consequent NDDs[6]. BCL-2 family proteins, including BCL-2, MCL-1, BAX, BIM, BAD, were typical MMPs mediating the integrity of mitochondria outer membrane. BCL-2 and MCL-1 represented anti-apoptotic proteins, whereas BAX, BIM, BAD represented pro-apoptotic proteins, and imbalance of them could result in cell apoptosis[37]. Analogues of BCL-2/MCL-1/BCL-xL or inhibitors of BAX/BAD/BIM are widely applied in the treatment of NDDs.

Lopez et al.[39] designed a BAX-inhibiting peptide V5 (Bip-V5) which displayed anti-apoptotic effect in rat 6-OHDA induced PD model. Mesencephalic astrocyte-derived neurotrophic factor (MANF) was proved to be an inhibitor of proapoptotic BAX protecting neurons in rat 6-OHDA model[40]. In addition, kukoamine A (KuA) also reduced the BAX/BCL-2 ratio, thereby inhibiting the apoptosis of neurons in MPP^+ induced animal models[41]. Voltage dependent anion channel 1 (VDAC1) is a mitochondria outer membrane protein and overexpression of VDAC1 enhances mitochondria permeability and subsequent cell apoptosis. VDAC1 inhibitors, VBIT-4, were reported to prevent the cell apoptosis *via* decreasing cellular ROS and Ca^{2+}[42]. Shteinfer-Kuzmine et al.[43] found that inhibitory peptide of VDAC1 could ameliorate phenotype of Amyotrophic Lateral Sclerosis (ALS).

3.2.2. Drug targeting electron transport chain (ETC) proteins

ETC proteins are key mediator of oxidative phosphorylation and energy conversion in mitochondria, which are classified as complexes I, II, III and IV. Complexes I and II catalyze the transfer of electrons from nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide (FADH2) to ubiquinone (UQ), complexes III transfer electrons from ubiquinone to cytochrome *c* (Cyt *c*), and complexes IV transfer electrons from Cyt *c* to O_2. Dysfunction of ETC protein would compromise mitochondrial oxidative phosphorylation and lead to NDDs[57]. Hence,

Table 2 The list of drugs targeting mitochondria for the therapeutics of NDDs.

No.	Drug	Application	Target	Mechanism	Ref.
1	MtZFNs	Zebra fishes Hek 293T cells	mtDNA	Bind and cleave the mutated DNA and retains the wild type mtDNA	31
2	Mito TALENs	iPSC cells	mtDNA	Deletion sites for selective cleavage of mtDNA	32
3	Mito CRISPR/Cas9	HEK-292T cells	mtDNA	Mitochondrial specific CAS 9 editing mtDNA m.3243A>G gene sites or knock in homologous recombination strategy to repair mutated mtDNA	33,34
4	DdCBE	HEK293T cells	mtDNA	Specifically catalyzes the transformation of CG base pairs of mtDNA into AT	35
5	Mito-Porter	Hela cells G625A cells	mtRNA	Knockdown the mRNA encoded complex II or introduce mito codon regulation system	27
6	ZFNs	iPSC cells	*SNCA* gene	Reprogram A53T mutation in iPSCs	36
7	TALENs	iPSC cells	CAG gene	Replace pathogenic CAG repeats with normal repeats gene sequences through homologous recombination	37
8	CRISPR/KamiCas9	HD mice model	hHTT-82Q gene	sgHTT1 targeting *HTT* gene with the aim of permanently blocking leading to HTT silencing	38
9	Bip-V5	Rat 6-OHDA PD model	BAX/BAK	Inhibit BCL-2	39
10	MANF	Rat 6-OHDA PD model	BAX	Inhibit BAX activation	40
11	KuA	Mice MPP^+ model	BAX/BCL-2	Reduce BAX/BCL-2 ratio	41
12	VBIT-3, VBIT-4	MEF cells	VDAC1	Inhibition of VDAC1 oligomerization	42
13	VDAC1 N-terminal peptides	NSC-34 cells	VDAC1	Interact with VDAC1, reduce channel conductance	43
14	Nobiletin	Rat model	Complex I	Restored the activity of complex I	44
15	NaHS	Animal model	ETC	Mediated oxygen consumption rate	45
16	Compound A	Rat PD model	Complex II	Blocked BIM-induced apoptosis after BAX is activated on the mitochondria	46
17	rT1	COS-7 cells	ATP synthase	Through TOM20 targets mitochondrial membrane ATP synthase	47
18	Nicotinamide riboside/NR	A-T fibroblasts, $Atm^{-/-}$ mice	NAD^+	Boosted NAD^+ levels by promoting mitophagy in a PINK1-dependent manner	48
19	Rapamycin	SCIRI mice model	mTOR	Promoted mitophagy and attenuating SCIRI-induced apoptosis	49
20	Urolithin A	*C. elegans* AD models	PINK1	Stimulated mitophagy, suppressed of neuroinflammation	50
21	Rilmenidine	ALS mice	SOD1	Induced autophagy, promoted autophagic clearance of mutant SOD1 and efficient mitophagy	51
22	Mito Q	Rat LTP model	ROS	Mitochondria-targeted antioxidants	52
23	Mito-TEMPO	Primary microglia	ROS	Inhibit the increase of TNF-α, IL-1β, IL-6 and iNOS	53
24	SS peptides	Caco2 cells	ROS	Targeted delivery of antioxidants to the inner mitochondrial membrane	54
25	Pyridoxine	PC12 cells	GSH	Alleviated the significant decrease of GSH to maintain the activity of complex I	55

compounds that could destroy ETC were widely utilized to create *in vitro* or *in vivo* NDDs models. Whereas, drugs that could protect the ETC exhibited rescue effects on NDDs. Amarsanaa et al.[44] reported that nobiletin restored the activity of rat brain neurons by restoring complex I activity. Kumar et al.[45] proposed that NaHS could increase the mitochondrial complex I, II and IV mediated oxygen consumption rates in hyperhomocysteinemia (HHCY) animal model. Jiang et al.[46] designed and synthesized a small molecule compound A which targeted the succinate dehydrogenase subunit B (SDHB) of complex II, and prevented the death of dopaminergic neurons in rat PD model. Kam et al.[47] extracted a cysteine rich peptide, rT1, from hibiscus syriacus, and proved that rT1 could prompt ATP synthase and cell survival by targeting ETC.

3.2.3. Drugs targeting mitochondria quality control related proteins

Mitochondria maintain the dynamic balance between biogenesis and clearance, which is important for cell survival. Mitochondrial biogenesis is a process of new mitochondria producing which is mainly regulated by PGC-1α and its downstream NRF1/2. The damaged or dysfunctional mitochondria are degraded by autophagy, termed as mitophagy, and deficiency of mitophagy is thought to be one causative factors of NDDs. So far PINK1/PARKIN mediated mitophagy was well defined. As a kinase, the activation of PINK1 causes the phosphorylation of ubiquitin, further leads to the activation of parkin, so that mitochondrial proteins are ubiquitinated, and finally cause mitochondrial degradation. Triggering mitochondria biogenesis or prompting

mitophagy is one therapeutic strategy. Curcumin or pigment epithelium-derived factor (PEDF) rescued PD models *in vitro* and *in vivo via* stimulating mitochondria biogenesis[58]. Adiponectin (APN), a multi-functional adipokine which sensitizes the insulin signals, was proved to stimulate mitochondria biogenesis in AD[59]. Yang et al.[48] showed that nicotinamide riboside (NR) prevented neurodegeneration by promoting mitophagy in PINK1-dependent manner. Memantine was found to enhance mitophagy in pluripotent stem cell derived neurons from PD patients. Li et al.[49] demonstrated that rapamycin, a pharmacological inhibitor of mTOR, attenuated spinal cord injury by promoting mitophagy. Urolithin A or actinonin treatment stimulated mitophagy and reversed memory impairment in AD models[50]. Rilmenidine, an anti-hypertensive agent, administration promoted mitophagy in mutant SOD1 induced ALS mice[51].

3.3. Drugs targeting redox of mitochondria

Mitochondria provide most of the energy to maintain the cells survival and function. Producing together with ETC, the majority of mitochondria ROS takes part in various cellular physiological processes, such as stimulating lipid peroxidation, stimulating cellular calcium signal and regulating mitochondrial calcium uptake[60]. The healthy cells have its own antioxidant system, including superoxide dismutase, glutathione peroxidase and peroxidase, to keep the redox balance. However, when genetic or environmental factors lead to excessive ROS generation or deficient antioxidant, the redox balance will be broken and diseases such as NDDs occur. So, reducing ROS or increasing antioxidant represent one way for conquering NDDs.

A variety of antioxidants targeting ROS have been reported for the treatment of NDDs. Mito-Q was synthesized by covalently linking ubiquinone to mitochondria targeting moiety TPP^+, and the later one facilitated its penetration to mitochondria. Mito-Q displayed rescue effect in AD, PD and Huntingtin diseases models by scavenging ROS[61]. SkQ1 is also a quinone based antioxidant with mitochondria targeting features, and its scavenging rate of lipid peroxidation was four times that of Mito-Q and showed neuroprotective effects[52]. Mito-TEMPOL is another widely used mitochondrial targeted antioxidant. It cannot only scavenge ROS, but also oxidative iron. Kang et al.[53] computationally designed and prepared peptide-based antioxidant, CAMP-HMT1A by fusing protein human metallothionein 1A (HMT1A) with a cell penetrating artificial mitochondrial targeting peptide (CAMP). CAMP-HMT1A alleviated mitochondria damage and movement impairment of PD model. SS-31 is also one antioxidant peptides which can target the delivery of antioxidants to the mitochondrial inner membrane, the site of ROS production[54]. Natural products, such as naringenin, bruceine, and tanshinone, conferred neuroprotection in NDDs models by stimulating glutathione (GSH) production. Pyridoxine, also known as vitamin B_6, was found to promote GSH biosynthesis and alleviate manifestation of MPTP induced PD mice[55] (see Fig. 2).

4. Evaluation the rescue effect of mitochondria targeting drugs for NDDs

After administration of the drugs, its rescue effects on NDDs need to be evaluated according to outcome of models or patients. Given that there is no one standard program applicable to assess all the subjects, we will address the evaluation from three aspects: molecular level, cellular level and organism level (Table 3).

4.1. Molecular level evaluation

When drugs exert its therapeutic effects on NDDs, it would inevitably lead to the change of mitochondria related molecules, such as proteins, mtDNA, ATP, radicals, mitochondrial membrane potential (MMP).

4.1.1. Adenosine triphosphate (ATP)

The fundamental role of mitochondria is to produce ATP. So, ATP is the key parameter to assess the effect of mitochondria targeting drugs. ATP level could be directly assessed by ATP fluorescence detection kit with the help of microplate reader[62]. Cell Titer Glo Luminescent reagent can detect intracellular ATP concentration by luciferin-luciferase reaction[63]. ATP level in different cellular compartments could be monitored with the fluorescence resonance energy transfer (FRET)-based genetically encoded indicators, such as AT1.03 composed of the epsilon subunit of the bacterial F_oF_1-ATP synthase[64]. Extracellular and intracellular ATP could be visualized by the single-wave length genetically encoded fluorescent sensors (iATPSnFRs). $iATPSnFR^{1.0}$ responded to relevant ATP with enhanced fluorescence of specific cell types and subcellular compartments[65]. Wu et al.[66] reported a new series of genetically encoded G protein-coupled receptor activation-based (GRAB) sensors for monitoring the ATP in targeted cells, called $GRAB_{ATP1.0}$. $GRAB_{ATP1.0}$ realized ATP detection with high sensitivity, selectivity, and spatiotemporal resolution. Recently, an aptamer-based fluorescence ATP probe was reported, which could sensitively detect ATP in cells as well as cardio-tissue[67]. ATP could also be indirectly determined by measuring the oxygen consumption rate (OCR)[68] and the extracellular acidification rate (ECAR)[69].

4.1.2. Free radicals

Free radicals, partially generated from mitochondria, are endowed with strong oxidation and high chemical reaction activity. Free radicals could be detected by spectrophotometry. Moreover, fluorescent probes such as DCFH-DA[70,71], H2DCFDA[72,73], DHR[74], DCF[75] could be used to detect ROS and reactive nitrogen species. With the help of flow cytometry or confocal microscopy, free radicals could also be quantified. ROS production was also determined by Amplex Red horseradish peroxidase and Electron Paramagnetic Resonance (EPR) spectroscopy[70,71]. GSH was one of the most abundant antioxidants in cells, and was essential to maintain redox balance of mitochondria. The content of GSH was determined by GSH kit, which indirectly reflected the ROS level[72].

4.1.3. Oxidoreductases

Oxidoreductases such as superoxide dismutase (SOD), lactate dehydrogenase (LDH), cytochrome oxidases (CCO) are the enzymes that catalyze the redox reaction. Activity or level of SOD/LDH reflects the mitochondria function as well as the effect of drugs. SOD could be detected by Western blot[62] or fluorescent probes such as MitoSox[59,72]. LDH levels can be determined directly using the LDH assay kit and CCO could be detected by Western blot or histochemistry staining[62].

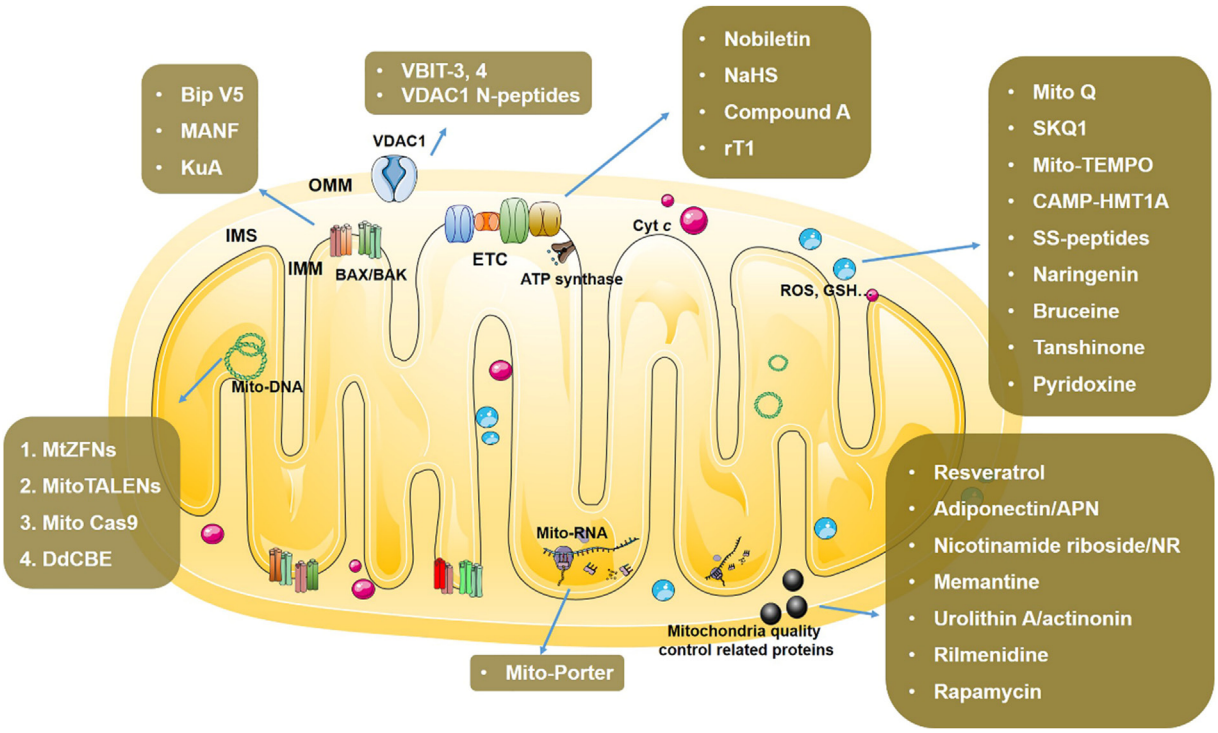

Figure 2 NDDs drugs with specific mitochondrial targets.

4.1.4. *Mitochondrial membrane potential (MMP, ΔΨm)*

MMP is an important indicator of mitochondrial function and integrity. Physiological MMP (ΔΨm 120−140 mV) allowed routine ATP production and loss of MMP led to apoptosis by efflux of macromolecules like Cyt *c* and caspase-9/caspase-3 cascade activation[76]. MitoTracker (Invitrogen), tetramethylrhodamine methyl ester (TMRM)[77], and other dyes can be used to assess MMP by fluorescence spectrophotometry[78]. Noteworthy, some dyes such as rhodamine B methyl ester (RhB-ME) was thermochromic, which might interfere the detection of MMP. The temperature-insensitive dyes such as rhodamine 800 were preferred[79]. In addition, fluorescent probes such as JC-1/JC-10 can also assess MMP by bioimaging or flow cytometry[72].

4.1.5. *Mitochondria DNA (mtDNA)*

Mutation or deletion of mtDNA contributes the pathogenesis of NDDs. mtDNA sequence and copy numbers can be checked by quantitative RT-PCR. Moreover, there are enzymes involved in mitochondrial DNA replication, such as DNA polymerase γ, mtDNA single-stranded DNA-binding protein 1 (SSBP1) and TWINKLE. These enzymes can be determined by Western blot to reflect the ability of mtDNA replication[62].

4.1.6. *Mitochondrial complex*

Mitochondria complexes are the electron carrier in charge of electron transfer during mitochondrial oxidative phosphorylation. The expression level of the mitochondrial respiratory chain complexes could be detected by the immunohistochemical analysis. The activity of the mitochondrial respiratory chain complexes could be examined by specific kit, and spectrophotometric assays[80]. Besides the classical spectrophotometric analysis, the blue native polyacrylamide gel electrophoresis (BN-PAGE) was also a useful tool for visualizing the activities of mitochondrial phosphorylation enzymes[81].

4.2. *Cellular level evaluation*

Besides biomolecules, the structure of mitochondria, the cell viability and morphology can also reflect the effects of mitochondria targeting drugs.

4.2.1. *Cell viability and the cell morphology*

Progressive neurodegeneration is the feature of NDDs. The neurons viability assessment tells the effectiveness of mitochondria drugs, which can be detected by CCK8 or MTT assay[59]. Meanwhile, Annexin V-fluorescein isothiocyanate (Annexin V-FITC)/propidium iodide (PI) double-staining can be adopted to determine the cell viability *via* flow cytometry or fluorescent microscope. Annexin-V positive and PI negative cells were defined as early necrotic cells while Annexin-V negative and PI positive cells were regarded as late necrotic cells[82]. TUNEL assay also detects apoptotic neurons[83]. Cytoplasm atrophy and nucleus condensation are typical morphology of neurons under apoptosis. Rhodamin and Hoechst 33258 staining, haemotoxylin and eosin (H&E) staining, immunostaining for neuronal markers[84], rhodamine-phalloidin staining[85], Golgi staining[86], silver staining[87], Nissl staining[88], Luxol fast blue (LFB) staining and Masson staining[171], and Virus-based neural tracer technology[89] are used to display cell morphology more intuitively.

4.2.2. *Mitochondrial morphology*

Mitochondria are highly dynamic organelles that constantly undergo fission and fuse to ensure mitochondria quantity control, which is regulated by proteins, such as DRP1 and MFN2. If this process goes wrong, cells may subject to apoptosis as happened in

Table 3 Table of assessment.

Level	Target	Assay
Molecular level	ATP	Luminescent ATP detection assay kit; the O_2 consumption rate (OCR); the extracellular acidification rate (ECAR); cell titer Glo luminescent reagent; luciferin−luciferase reaction; fluorescence probes
	Free radicals	Specific fluorescent probes (DPPH, DCFH-DA, H2DCFDA, DHR, DCF); flow cytometry analysis; ESR; confocal microscope; spectrophotometry; chemiluminescence
	GSH	GSH assay kit; spectrophotometry
	SOD	Western blot; MitoSOX (specific fluorescent probe); immunoblotting
	LDH	LDH assay kit
	CCO	Spectrophotometer; Western blot; quantitative cytochrome oxidase (CCO); histochemistry
	MMP	Tetramethylrhodamine methyl ester (TMRM); 123-rhodamine; flow cytometry analysis; rhodamine 800; JC-1/JC-10
	mtDNA	Western blot; DNA polymerase γ; mtDNA single-stranded DNA-binding protein (SSBP1); twinkle
	Mitochondrial complex	Spectrophotometer; immunohistochemistry; spectrophotometric enzyme assays; microplate assay kit; BN-PAGE
Cellular level	Cell viability and cell morphology	CCK8; MTT; TUNEL assay; AnnexinV-FITC/PI staining; Co-IP; flow cytometry; immunofluorescence; Hoechst and rhodamin staining; Golgi staining; silver staining; Nissl's staining; virus-based neural tracer technology; LFB and Masson staining
	Mitochondrial morphology	Transmission electron microscopy (TEM); Mito trackers; JC-1; DRP1 or MFN2 immunofluorescent imaging; immunofluorescent staining of TOM20/TIM23
	Axons, dendrites and synapses	Immunofluorescence analysis; TEM; MAP-2 or β-tubulin staining; Golgi's staining; rhodamine-phalloidin; Nlgn1 or Nrxn3 staining
	Electrophysiological Feature	Whole-cell patch-clamp; brain slices patch-clamp; multichannel electrophysiological
Organism level	Memory assessment	Y-maze test; Barnes; Morris water maze; visual water task; water maze reversal; NOL test; NOR test; long-term potentiation (LTP)
	Locomotor assays	Open field test; pole test; beam hang test; catalepsy measurement; passive avoidance test; rotarod task; forced swimming test; gait analysis; balance beam test; climb test; curling; BBB scale
	Brain imaging	CT; FMRI; DTI

NDDs. The morphology mitochondria can be observed using a transmission electron microscope (TEM). There are many mito-trackers developed so far, which can label mitochondria specifically and confer the morphology visible under microscope. DRP1 or MFN2 immunofluorescent imaging can also be used to observe the dynamic change of mitochondria. Swelling is one morphological manifestation of mitochondria suffered in NDDs, which was checked by immunofluorescent staining of TOM20/TIM23[90].

4.2.3. Axons, dendrites and synapses

Neurons have some specific structures, such as axons, dendrites and synapses, to maintain their physiological functions. Synapses lose and axon degeneration often occurs in neurons of NDDs, and neurite deficit impairs neural connectivity and brain function. MAP-2 or β-tubulin are often utilized to label the axons so as to determine their structures and integrity in NDDs. Golgi's staining is commonly used to demonstrate the entire neuronal morphology including its axon, dendrite, and synaptic spine. Monomeric fluorescent protein co-transfected with specific dendritic spine protein is utilized to label the dendritic spine of dissociated neurons. Immunofluorescent assay of rhodamine-phalloidin can be applied to detect the neurite length[91]. Synapses stained with postsynaptic neuroligin-1 (Nlgn1) or presynaptic neurexin-3 (Nrxn3) can be observed under fluorescent microscope or transmission electron microscope.

4.2.4. Electrophysiological feature

In NDDs, the electrophysiological features of neuron are often altered. So recording and analyzing the electrophysiological activity of neurons provides one window to assess the therapeutic effect of drugs on NDDs. Various techniques are developed to record the electrophysiological features *in vitro* and *in vivo*. Whole-cell patch-clamp recording can observe the electrophysiological properties of single neuron, which help to determine the function and classification of neurons[92]. The patch-clamp recorded action potentials of brain slices representing the plasticity of neurons in learning and memory. Local field potentials (LFPs) recorded from the brain reflected the changes of membrane potential derived from population of neuronal somata located nearby the recording electrode[93]. Multichannel electrophysiological recording technique can be used *in vivo* to monitoring the activity of different neuron populations in real-time with drug administration.

4.3. Organism level

After administration of mitochondria drugs, subjects, including animal or patients, with NDDs will demonstrate manifestations in organism level which can be used to assess the effect of drugs. Mental, memorial, locomotorial indicators are often adopted.

4.3.1. Memory assessment

Y maze test, Morris water maze, visual water task, water maze reversal, the novel object recognition test, and the novel object location test can be used to test the learning and memory abilities subjects. If the mitochondria targeting drugs display rescue effect, those parameters reflecting learning and memory will be improved. The long-term potentiation (LTP) is a long-lasting strengthening of the response of a postsynaptic nerve cell to

stimulation across the synapse that occurs with repeated stimulation and is thought to be related to learning and long-term memory. It is a persistent enhancement in signal transduction between two neurons, which is widely accepted as one of the main molecular mechanisms underlying learning and memory. So LTP assay showed the effect of drugs mechanically.

4.3.2. *Locomotor assays*

Open field test, pole test, beam hang test, step-by-step passive avoidance test, step-down passive avoidance test, rotarod, forced swimming test, gait analysis for stride length, balance beam and climb test are often applied to determine the capability of subjects in sensing of balance, motor coordination, muscle tone and depressive symptoms[94]. Curling was once used to monitor motility defects of *Caenorhabditis elegans* with PD[95]. The Basso, Beattie and Bresnahan locomotor rating scale is currently used to evaluate the functional recovery of locomotor capacity in rats[83]. Locomotor assays are essential to assess the effect of mitochondria targeting drugs for NDDs.

4.3.3. *Brain imaging*

The rescue effect of mitochondria targeting drugs to NDD can also be validated with brain imaging, such as computed tomography (CT), functional magnetic resonance imaging (fMRI), and diffusion tensor imaging (DTI). Those imaging techniques provide solid evidence to show the structure, and function recovery after treatment.

5. Perspective

Mitochondria-targeting drugs have brought hope for effective treatment of NDDs. But there is still a big gap between what are achieved and expected. Novel therapeutic methods are required to better tackle NDDs. Firstly, better understanding the mechanisms underlying NDDs pathogenesis is needed, so as to identify targets of intervention. Secondly, response triggered release of mitochondria targeting drugs is one trend of drug development for improving its specificity and reducing undesired effect[96]. Thirdly, mitochondria transplantation represents alternative strategy for therapeutic of NDDs[97], though the way to harvest functional mitochondria remains to be settled. PROteolysis TArgeting Chimeras (PROTACs), one technique recently designed for specific targeting protein degradation, attracted increasing attention in drug development[98]. Given the characterized protein aggregation in NDDs, PROTACs combined with mitochondria targeting strategy is worthy of expectation for treatment of NDDs. In the future, great efforts need to be taken to combine advantages of different fields such materials, chemistry, and biology and propose better solution strategy of drug development toward NDDs.

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Author contributions

Lin Li, Chengwu Zhang and Li Lu proposed the conception for the review. Jiajia Xu, Wei Du and Yunhe Zhao wrote the manuscript and prepared the tables and figures. Kahleong Lim provide critical suggestion on manuscript preparation. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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