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Power of mitochondrial drug delivery systems to produce innovative nanomedicines

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ABSTRACT

Mitochondria carry out various essential functions including ATP production, the regulation of apoptosis and possess their own genome (mtDNA). Delivering target molecules to this organelle, it would make it possible to control the functions of cells and living organisms and would allow us to develop a better understanding of life. Given the fact that mitochondrial dysfunction has been implicated in a variety of human disorders, delivering therapeutic molecules to mitochondria for the treatment of these diseases is an important issue. To date, several mitochondrial drug delivery system (DDS) developments have been reported, but a generalized DDS leading to therapy that exclusively targets mitochondria has not been established. This review focuses on mitochondria-targeted therapeutic strategies including antioxidant therapy, cancer therapy, mitochondrial gene therapy and cell transplantation therapy based on mitochondrial DDS. A particular focus is on nanocarriers for mitochondrial delivery with the goal of achieving mitochondria-targeting therapy. We hope that this review will stimulate the accelerated development of mitochondrial DDS.

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Abbreviations: ACAD9, acyl-CoA dehydrogenase family member 9; AIFM1, apoptosis-inducing factor 1 mitochondria associated 1; ALT, alanine transaminase; ASO, antisense RNA oligonucleotide; ATP, adenosine triphosphate; CoQ_{10}, coenzyme Q_{10}; COX2, cytochrome *c* oxidase subunit II; CPD, cell-penetrating poly(disulfides); CPP, cell-penetrating peptide; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats/CRISPR; DDS, drug delivery system; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DLCs, delocalized lipophilic cations; DMA, 2, 3-dimethylmaleic anhydride; DQA, dequalinium; DQAplex, DQAsome-pDNA complex; DSPE, 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine; EPR, enhanced permeability and retention; ES cells, embryonic stem cells; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GFP, green fluorescence protein; GSH, glutathione; HA, hyaluronic acid; $HHG2C_{18}$, synthetic zwitterionic oligopeptide lipid; HIV, human immunodeficiency virus; IC_{50}, half maximal inhibitory concentration; iLiNP, invasive Lipid Nanoparticle Production; iPS cells, induced pluripotent stem cells; KH peptides, lysine-histidine peptides; LCST, lower critical solution temperature; LHON, leber's hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes; MEND, Multifunctional Envelope-type Nano Device; MNGIE, mitochondrial neurogastrointestinal encephalopathy; mtDNA, mitochondrial DNA; MTD, mitochondrial transduction domain; MTS, mitochondrial targeting signal peptide; MPP, mitochondria penetrating peptide; NAC, *N*-acetyl-l-cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NDUFV1, NADH dehydrogenase flavoprotein 1; NER, nucleotide excision repair; PAM, polyacrylamide; PBN, phenyltert-butylnitrone; pDNA, plasmid DNA; PDT, photodynamic therapy; PEG, polyethylene glycol; Pgp, P-glycoprotein; Platin-M, Pt(IV)-prodrug of cisplatin; PLGA, poly(lactic-*co*-glycolic acid); PNIPAM, poly(N-isopropyl acrylamide); PTD, protein transduction domain; PTX-*ss*-BBR, berberine with paclitaxel through a disulfide bond; R8, octaarginine; RES, reticuloendothelial system; ROS, reactive oxygen species; SDHA, succinate dehydrogenase flavoprotein subunit; SM, sphingomyelin; SOD, super oxide dismutase; SPC, soy phosphatidylcholine; SS-peptide, Szeto-Schiller peptide; TALEN, transcription activator-like effector nuclease associated proteins; TCA cycle, tricarboxylic acid cycle; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxy; TFAM, mitochondrial transcription factor A; TPP, triphenylphosphonium; TRITC, tetramethylrhodamine-5-isothiocyanate; tRNA, transfer RNA; ZFN, zinc-finger nuclease.

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1. Introduction

Mitochondria carry out vital and lethal functions for cells that are relevant to the pathophysiology of diseases. Mitochondria are responsible for providing a significant portion of cellular energy in the form of adenosine triphosphate (ATP), for controlling the level of reactive oxygen species (ROS), buffering cytosolic calcium levels, and regulating programmed cell death (apoptosis) [1]. To support their functions, mitochondria are supplied by several proteins that are encoded either by mitochondrial DNA (mtDNA) or nuclear DNA. Fig. 1 is shown to summarize our current understanding of mitochondrial structure and their various functions [2]. The mitochondrion possesses a double membrane consisting of an outer membrane, which includes important proteins related to apoptosis regulation, and an inner membrane containing the mitochondrial oxidative phosphorylation system, including proteins related to the electron transport chain and ATP synthase. The very inner space, the matrix, contains pooled mtDNA and major metabolic pathways, including the tricarboxylic acid (TCA) cycle, the urea cycle and fatty acid oxidation (β-oxidation). Thus, by delivering target molecules to mitochondria, it would be possible, in theory, to control the functions of cells and living organisms, which could be useful for our fundamental understanding of life phenomena.

It has often been reported that mitochondrial dysfunction can cause a variety of human disorders, including neurodegenerative and neuromuscular diseases, heart failure, ischemia/reperfusion (I/R) injuries, cancer and a variety of inherited mitochondrial diseases [3–6]. For example, inherited mitochondrial diseases are caused by mutations and defects of mtDNA. Thus, if it were possible to deliver therapeutic compounds to the mitochondrial matrix where the mtDNA is located, the condition caused by such mutations and defects of mtDNA in mitochondria in diseased cells could be improved. While, the mitochondrial delivery of compounds that are toxic to mitochondria and destroy them, the energy plant of cancer cells could be destroyed. Such a mitochondrial targeting strategy would be useful for cancer therapy [7].

Based on the above information, mitochondria would be expected to be promising organelles for targeting. The technology of delivering the target molecule to mitochondria should have a substantial impact on our understanding of life processes. Moreover, delivering therapeutic molecules to mitochondria for the treatment of a variety of human disorders promises to be a useful innovative therapeutic strategy. It should be noted here that the transport system of naïve mitochondria is strictly controlled as shown in Fig. 2 [2]. The outer membrane is only permeable to small molecules with molecular weights of less than 5 kDa, with passage through a membrane-spanning protein, namely porin.

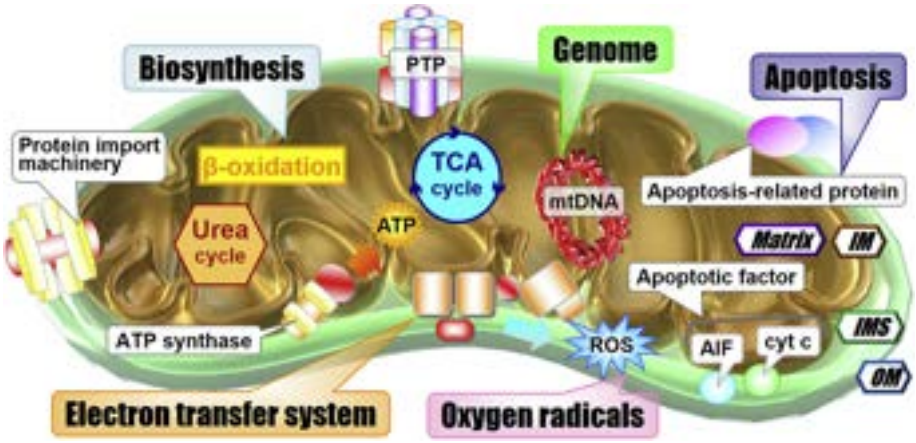

Fig. 1. Mitochondria with various functions. Mitochondria possess double membrane structures. The OM includes the main proteins related to apoptosis and the IM contains the electron transport system and ATP synthase. The matrix space contains pooled mtDNA and major metabolic pathways including the tricarboxylic acid (TCA) cycle, the urea cycle and β - oxidation, while the space between the membranes, the IMS, contains apoptosis-inducing factor (AIF) and cytochrome *c* (cyt c). IM, inner membrane; IMS, intermembrane space; OM, outer membrane; PTP, mitochondrial permeability transition pore; ROS, reactive oxygen species.

Macromolecules, such as proteins, are taken up by mitochondria *via* a protein transport machinery by a special route. The mitochondrial protein import machinery is involved in mitochondrial transport of a variety of proteins linked with mitochondrial targeting signal peptide (MTS). Specific compounds reach the matrix space *via* a number of transport proteins that are imbedded within the inner membrane—each of which is responsible for the transport of a specific ligand.

For the treatment of a mitochondrial disease, the molecular mechanism and pathway of mitochondrial diseases needs to be elucidated and a drug delivery system (DDS) for mitochondria in diseased cells is required. First, methodology for encapsulating drugs in nanocarriers independent of the physicochemical characteristics of the drugs are needed. Second, the nanocarriers should be internalized into the target cells of a diseased tissue. Finally, the precise control of the intracellular trafficking of a nanocarrier is required to deliver the cargo to the mitochondria. Therefore, the development of DDS technology for delivering cargoes to mitochondrial sites, irrespective of the size and type of molecule, would be highly desirable. To date, several mitochondrial DDS developments have been reported [2,8,9], but a generalized DDS leading to therapy targeting mitochondria has not been established. The

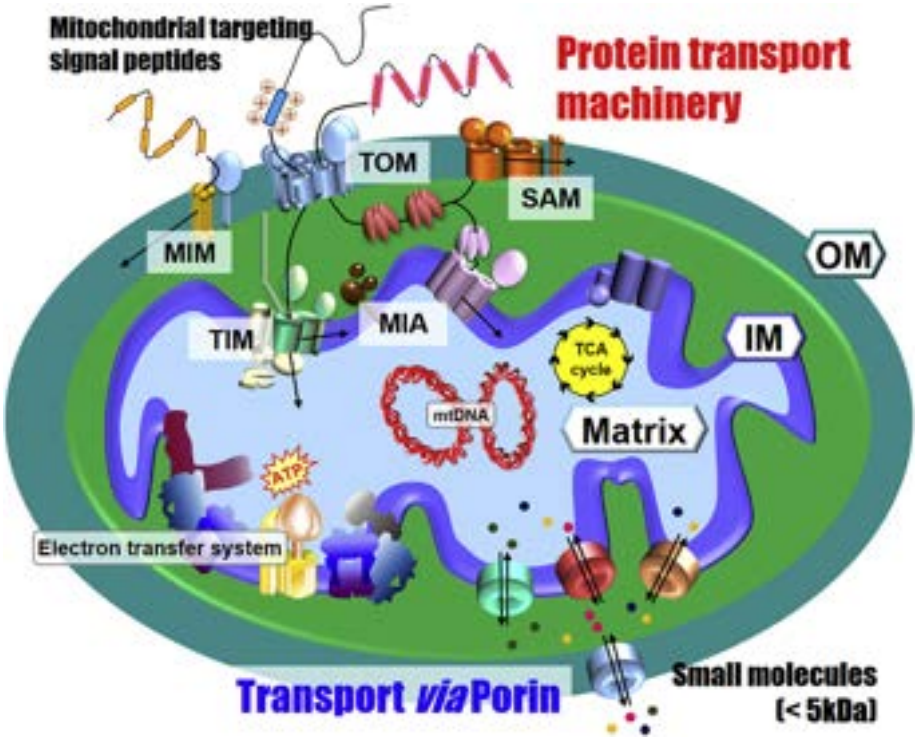

Fig. 2. Mitochondrial transport system. The outer membrane (OM) is only permeable to small molecules with molecular weights of less than 5 kDa, with passage through a membrane-spanning protein, namely porin. Macromolecules are taken up by mitochondria *via* a protein transport machinery by a special route. The mitochondrial protein import machinery is responsible for the mitochondrial transport of a variety of proteins linked with the mitochondrial targeting signal peptide. IM, inner membrane; MIA, mitochondrial intermembrane space import and assembly; MIM, mitochondrial import; SAM, sorting and assembly machinery; TIM, translocator of the mitochondrial inner membrane; TOM, translocator of the mitochondrial outer membrane.

mitochondrial delivery of macromolecules such as nucleic acids and proteins is particularly difficult using the currently available technology for targeting mitochondria.

This review focuses on mitochondria-targeted therapeutic strategies including antioxidant therapy, cancer therapy, mitochondrial gene therapy and cell transplantation therapy based on mitochondrial DDS. In particular, we discuss nanocarriers for mitochondrial delivery to achieve mitochondria-targeting therapy. In the section on antioxidant therapy targeting mitochondria, we summarize the current state of knowledge of mitochondrial delivery of anti-oxidant molecules, including chemicals, peptides. The discussion of cancer therapy includes the mitochondrial delivery of anticancer drugs and mitochondrial targeted photodynamic therapy (PDT). In the section of mitochondrial gene therapy, we summarize current therapeutic methods that are available for the treatment of mitochondrial inherited diseases and discuss the mitochondrial delivery of small nucleic acids and circular DNA for an innovative mitochondrial gene therapy. Finally, we introduce cell plantation therapy using mitochondria activated cells. This review also summarizes our current efforts regarding a liposome-based carrier for mitochondrial delivery, MITO-Porter that delivers cargoes to mitochondria *via* a membrane fusion mechanism.

2. Antioxidant therapy using mitochondrial DDS

In this section, research related to antioxidant therapy targeting mitochondria is described. Current reports of the use of mitochondrial DDS in antioxidant therapy are mainly classified into three groups: mitochondrial delivery *via* triphenylphosphonium (TPP), Szeto-Schiller (*SS*)-peptides, and mitochondrial targeting nanocarriers (Fig. 3).

2.1. Strategies for delivering antioxidants to mitochondria using TPP

Among the reports of antioxidant therapy targeting mitochondria, strategies involving the use of TPP have been the most heavily reported to date. TPP, a cationic compound with hydrophobic properties, has been most commonly used as a mitochondrial targeting device, and is used to deliver mainly lipid-soluble small molecules to mitochondria. Because TPP is cationic, it can accumulate at high levels in the mitochondria by virtue of electrostatic interactions with negatively charged mitochondria (−160 to −180 mV) [61]. On the other hand, TPP could also act as an uncoupler in mitochondria, increasing the leakage of protons and possibly causing toxicity, when it is used at concentrations in excess of 10 μM [11]. It was also reported that, after the intravenous injection of TPP, it disappeared from the blood within 5 min and accumulated in the kidneys and liver [12]. Mitochondrial targeting strategies using TPP are roughly divided into three types (Fig. 4), where a therapeutic molecule is conjugated to TPP or chemically modified derivatives of TPP (TPP analogues), TPP analogs are encapsulated in nanoparticles, and TPP is modified on the surface of nanoparticles as ligand. Table 1 summarizes the strategies that are currently in use concerning the mitochondrial targeting of antioxidants by TPP.

2.1.1. Conjugation of TPP with antioxidants for mitochondrial delivery

Numerous reports dealing with the conjugation of TPP with various antioxidant molecules have appeared (Fig. 4A), and most of these are lipophilic antioxidant molecules with very few being water-soluble molecules [13]. MitoQ is one of the most popular compounds in which coenzyme Q_{10} (CoQ_{10}) chemically conjugated with TPP, and a number of reports on the use of MitoQ for antioxidant therapy in mitochondria have appeared. In the case of ferrotosis, a form of Fe- and ROS-

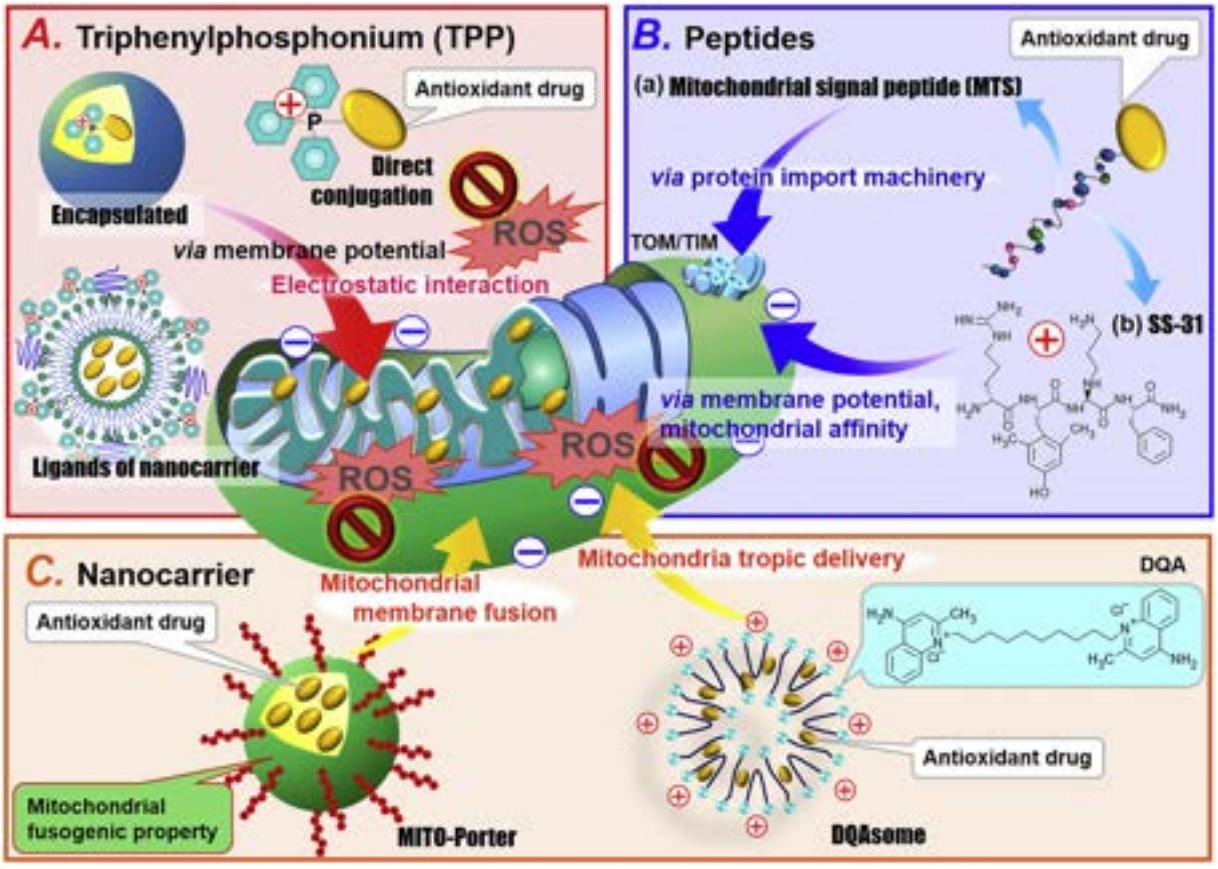

Fig. 3. Schematic showing the delivery of antioxidants to mitochondria. Strategies for the mitochondrial delivery of antioxidants are summarized. Three strategies include mitochondrial delivery using TPP (A), peptide based delivery targeting mitochondria (B) and mitochondrial targeting nanocarriers (C). ROS, Reactive oxygen species; SS-31, the Szeto-Schiller peptide 31.

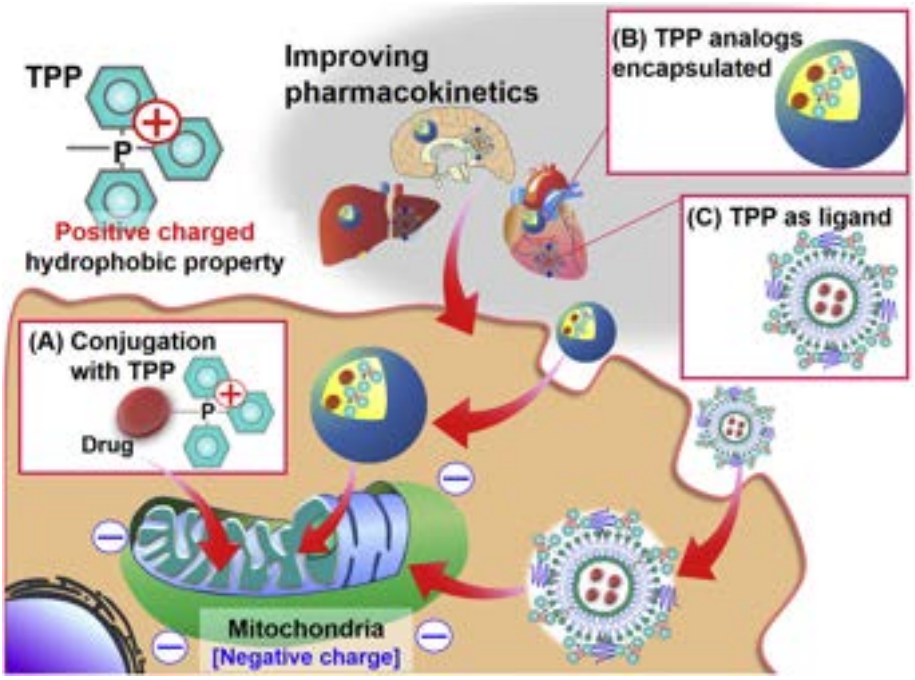

Fig. 4. Strategies for mitochondrial delivery of antioxidant using TPP. Three types of mitochondrial targeting strategies using TPP are shown. (A) Drugs conjugated with TPP, where the drug is conjugated to TPP or a chemically modified derivative of TPP (TPP analogues). (B) TPP analogs are encapsulated in nanocarriers. (C) The surface of the nanocarrier is modified with TPP as a ligand.

dependent cell death in neuronal HT22 cells and mouse embryonic fibroblasts, the addition of MitoQ to the cells reduced ROS production and significantly inhibited mitochondrial fragmentation compared to untreated groups [14].

MitoQ is also being evaluated in clinical trials, and when 20 healthy elderly people (60 to 79 year old) used MitoQ for 6 weeks, the findings suggested that arteriosclerosis was suppressed and vascular function was improved [15]. MitoQ is also used supplements aimed at anti-aging and in cosmetics for skin care. Mito-TEMPO is a compound in which TPP and 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) are combined. The intravenous injection of Mito-TEMPO decreased Alanine transaminase (ALT) levels in acetaminophen liver injury model mice in a dose-dependent manner, resulting in a reduction in liver injury [16].

2.1.2. Delivery of TPP analogs when encapsulated in nanocarriers

Since controlling the pharmacokinetic of the TPP analog alone is limited, a nanocarrier such as a liposome would be expected to be useful for effective delivery to a target tissue (Fig. 4B). Mito-Apo is a compound in

Table 1
Summary of reports of mitochondrial antioxidant therapeutic strategies using TPP.

Name	Strategies using TPP	Cargo antioxidant	Nanocarriers		Outcomes	Refs.
			Composition	Properties		
MitoQ	Direct conjugation	Coenzyme Q_{10}	–	–	Decreasing mitochondrial oxidative stress in neuronal HT22 cells and mouse embryonic fibroblasts	[14]
Mito-TEMPO		2,2,6,6-tetramethylpiperidine 1-oxyl	–	–	Preventing peroxynitrite formation and the subsequent mitochondrial dysfunction of APAP-induced liver injury mouse	[16]
Mito-Apo	TPP analogs encapsulated in nanocarriers	Apocynin	Brain targeting nanoparticle (CPH:SA = 20/80) with FA	Brain targeting/biodegradable to control drug release/uptake to neurons *via* FA receptor	Protection against mitochondrial dysfunction induced by oxidative stress and neuronal damage.	[17]
MitoPBN		PBN	Liver targeting nanoparticle (Cholesterol:lecithin = 1/2)	Liver targeting	Alleviating ROS-induced mitochondrial dysfunction of diabetic mouse model	[18]
TPP-PLGA-Nanoparticle	The use of TPP as ligand of nanocarriers	Coenzyme Q_{10}	Biodegradable polyanhydride nanoparticle (Pegylated polymer:CoQ_{10} = 5/1(w/w)) modified TPP	Brain targeting through BBB to accumulate in brain	Decreasing mitochondrial oxidative stress in neuronal progenitor cells	[19]
TPP-dendrimer-NAC		*N*-acetyl-l-cysteine	Dendrimer conjugated TPP and NAC (11 TPP and 7 NAC/dendrimer)	Efficient accumulation in brain and diseased site	Accumulation of the carriers in injured brain	[20]

APAP, acetaminophen; Apocynin, 4-hydroxy-3-methoxyacetophenone; BBB, blood brain barrier; CoQ_{10}, coenzyme Q_{10}; CPH, 1,6 bis(p-carboxyphenoxy)hexane; FA, folic acid; NAC, *N*-acetyl-l-cysteine; PBN, phenyl tert-butylnitrone; PLGA, poly (D,L-lactide-*co*-glycolide); TPP, triphenylphosphonium.

which TPP is combined with Apocynin, which has an antioxidant activity and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitory activity. Apocynin has been used in research to validate therapeutic strategy for Parkinson's disease and Alzheimer's disease. A previous study reported that Mito-Apo encapsulated in folate-modified nanoparticles for targeting the brain showed an enhanced uptake into neurons and significant neuroprotective effects in *in vitro* experiments [17]. They reported that the cellular uptake of the nanoparticles occurred *via* receptor-mediated endocytosis *via* folate receptors, because these receptors were present on neurons [17].

MitoPBN is a compound in which phenyltert-butylnitrone (PBN) is conjugated with TPP. Wu et al. developed a liposome composed of cholesterol and lecithin for targeting encapsulated MitoPBN to the liver, and validated the therapeutic effect by the intraperitoneal administration of the liposome to a type 2 diabetes model mouse. They specifically targeted hepatocytes in the liver, because mitochondria, the source of ROS, are located in hepatocytes. As a result, the mitochondrial cristae structure was arranged in an orderly manner, and glucose metabolism was normalized by improving the mitochondrial redox balance [18].

2.1.3. The use of TPP for ligands of nanoparticles for mitochondrial targeting

Several researchers have reported on the use of TPP as mitochondrial targeting ligand for nanoparticles, in which the surface of the nanoparticles are modified with TPP (Fig. 4C). Velichkovska et al. developed CoQ_{10}-encapsulated polyethylene glycol (PEG)ylated TPP-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles, and attempted to validate a therapeutic strategy for the prevention of neurocognitive impairment, which is a symptom of the Human immunodeficiency virus (HIV) and a side effect of multi-drug therapy. Treatment of human neural progenitor cells with these nanoparticles significantly decreased ROS production in mitochondria and reduced telomere shortening compared to the control group [19].

In most studies of antioxidant therapy using TPP conjugated drugs, TPP and antioxidant molecule were conjugated at 1:1 (molar ratio), making it difficult to deliver multiple therapeutic molecules by one TPP. Sharma et al. synthesized TPP-dendrimer-NAC, in which 7 molecules of TPP and 11 molecules of *N*-acetyl-l-cysteine (NAC) were conjugated to 1 molecule of dendrimer. Intravenous injection of this dendrimer into a model rabbit with a brain injury showed that the activated microglia had successfully accumulated in mitochondria at the injury site, suggesting a potential therapeutic effect [20].

The mitochondrial delivery of antioxidant molecules *via* TPP is an attractive strategy, and there are a large number of reports at the preclinical stage. However, these strategies should overcome their pharmacokinetics and safety to obtain a sufficient therapeutic effect.

2.2. Antioxidant therapy using SS-peptides

MTS is the most famous peptide-based tool in mitochondrial delivery (Fig. 3B (a)). This peptide is used for targeting allotopic expression in mitochondria, where a gene coding target protein linked with MTS is delivered to the nucleus and the target protein with MTS expressed in cytosol, and the protein finally is delivered to mitochondria *via* the mitochondrial protein transport machinery. However, it would be difficult to exert an antioxidant effect using MTS conjugated drugs, because MTS has no cellular uptake ability.

On the other hand, the SS peptide is a well known peptide that exerts an antioxidant effect and has both cellular uptake and mitochondrial abilities (Fig. 3B (b)) [21]. The SS peptides were discovered to be cell-permeable peptides that are derived from a delmorphine-derived tetrapeptide that exerts a central analgesic effect, and one of these tetrapeptide derivatives has mitochondrial-targeting ability, which led to the creation of SS peptides [22,23]. The SS peptide is a water-soluble tetrapeptide composed of tyrosine (or dimethyltyrosine), arginine, phenylalanine and lysine, and various types of SS peptides from SS-01 to SS-31 have been developed to date. The SS peptide can accumulate in the cardiolipin-rich inner mitochondrial membrane *via* electrostatic and hydrophobic interactions [24]. In addition, non-reactive tyrosyl or dityrosine radicals produced through aromatic amino acid residues such as tyrosine and or dimethyltyrosine form tyrosine hydroperoxides, which can react with superoxide radicals and remove them, showing antioxidant effects [25]. Furthermore, the fact that the SS peptide did not depolarize mitochondria and has a low toxicity even when used at a concentration of 1 mM [26] is an attractive feature and because of this, this peptide has been used in various studies [27,28]. Elamipretide (also known as SS-31, MTP-131 and Bendavia™) improved I/R injury after percutaneous transluminal renal angioplasty [29].

Because SS-31 is a peptide formulation, there is a concern that the peptide would be degraded by proteases, structurally changed, and form complexes with blood components in the blood circulation, which might shorten the half-life and decrease bioavailability [30]. To overcome this issue, Liu et al. encapsulated SS-31 in pH-responsive nanopolyplexes composed of hyaluronic acid (HA) and chitosan, and validated the utility of this carrier for a therapeutic effect on acute renal injury caused by ROS. Since HA shows selectivity for the CD44 receptor, which is upregulated in the kidney upon injury, when SS-31 was encapsulated in nanopolyplexes, it was efficiently internalized into the cells of the injured kidney. Kidney injury in particular, leads to damage to proximal tubule epithelia cells. Thus, SS-31 was selectively delivered to mitochondria in the proximal tubule epithelia cells, indicating that is has antioxidant and antiapoptotic effects [31].

2.3. Mitochondrial delivery using nanocarriers to achieve antioxidant therapy

Nanocarriers such as liposomes can be used to encapsulate a wide variety of therapeutic molecules (Fig. 3C). Surface-modification of the nanocarrier with ligands and optimization of the composition of the carrier not only controlled drug release, but also regulated bio-distribution and intracellular trafficking of the nanocarrier. Furthermore, optimized nanocarriers are resistant to biodegradation and are biocompatible, and therefore have low toxicity and antigenicity. However, considering clinical use, there are many problems that need to be overcome, including the scale up risk of nanocarrier preparation, aseptic methods, efficient drug encapsulation and the homogeneity of the preparations. We also should face important issues, such as the stability of the nanocarriers in the body, pharmacokinetic and intracellular trafficking control and therapeutic effects when the nanocarrier is administered [32,33].

Considering mitochondria targeting, although it is necessary to deliver therapeutic molecules to target tissues, there seems to be a large barrier to the delivery of nanocarriers to mitochondria. In the field of antioxidant therapy targeting mitochondria, many passive strategies for targeting mitochondria for improving the mitochondrial accumulation of drugs by improving tissue targeting and cellular uptake have been reported rather than targeting mitochondria [34–36]. On the other hand, there are only limited reports of the development of active strategies for increasing mitochondrial targeting by nanocarriers. The target tissues in these reports are the brain, heart, liver and kidney with severe symptoms due to ischemia. In this section, we introduce validating mitochondrial targeting antioxidant therapeutic strategy using nanocarriers.

2.3.1. Mitochondrial delivery by DQAsomes for antioxidant therapy

Dequalinium (DQA) was approved by the FDA as an antimicrobial agent for the treatment of oral diseases more than 50 years ago. Since DQA is a single-chain bola-amphiphile molecule, it self-associates in aqueous solution to form liposome-like vesicles called DQAsomes [37]. DQAsomes have been used to deliver drugs and DNA to mitochondria, and positively charged DQAsomes accumulate in negatively charged mitochondria *via* electrostatic interactions (Fig. 3C (a)) [38]. Over the past decade, DQAsomes have been used in research on cancer therapy, where they are often derivatized [39,40] or used in combination with lipids rather than being used alone, thereby enhancing mitochondrial

targeting ability [41]. However, there is a concern that DQA induces apoptosis and acts as a mitochondrial toxin at high concentrations [42,43].

In antioxidant therapy targeting mitochondria using DQAsome, Zupancic et al. developed DQAsomes encapsulating curcumin, an antioxidant, for use as an inhalant for acute lung injury. The DQAsome formulation had a particle size of 170 to 200 nm and a ζ potential of +50 mV, and successfully accumulated in mitochondria [44]. By using DQAsome, it was confirmed that curcumin, when packaged in a nanocarrier, targeted mitochondria more efficiently than free curcumin. *In vivo* and *in vitro* therapeutic effects will need to be verified in the future.

2.3.2. Mitochondrial delivery via MITO-Porter and antioxidant therapy targeting mitochondria

Over the last decade, our laboratory has been focusing on the development of a versatile mitochondrial targeting liposomal-based nanodevice, namely a MITO-Porter system (Fig. 3C (b)). We developed a nanocarrier composed of a mitochondrial fusogenic lipid of 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine (DOPE) and sphingomyelin (SM) with the cell-penetrating peptide (CPP) of octaarginine (R8) on the exterior of the liposomes [2,45–48]. We identified four essential cellular events during the mitochondrial delivery of the MITO-Porter system. The first is the cellular uptake process, facilitated by the high-density R8 moieties that interact with the cell surface proteoglycans to activate the macropinocytosis pathway. The effective endosomal escape of the particles was identified as the second cellular process, and is driven by the combination of R8 and DOPE through the membrane fusion process. The third is electrostatic interactions with the mitochondrial membrane, followed by the membrane fusion process as the final step of mitochondrial delivery (Fig. 5).

We previously attempted to validate antioxidant therapy by the mitochondrial delivery of CoQ_{10} using a MITO-Porter system in the case of hepatic I/R injury model mice [49]. Under an ideal scenario, the MITO-Porter encapsulating CoQ_{10} (CoQ_{10}-MITO-Porter) reaches the liver tissue *via* systemic injection, and the nanocarrier then delivers CoQ_{10} to mitochondria in hepatocytes, exerting a therapeutic effect (Fig. 6A). Experiments including histological observations of liver tissue by confocal laser scanning microscopy confirmed that the CoQ_{10}-MITO-Porter accumulated in the liver and mitochondria after mice were intravenously administered the nanocarrier. Furthermore, when the CoQ_{10}-MITO-Porter was pre-administered to the hepatic I/R injury model mice, ALT levels were significantly reduced probably by decreasing mitochondrial-derived ROS levels by the action of the CoQ_{10} delivered in liver mitochondria (Fig. 6B) [49].

The use of liposomes and lipid nanoparticles has attracted attention as a technique for solubilizing poorly water-soluble drugs such as CoQ_{10}. However, it is necessary to determine an appropriate preparation method needed in order to achieve the efficient encapsulation of drugs in nanocarriers. We previously investigated the encapsulation of CoQ_{10} in a MITO-Porter using various preparation methods, we confirmed that the ethanol dilution method was optimal for preparing a CoQ_{10}-MITO-Porter in which CoQ_{10} was efficiently encapsulated [50]. Electron microscopy observations confirmed that this CoQ_{10}-MITO-Porter appears to have a unique onion-like structure (Fig. 6C). As next process to scale up liposome formulations from the laboratory to clinical use, changes in the manufacturing process would be required.

In recent years, considerable interest had developed regarding methods for preparing lipid-based nanoparticles using a microfluidic device as a means of avoiding the risk of scale up. This preparation technique was applied to the manufacturing of "Patisiran (ONPATTRO (R))", the nucleic acid medicine *via* RNA interference that was approved by the FDA in 2018. Most recently, we attempted to prepare a CoQ_{10}-MITO-Porter [μ] using an original microfluidic device [invasive Lipid Nanoparticle Production (iLiNP) device] developed by Tokeshi and coworkers [51], and successfully achieved large-scale preparation with acceptable reproducibility. Furthermore, we succeeded in finely controlling the particle size of the CoQ_{10}-MITO-Porter [μ], the diameter of which was half the size of the conventional MITO-Porter (Fig. 6D) and improved the intracellular cellular trafficking [52]. These results indicate that the CoQ_{10}-MITO-Porter [μ] represents a potential candidate that could be used in mitochondrial nanomedicine.

3. Cancer therapy targeting mitochondria

In the early 19th century, a German physiologist and Nobel laureate, Otto Heinrich Warburg hypothesized the existence of a close relationship between defects in mitochondrial function with tumorigenesis [53]. He observed that tumors rely on aerobic glycolysis, even in an oxygen-rich environment, by taking up more glucose and secreting increased levels of lactate to the tumor microenvironment (termed the Warburg effect). In current studies, Chandel et al. proposed that aerobic glycolysis is essential for nucleotide and phospholipid synthesis in rapidly proliferating cells such as cancer. At the same time, ATP generation from this pathway is only essential for survival under hypoxic conditions. Furthermore, tumor cells require glutamine to activate mitochondrial metabolism for the generation of ATP, ROS, NADPH, amino acids, nucleotides, and lipids, which could support tumor growth [54,55].

Other reports have also shown the existence of an interconnection between multiple hallmarks of cancer cells with mitochondrial dysfunction, particularly limitless proliferative potential, impairment of apoptosis cell death, and insensitivity to anti-growth signals, as well as the capacity of mitochondria in supporting tumorigenesis at multiple stages [56]. It has also been reported that mitochondria are involved in the metastatic dissemination of cancer cells by altering the balance of mitochondrial dynamics, moderately increasing ROS production, overexpressing anti-apoptotic proteins of the Bcl-2 family, and changing the metabolic process [57–59].

Therefore, directing antineoplastic agents to the mitochondria of tumors could be a promising strategy for achieving better therapeutic outcomes and to address several problems associated with the current cancer therapy options, particularly regarding multi-drug resistance and metastasis. However, the morphology of mitochondria caused by the delivery process becomes more complicated and challenging. In this session, we summarize several currently developed drug delivery technologies for mitochondrial targeting in cancer therapy (Fig. 7).

3.1. Mitochondrial delivery by delocalized lipophilic cations (DLCs) for cancer therapy

Mitochondria are composed of four distinct structures, namely, the outer membrane, the intermembrane space, the inner membrane and the mitochondria matrix, thus making in a perfect barrier for many molecules to penetrate inside the mitochondria, as shown in Fig. 2. The occupancy of the unique phospholipid structure of cardiolipin and the strong negative membrane potential on the inner membrane further provide more complexities for the drug delivery process. On the other hand, this substantial transmembrane potential can be harnessed to attract cationic molecules and cause them to strongly bind with the mitochondrial membrane through electrostatic interactions.

To effectively cross the phospholipid bilayer of the inner membrane, the cationic molecule should carry a suitable level of lipophilicity to advance the equilibration process with the membrane [60]. Therefore, such compounds are widely known as DLCs (Table 2). According to the Nernst equation, every 61.5 mV increment in membrane potential results in a ten-fold increase in the concentrations of DLCs within mitochondria [61,62]. Assuming a mitochondrial membrane potential of approximately 200 mV, the concentration of DLCs could be up to 1000-fold inside the mitochondrial matrix. In addition, the plasma membrane has a negative potential, thus allowing the DLCs to be easily internalized into the cells. Thus, several DLCs, including TPP, DQA and rhodamine-123, have been proposed as active moieties for the mitochondrial accumulation of a wide range of chemotherapeutics.

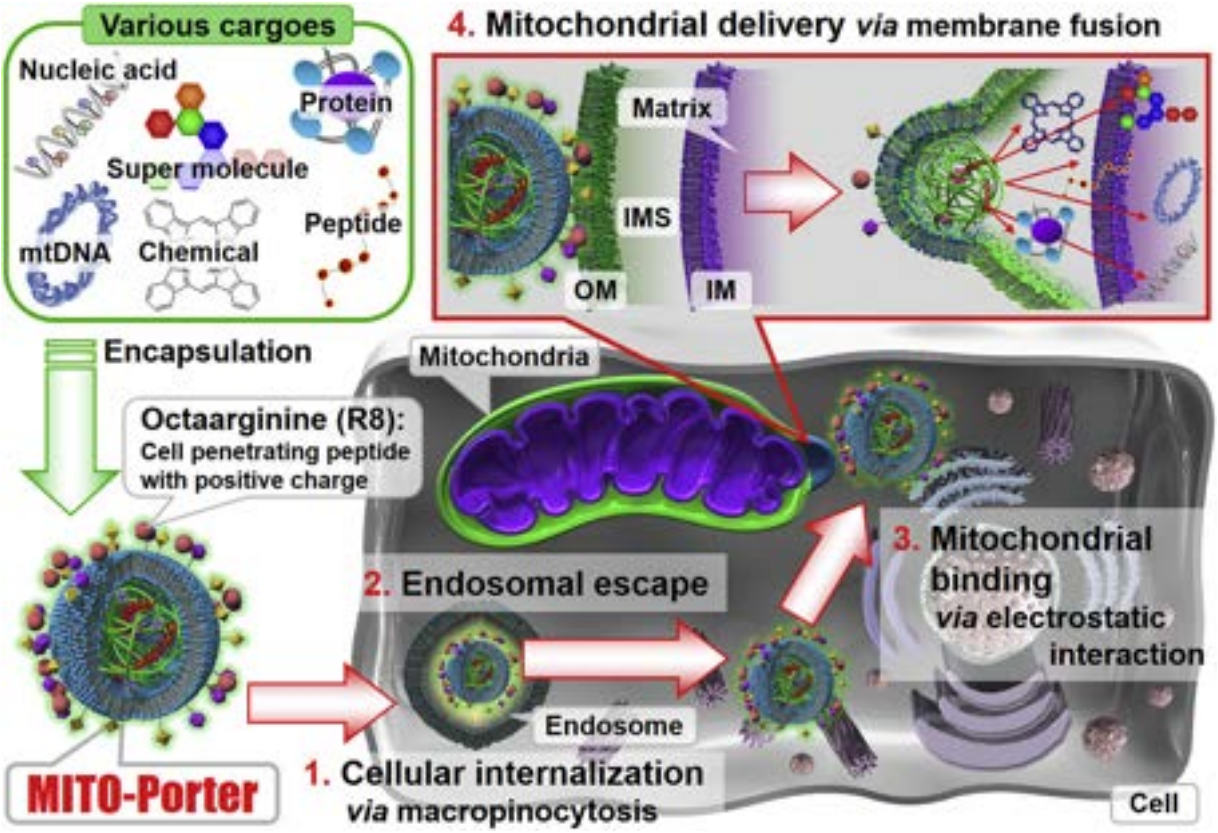

Fig. 5. Mitochondrial delivery using a MITO-Porter system. We identified four essential cellular events that are involved in the mitochondrial delivery of the MITO-Porter system. First, the MITO-Porter is efficiently internalized *via* macropinocytosis, a process that is facilitated by the high-density of R8 moieties. The effective endosomal escape of the MITO-porter was identified as the second cellular process. The third is the electrostatic interaction of the MITO-Porter with the mitochondrial membrane, followed by the membrane fusion process as the final step. Finally, the cargoes are delivered into mitochondria *via* membrane fusion. This membrane fusion mechanism-based strategy permits a cargo to be delivered to mitochondria independent of its size and physical properties. IM, inner membrane; IMS, intermembrane space; outer membrane.

3.1.1. Conjugation of mitochondrial toxins with TPP

The conjugation of an antiglycolytic drug, lonidamine, to TPP *via* an aliphatic chain (Mito-LND) significantly improved the cell-killing ability against human lung cancer cells, as indicated by a low half maximal inhibitory concentration (IC_{50}) value of less than 1 μM. This mitochondrial-targeted lonidamine potently inhibited the progression of lung cancer in an orthotopic mouse model and brain metastasis by inhibiting the mitochondrial bioenergetic, generation of ROS, and the induction of autophagic cell death [63].

Another report strongly suggested that redirecting doxorubicin to mitochondria by the direct conjugation with TPP *via* an amide bond (DOX-TPP) lowered the resistance index by 2.2-fold in MDA-MB-435 cell lines as opposed to a solution of free doxorubicin [64]. However, this conjugate system showed inefficient antitumor activity against a multidrug-resistant breast cancer, an MCF-7/ADR, bearing mouse model. Thus, additional conjugation of DOX-TPP with HA using a pH-sensitive hydrazone bond (HA-*hydra*-DOX-TPP) improved tumor accumulation and antitumor activity, as well as the safety profile of doxorubicin [65]. The presence of HA could facilitate tumor accumulation *via* the enhanced permeability and retention (EPR) effect and interaction with the CD44 receptor that is overexpressed on the surface of malignant cells, as evidenced by observations of tumor tissue at the end of several sequences of administration using confocal laser scanning microscopy. At the same time, the hydrazone bond promotes the release of DOX-TPP during the cell internalization process triggered by the acidic lysosomal environment.

3.1.2. The use of TPP-modified nanocarriers

Besides being directly conjugated to the therapeutic molecules, TPP can also be used to modify a nanoparticle system, such as polymeric-based nanocarrier including TPCL NPs and PEI-TPP-DOX [66,67], a dendrimer system [68], and a lipid-based nanoparticle including TPP-IR780/Ce6-TNS and TPP-PEG-L [69,70]. The involvement of a nanoparticle platform could be a benefit for improving the solubility and stability of encapsulated drugs, as well as improved selective tumor accumulation.

A systematic study regarding the use of TPP in combination with a nanoparticle platform was conducted using biodegradable PLGA [71]. This study revealed the effect of nanoparticle size and surface charge on mitochondrial accumulation and the results suggested that 80–100 nm diameter particles and more positively charged particles are more favorable for mitochondrial delivery. Moreover, the TPP-modified PLGA nanoparticles (PLGA-*b*-PEG-TPP) showed an efficient lysosomal escape ability by exploiting the proton sponge effects.

The axial conjugation of two TPP moieties on the Pt(IV)-prodrug of cisplatin (Platin-M) encapsulated on the TPP-modified PLGA system (T-Platin-M-NPs) exhibited an efficient distribution on the mitochondria matrix to attack mtDNA, which was lacking in the nucleotide excision repair (NER) machinery, for overcoming the cancer multidrug-resistant mechanism [72]. Interestingly, this dual-targeting strategy displayed a prolong blood circulation profile with a high brain accumulation level as opposed to the non-encapsulated Platin-M, making it a promising tool for treating brain cancers.

Although TPP, the most popular DLCs, has several drawbacks, especially negative impact on the mitochondrial membrane potential and respiratory chain complexes [73]. Additionally, the density of TPP and the type of linker exerted a significant effect on the TPP-induced cytotoxicity profile [68]. Therefore, a comprehensive cytotoxic evaluation, especially on normal cells, should be considered during the development of TPP-based chemotherapeutic drugs.

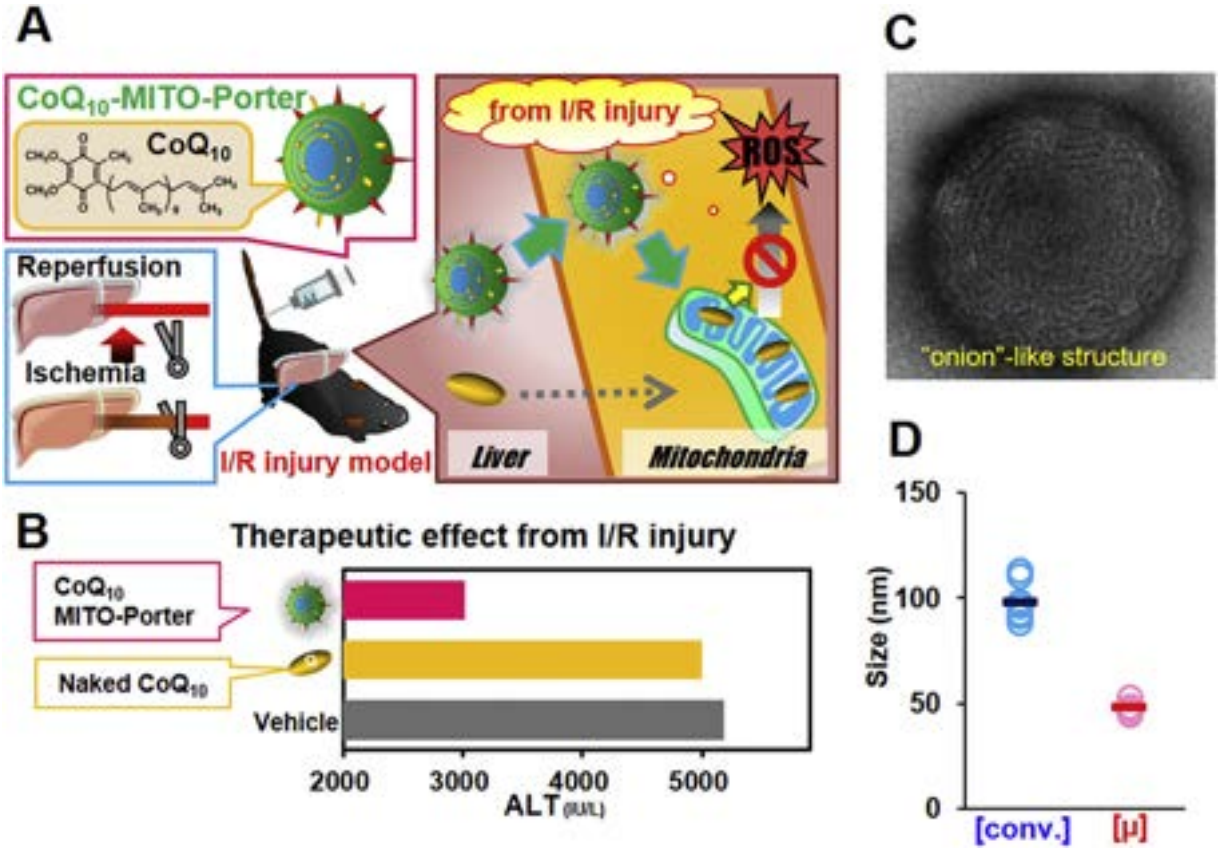

Fig. 6. Research outcome of CoQ_{10}-MITO-Porter. (A) Concept of antioxidant therapy by the mitochondrial delivery of CoQ_{10} by a MITO-Porter. (B) Measurement of serum ALT activities (marker of liver damage) of the hepatic I/R injury mice treated with CoQ_{10} by MITO-Porter [49]. (C) Electron microscopic image [50]. (D) Sizes of conventional MITO-Porter [conv.] and MITO-Porter [μ] [52]. ALT, alanine transaminase; I/R, ischemic reperfusion. These figures are reproduced with permission from Elsevier.

3.2. Peptide-based carriers for mitochondria targeting to achieve cancer therapy

3.2.1. Mitochondrial delivery by MPP

Inspired by a CPP, a short peptide sequence that has a robust capacity to facilitate the cellular internalization of a wide variety of cargos [74], a research group from the University of Toronto came up with the idea to design a peptide-based mitochondrial delivery system, namely a mitochondria-penetrating peptide (MPP) [75–77]. By combining cationic and lipophilic amino acid sequences, the MPP was reported to be a useful tool for selectively transporting various anticancer agents to mitochondria of tumors (Table 3).

The positively charged amino acid residues, such as lysine and arginine, promote the internalization process through the plasma and mitochondria membrane by exploiting the potential gradient that exists across both membranes. Furthermore, the lipophilic units, like phenylalanine and cyclohexyl alanine, preserve the partition process through the highly hydrophobic double-layered mitochondrial membrane. This short peptide sequence is dominantly taken up by cells through direct penetration pathways that could circumvent endocytic uptake, in combination with membrane potential-dependent manner, which could greatly improve tumor selectivity [75,78,79].

Due to the simple synthesis process and ease of conjugating with several low molecular weight compounds, MPP has been utilized to direct several antineoplastic agents into the mitochondrial compartments of cancer cells [80]. A platinum-based anticancer agent has been linked to the N terminus of the MPP consisting of D-arginine and L-cyclohexyl alanine residues (mt-Pt) to result in specific mitochondria accumulation and an improvement in the extent of cytotoxicity against a cisplatin-resistant ovarian cancer cell line through the harmful effect on the mtDNA [81].

Moreover, the conjugation of an alkylating agent, chlorambucil, to MPP (mt-Cbl) has also been reported result in a significant potency toward a panel of leukemia cell lines in comparison to the parent compound. This system has been proven to effectively overcome the drug resistance that develops through a combination of chemical and biochemical mechanisms by evading the drug inactivation process and circumventing the overexpressed anti-apoptotic proteins, respectively [82].

A similar MPP sequence has been coupled with doxorubicin through a succinic anhydride connector (mtDox), causing the alteration of subcellular drug distribution within the cell without affecting the cell-killing ability of doxorubicin. A cytotoxic evaluation against the multidrug-resistant osteosarcoma and ovarian cancer cell revealed that the redirection of doxorubicin to the mitochondria resulted in the efficient evasion of drug efflux mediated by the P-glycoprotein (Pgp) [83,84], the predominant factor in drug resistance mechanisms. Significant antitumor activity of the mtDox was also obtained in a murine osteosarcoma xenografted on the mouse model with a negligible cardiotoxicity effect [84,85], which is one of the most severe adverse reactions of doxorubicin.

In a very recent report, the application of MPP was extended to transporting a rhodamine-based fluorescent dye (TAMRA-rFrFrF) with a low cytotoxicity and prolong mitochondrial tracking capacity for up to 3 days, which could not be achieved when using the commercially available mitochondrial probe [86]. Although applications of MPP technology are promising, they may not be applicable for transporting macromolecules to the mitochondria compartment [80]. In addition, the drug distribution and systemic cytotoxicity profile needs to be further investigated in more detail to assess the risk and benefit ratio of this system, especially for purposes of clinical translation.

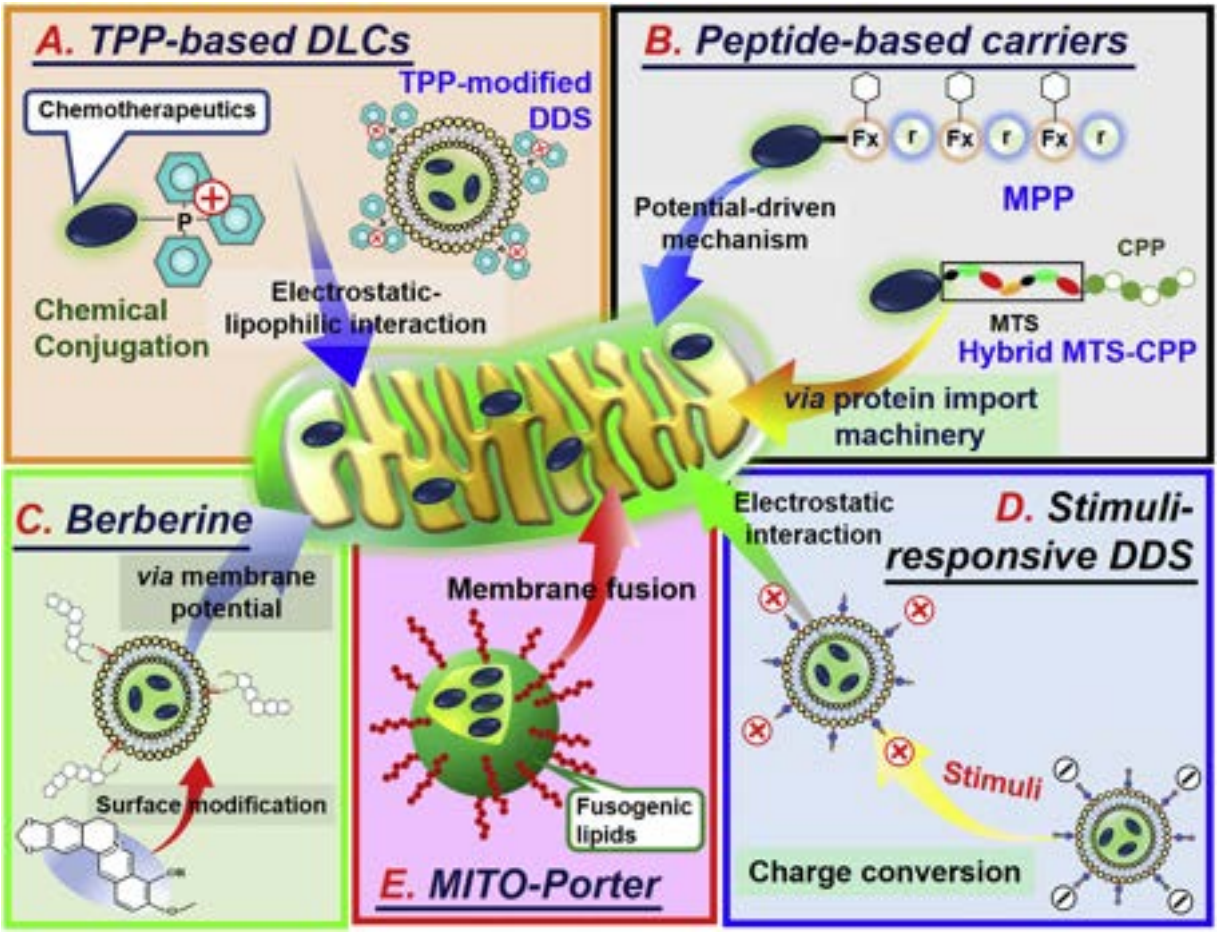

Fig. 7. Schematic illustration of a mitochondria-targeted strategy for cancer therapy. Five different strategies for the selective transport of chemotherapeutics into the mitochondria compartment of tumors consisting of: (A) Triphenylphosphonium (TPP)-based delocalized lipophilic cations (DLCs); (B) Peptide-based carriers; (C) Berberine as mitochondriotropic; (D) Stimuli-responsive drug delivery system (DDS); and (E) MITO-Porter system.

3.2.2. Targeting cancer mitochondria via MTS

A peptide-based vector for mitochondria targeting was developed based on the natural protein import process in mitochondria (Table 3). As mentioned above, more than 99% of mitochondrial proteins are encoded by nuclear genes, synthesized as precursors by cytoplasmic ribosomes, and are then imported to mitochondria through protein translocator complexes. The transported-proteins should carry a specific amino acid sequence (known as MTS) that is recognized by the protein import machinery [87,88].

Although it has a high specificity of mitochondrial delivery, MTS exhibits limited cellular uptake efficiency through the plasma membrane, resulting in a substantial reduction in intracellular transport [89]. The

Table 2
Summary of TPP-based DLCs for cancer therapy.

System name	Drug	Type	Mode of actions	Refs.
Conjugation of drugs with TPP				
Mito-LND	Lonidamine	Chemical conjugation	Inhibiting lung tumor progression and brain metastasis	[63]
DOX-TPP	Doxorubicin	Chemical conjugation	Inducing apoptosis cell death in drug resistance in tumors	[64]
HA-hydra-DOX-TPP	Doxorubicin	Chemical conjugation and self-assembled nanoparticle	Improving the *in vivo* tumor accumulation and antitumor activity of DOX-TPP	[65]
TPP-modified nanocarriers				
TPCL NPs	Doxorubicin	Polymeric nanoparticle	Enhancing tumor-killing ability through the mitochondrial accumulation	[66,67]
PEI-TPP-DOX	Doxorubicin	Hyperbranched polymeric nanoparticle	Triggering rapid and severe cytotoxicity using a low concentration	[66,67]
TPP-IR780/Ce6-TNS	IR780 & chlorin e6	Lipid-based nanoparticle	Inducing the production of thermal and singlet oxygen by photoirradiation	[69,70]
TPP-PEG-L	Paclitaxel	Liposome	Improving the tumor inhibition capacity of paclitaxel by the activation of apoptotic pathway	[69,70]
PLGA-*b*-PEG-TPP	Lonidamine & α-tocopheryl succinate	Polymeric nanoparticle	Enhancing cytotoxicity through preferential localization in specific organelle	[71]
T-Platin-M-NPs	Platin-M	Polymeric nanoparticle	Altering biodistribution profile and inhibiting mitochondrial biogenesis in cisplatin-resistant cells	[72]

Table 3
Summary of peptide-based carriers for mitochondria targeting.

System Name	Drug	Targeting moiety	Purposes	Refs.
Mitochondrial Penetrating Peptide (MPP)				
The combination of cationic and lipophilic amino acid sequences				
mt-Pt	Platinum-based anticancer drug	r Fx r Fx r Fx r	Improving the effects of cytotoxicity toward a cisplatin-resistant ovarian cancer cell line	[81]
mt-Cbl	Chlorambucil	Fx r Fx r Fx r	Overcoming drug resistance in leukemia cell lines	[82]
mt-Dox	Doxorubicin	Fx r Fx r Fx r	Combating Doxorubicin-resistant osteosarcoma *in vivo*	[83,84]
TAMRA-rFrFrF	TAMRA	r F r F r F	Mitochondria imaging with prolonged tracking capacity	[86]
Hybridization of the Cell-Penetrating Peptide (CPP) and Mitochondria Targeting Sequence (MTS)				
MTS-(5-FAM)-H_3R_8	5-FAM	CPP: R_8H_3 MTS: MLRAALSTARRGPRLSRLL	Developing dual peptide conjugation for mitochondria targeting	[90,91]
ALD5-sC18	Chlorambucil	CPP: sC18 MTS: LSRTRAAAPNSRIFTR	Potentiating chlorambucil activity	[92]

insertion of the CPP into MTS (MTS-(5-FAM)-H_3R_8) has been proposed to overcome the limitations of MTS, with the expectation that CPP would facilitate efficient cytosolic delivery, while the MTS would direct the complex for mitochondrial internalization [90,91]. Furthermore, the direct conjugation of MTS derived from the yeast aldehyde dehydrogenase 5 with sC18 CPP (ALD5-sC18) resulted in the efficient delivery of chlorambucil to the mitochondrial matrix within 30 min, thus increasing the cytotoxicity as opposed to the parent drug [92]. Additionally, the selection of MTS is the most crucial aspect to be considered for optimum mitochondrial delivery.

3.3. Berberine as an alternative of mitochondriotropics

Over the last several years, berberine, an isoquinoline alkaloid extracted from a plant and a traditional Chinese herbal medicine, has attracted considerable attention as a promising mitochondrial targeting ligand. The combination of the amphiphilic characteristics and delocalized positive charge of berberine is considered to be the main factor for its selective mitochondria accumulation [93,94]. The chemical conjugation of berberine with paclitaxel through a disulfide bond (PTX-*ss*-BBR) has been reported to ameliorate the mitochondrial delivery of paclitaxel, resulting in depolarization of the mitochondrial membrane, with an increased production of ROS, and further induction of apoptosis cell death. Based on molecular dynamics simulations, it was revealed that PTX-*ss*-BBR could form supramolecular self-assembly in water through the non-covalent hydrophobic interactions and π-π stacking with a critical micelle concentration of 1.26 μM [95].

To further advance the mitochondrial targeting ability of berberine, a 16-carbon aliphatic chain was introduced into the C-9th of berberine (9-C16-berberine) and inserted onto the surface of doxorubicin-loaded folic acid-conjugated PEGylated liposomes. A substantial antitumor activity toward multidrug-resistant MCF-7 human breast cancer cell bearing mice was observed as a result of the high-level tumor accumulation through the EPR effect and the interaction with folate receptors in combination with selective mitochondrial localization [96].

Berberine has also been reported to possess antineoplastic activity against a wide variety of tumor cells through its ability to suppress cell proliferation and activate the apoptosis pathway by up-regulating the expression of the pro-apoptotic BAX and down-regulating the level of anti-apoptotic Bcl-2 [97–99]. The insertion of a hydrophobic octadecyl group into berberine molecules resulted in the formation of self-assembled nanoparticles, which could improve its antitumor activity. The nanoparticles were further modified with PEG and HA to intensify the stability and cancer-targeting ability of the preparation. This system was actively be taken up by CD44 positive tumor cells *via* endocytosis followed by the proton sponge effect and electrostatic interactions to facilitate endosomal escape and mitochondrial accumulation, respectively. A significant improvement in cell-killing ability was obtained, both *in vitro* and *in vivo*, through the activation of the intrinsic apoptosis pathway [100]. These findings imply that berberine has promise as an active mitochondrial targeting ligand, as well as an antineoplastic agent.

3.4. Stimuli-responsive drug delivery system targeting cancer mitochondria

The employment of cationic nanoparticles results in several disadvantages during systemic delivery, including the induction of toxicity, poor serum stability, and rapid clearance from the blood circulation by the reticuloendothelial system (RES), causing a low level of accumulated drug in a tumor region. On the other hand, the positively charged nanoparticles exhibit a robust cellular uptake efficiency by taking advantage of the presence of negatively charged membrane surface molecules such as sialic acid and proteoglycans. Furthermore, the cationic characteristics are also essential for interactions with the highly negative mitochondrial membrane thus further triggering the internalization process to the innermost regions of mitochondria. Thus, the utilization of a pH-sensitive drug delivery system would be expected to be a promising strategy for attaining an adequate blood circulation profile and considerable cellular interactions and mitochondria delivery by exploiting the acidic condition of the tumor microenvironment (Table 4).

Zhang and co-workers reported on the utilization of a pH-sensitive liposomal-based nanocarrier, namely $HHG2C_{18}$-L, for the mitochondrial delivery of temsirolimus, an FDA-approved drug for the treatment of renal cancer [101]. This system was constructed from a combination of soy phosphatidylcholine (SPC), cholesterol, and a synthetic zwitterionic oligopeptide lipid ($HHG2C_{18}$). The following lipid material was composed of glutamic acid, histidine, and a pH-cleavable hexahydrobenzoic amide as the hydrophilic portion linked with two stearyl alkane chains as the hydrophobic segment.

The surface charge conversion was initiated when the nanoparticles entered the tumor microenvironment to promote the cell internalization process through clathrin-mediated endocytosis. The presence of the imidazole group on the histidine structure drives the endosomal escape process *via* the proton sponge effect, followed by the hydrolysis of the hexahydrobenzoic amide group, which significantly extends the positive charge of the particles. Mitochondrial accumulation was subsequently accomplished by electrostatic interactions, leading to considerable antitumor activity against murine renal carcinoma bearing mice. To further improve the bioavailability and stability in the blood circulation, the PEG moiety was introduced ($PEGHG2C_{18}$-L), resulting in an enhanced tumor growth suppression activity [102].

A similar approach involving the synthesis of a dual-functional lipid consisting of 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) linked with a mitochondria-targeted peptide of $_D$[KLAKLAK]$_2$ was reported, which was further conjugated with a negatively-

Table 4
Summary of stimuli-responsive mitochondria-targeted DDS.

System name	Cargo	Triggering factor	Vehicle composition	Evaluation method	Outcomes	Refs
PEGHG2C_{18}-L	Temsirolimus	pH-sensitive zwitterionic oligopeptide (HHG2C_{18})	SPC:HHG2C_{18}:Chol (7.5:2.5:1 mass ratio) + 1 mol% PEGHG2C_{18}	Murine renal carcinoma cell bearing mice, i.v. 10 mg/kg BW	Improvement in bioavailability and blood persistence, leading to reduction on the tumor volume	[102]
DKD-Lip	Paclitaxel	pH-cleavage amide bond of KLA peptide and DMA (DKD)	SPC:Chol:DKD (8:1:2 mass ratio)	A549/Taxol cell bearing mice, i.v. 7.5 mg/kg BW	Suppression of tumor proliferation through the activation of the apoptotic pathway	[103]
PNIPAM@PTX	Paclitaxel	Thermo-responsive poly (N-isopropylacrylamide) (PNIPAM)	PNIPAM:TPP:iRGD	4T1 cell bearing mice, i.v. 5 mg/kg BW	Tumor growth inhibition with minimal effect on healthy tissues	[106]

charged 2, 3-dimethylmaleic anhydride (DMA) through the pH cleavage of an amide bond. In the physiological pH (7.4) range, the liposomes (DKD-Lip) had a negative surface charge and became highly positively charged in association with the decrease in pH due to the cleavage of the DMA portion. Furthermore, this liposomal system was actively taken up by cells *via* macropinocytosis and clathrin-mediated endocytosis. The delivery of paclitaxel using this system was found to be effective in producing antitumor activity against paclitaxel-resistant lung cancer cells that had been xenografted on the mouse model with a tumor inhibition rate of 86.7% [103].

Another innovative strategy for selectively transporting anticancer drugs to the mitochondrial compartment of tumors involves exploiting the relatively higher temperature of mitochondria in comparison to the other organelles, particularly in the case of tumor cells [104], using a thermoresponsive nanocarrier. By incorporation of PEG derivative into a thermoresponsive poly(N-isopropyl acrylamide) (PNIPAM), the lower critical solution temperature (LCST) of the polymer could be adjusted to be close to 50 °C. Drug release was found to be effective when it was in close proximity to mitochondria through the shrinkage of the polymers, leading to the mitochondrial accumulation of paclitaxel, as an anticancer drug model, and robust cytotoxicity against an MB49 carcinoma cell line with an IC_{50} of approximately 1 μg/mL [105].

The addition of iRGD, tumor-homing and penetrating peptide, and TPP moieties to the PNIPAM-based nanocarrier system for enhancing the tumor-targeting ability and lysosomal escape capacity, respectively, resulted in a higher level of tumor accumulation as opposed to the non-thermoresponsive polyacrylamide (PAM). This system potently inhibited the growth of tumors in 4T1 tumor-bearing mice with minimal systemic toxicity [106].

3.5. Mitochondrial-targeted photodynamic therapy

During the mitochondrial electron transport process, a small number of electrons can escape and react immaturely with oxygen molecules to form ROS, specifically superoxide ($O2^{\bullet-}$). Cells are basically equipped with a defense mechanism system to control the amount of ROS to below the concentration that could harm the cells. ROS, at certain levels, play an important role in intracellular signaling pathways to regulate several biological and physiological processes, including the transformation and progression of cancer [107,108]. Interestingly, the increment of the ROS level in malignant cells is accompanied by the expansion of the scavenging activity of endogenous antioxidant systems, such as glutathione (GSH) and thioredoxin [109,110].

On the other hand, the uncontrolled production of ROS may induce irreversible oxidative damage and further produce lethal effects for cells [111]. ROS are highly reactive with respect to numerous major biologically active molecules and this could be exploited as a powerful weapon for cancer therapy by promoting the massive production of ROS selectively in tumor cells. One of the most assuring approaches for promoting the generation of ROS is through photochemical reaction processes, namely PDT, which offers a highly spatiotemporal selectivity toward tumors [112]. A lethal level of ROS, mainly singlet oxygen, is generated through the dynamic interaction of a non-toxic light-activated molecule (photosensitizer) and harmless visible light in the presence of oxygen molecules [113].

However, the effectivity of PDT is restricted by the limited lifetime of singlet oxygen and its diffusion capacity [114]. Moreover, the shortage of oxygen supply inside the tumor region (hypoxia) is considered to be another challenging problem in applications of PDT. Therefore, the specific localization of the photochemical reaction, particularly at the subcellular level, is necessary to obtain a maximum PDT effect.

It has been reported that the inhibition of mitochondrial respiration, either by chemicals or the PDT process, causes an increase in the concentration of oxygen in mitochondria [115,116], which could be an advantage for the PDT process, particularly under hypoxic conditions. Furthermore, the increased level of ROS, specifically inside the mitochondrial compartment, would cause depolarization of the mitochondrial membrane, leading to the release of proapoptotic factors such as cyt c from the mitochondria to the cytosol, followed by activation of the caspase pathway to start the apoptosis process [117–119].

By selectively delivering a photosensitizer into the mitochondrial compartment, the damaging effects of ROS would be concentrated in the mitochondria, and this would be a distinct advantage for a mitochondrially selective targeting PDT system to reap the full benefits of PDT (Fig. 8). To date, several strategies for mitochondrial-targeted PDT have been developed and these have been comprehensively reviewed elsewhere [46]. For example, modifying the surface of the upconversion nanoparticles (UCNPs) carrying titanium dioxide with TPP was reported to successfully induce the production of an extensive amount of ROS in mitochondria during near-infrared laser irradiation [119,120]. Moreover, incorporating a mitochondrial-targeted photosensitizer, namely TPP-Pheophorbide-a, to a folate-modified cholesteryl-bovine serum albumin nanocarrier resulted in a significant inhibition in the growth of tumors in an orthotopic glioblastoma-xenografted mouse [119,120]. In addition, other mitochondriotropics such as the rhodamine B and $(KLAKLAK)_2$ peptide have been chemically conjugated to Si (IV)-phthalocyanine and protoporphyrin IX, respectively, resulting in apoptosis cell death [121,122]. The other a hydrophilic rhodamine derivative, tetramethylrhodamine-5-isothiocyanate (TRITC), has been shown to have mitochondrial targeting characteristics, and has been employed to deliver a hybrid nanoparticle consisting of UCNPs and graphene quantum dots specifically into the mitochondrial compartment of 4T1 cells [123]. The mitochondrial accumulation of a phthalocyanine photosensitizer could also be achieved by chemical conjugation with the specific ligand of the outer mitochondrial membrane translocator protein [124]. Furthermore, the mitochondria-targeted PDT system would be more effective as opposed to a non-mitochondrial targeting system, either in the case of a normal oxygen supply or under hypoxic conditions.

3.6. The use of a MITO-Porter for cancer therapy targeting mitochondria

As an integral part of our efforts to develop a versatile mitochondria-targeted DDS, we validated the application of a MITO-Porter system in

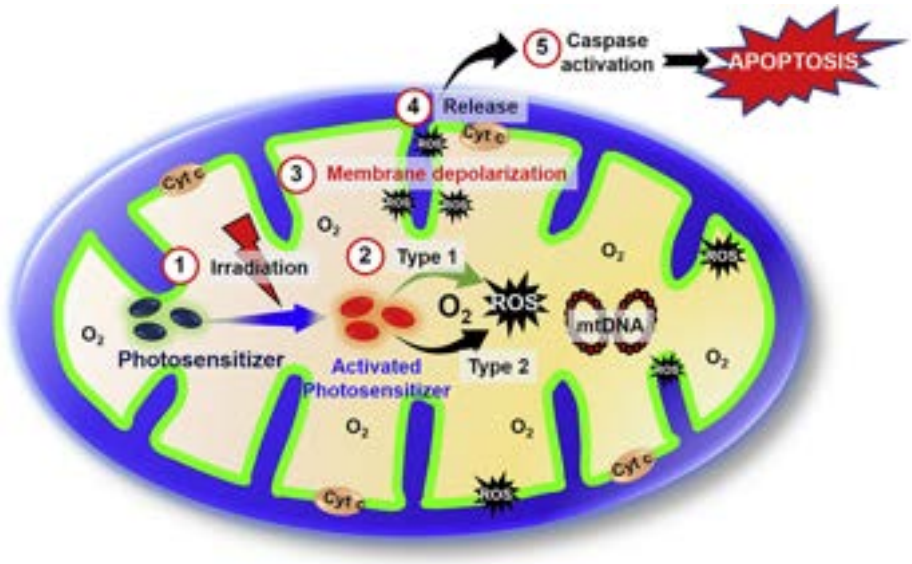

Fig. 8. Mitochondria-targeted PDT. The activation of a photosensitizer is triggered by a light irradiation process (1). The activated photosensitizer then interacts with the abundant levels of O_2 inside mitochondria to produce a lethal level of reactive oxygen species (ROS) through the Type 1 (electron transfer) or Type 2 (energy transfer) reaction (2). ROS attack the mitochondria membrane, causing membrane depolarization (3), subsequently releasing the cyt c (4) into the cytosol. This cyt c then activates the caspase pathway (5) to initiate the apoptosis process.

terms of cancer therapy. In the first attempt, we developed a MITO-Porter encapsulating gentamicin, an aminoglycoside antibiotic that has the potential ability to cause mitochondrial damage, to annihilate a human cervical cancer cell line [125]. As a result, the MITO-Porter system could have the cell-killing ability of gentamicin by facilitating the cellular internalization and mitochondrial delivery process.

We further verified the ability of our mitochondrial targeting technology to overcome cancer resistance phenomena. We selected doxorubicin as a model antineoplastic agent and developed an encapsulation strategy that involved the use of a pH gradient loading method (DOX-MITO-Porter) [126]. As expected, the DOX-MITO-Porter effectively eradicated DOX-resistant human renal cancer cells (OS-RC-2) through the suppression of ATP production and the reduction of the mitochondrial membrane potential. Taking advantage of the presence of PEG moieties on the surface of DOX-MITO-Porter, a prolong blood circulation profile was achieved, thus promoting tumor accumulation through the EPR effect. Subsequently, the DOX-MITO-Porter successfully inhibited the growth of OS-RC-2 cells xenografted on the mice model with negligible systemic toxicity [214].

In the phototherapy field, the MITO-Porter system was developed as a vehicle for a novel pi-extended porphyrin-type photosensitizer, namely rTPA [127]. This system has been confirmed to be effective in activating the apoptotic pathway by irradiation with a near-infrared region light, which results in the massive production of singlet oxygen inside the mitochondrial compartment. Furthermore, a MITO-Porter system was also employed for transporting an electron donor-acceptor linked molecule, resulting in the optically-controlled generation of superoxide, which could be used to induce the apoptosis process [128]. The nature of the MITO-Porter structure allows the encapsulation of both hydrophilic and hydrophobic molecules, without any significant effect on mitochondrial targeting efficiency. Therefore, the MITO-Porter system could be utilized to selectively deliver a wide range of molecules to the mitochondria compartment of tumors through a unique mitochondria membrane fusion mechanism.

4. Mitochondrial diseases and an attempt toward mitochondrial gene therapy

Mitochondrial diseases are defined as a group of genetic disorders that are characterized by defects in oxidative phosphorylation, which are caused by genetic mutations in the both the mtDNA and nuclear DNA that encode mitochondrial proteins or proteins that are related to mitochondrial function [129]. Mitochondrial diseases have wide heterogeneities and can occur at any age, resulting in various manifestations with a broad range of clinical phenotypes [129]. Mitochondrial diseases can occur in any tissues and organs involving multiple systems. In general, the affected organs related to a mitochondrial disease strongly depend on aerobic metabolism, which are usually slowly progressive. Both the diagnosis and management of mitochondrial diseases are challenging, even in modern medicine. To diagnose mitochondrial diseases, pathological analyses and biochemical tests using biopsy specimens are needed, in addition to genetic tests. There are no specific treatments for mitochondrial diseases. To lessen the morbidity and mortality of mitochondrial diseases, our goal was directed to the early intervention of disease-specific complications [129].

In this section, some palliative therapies such as vitamin supplementation are summarized as currently used clinical medicines. Novel therapies which are available only in preclinical or clinical studies are also summarized. Moreover, major genetic mutations related to mitochondrial diseases are described and several research projects regarding innovative mitochondrial gene therapy to achieve drastic treatment are introduced.

4.1. Current status of the clinical treatment of mitochondrial disease and potential therapeutic strategies to lessen the reduction in mitochondrial function

The current medication options for mitochondrial dysfunction-related diseases are restricted to supportive and symptomatic issues with uncertain effectivity such as increasing the production of mitochondrial ATP, reducing the generation of ROS, and inducing mitochondrial biogenesis [130,131], without significant effect on the leading cause of the malfunction. Vitamin supplements are often administered to patients with mitochondrial diseases: antioxidants such as α-lipoic acid, vitamin C and vitamin E [132,133], chemical compounds to modulate mitochondrial electron transfer flux such as vitamin B_2 [134], well-known as riboflavin, and CoQ_{10} [135], nitric acid precursors such as *l*-arginine and *l*-citrulline [136], energy buffers such as creatine [137], drugs involved in fatty acid uptake such as L-carnitine and mitochondrial biogenesis such as vitamin B_3 [138]. *l*-Arginine is reported to be

Table 5
Potential therapeutic strategies for the treatment of mitochondrial diseases.

Drug name	Therapeutic strategy	Target of disease	Trial phase	Clinical trials no.
Idebenone	Antioxidant effect by synthetic CoQ_{10} analogue	MELAS syndrome	Phase IIa	NCT00887562
		LHON syndrome	Phase IV	NCT02774005
EPI-743	NADP dehydrogenase modulator	Leigh syndrome, mitochondrial-related disease	Phase IIb	NCT01721733 /NCT01642056
Elamipretide (MTP-131)	Small peptide to stabilize mitochondrial inner membrane	Mitochondrial myopathy, Barth syndrome	Phase III	NCT02367014
Taurine	Taurine modification	MELAS syndrome	Phase III	UMIN000011908

beneficial for stroke-like episodes in the treatment of mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) patients [139]. In addition, most of the therapeutic modalities are based on the use of antioxidant agents such as CoQ_{10}, idebenone, EPI-743 [140]. The following drugs and therapies against mitochondrial disorders are thought to be promising although some are not commercially available (Table 5).

4.1.1. Scavengers

Some agents can be used to modify the metabolic course and therefore, block the progress of mitochondrial diseases, which involve the accumulation of toxic substances. Metronidazole and *N*-acetylcysteine can differently reduce the amounts of hydrogen sulfide that are accumulated in ethylmalonic encephalopathy. This combination is currently the most useful palliation for ethylmalonic encephalopathy [141].

4.1.2. Enzyme replacement therapy

Enzyme replacement therapy clinically has enjoyed great success in the treatment of many metabolic diseases. The mitochondrial neurogastrointestinal encephalopathy (MNGIE) syndrome is the first-in human mitochondrial disease to be approached. The administration of erythrocyte encapsulating thymidine phosphorylase turned out to be successful in a patient under a clinical research situation [142].

4.1.3. CoQ_{10} supplementation

Primary CoQ_{10} deficiency is a genetic disease characterized by defects in the CoQ_{10} biosynthetic pathway with a variety of manifestations such as systemic mitochondrial disorders during childhood, nephrotic syndrome, and cerebellar ataxia. CoQ_{10} supplementation can be used to improve the symptoms concerning a primary CoQ_{10} deficiency [143,144]. Moreover, CoQ_{10} supplementation has often been used to treat patients with mitochondrial diseases and disorders [145].

4.1.4. CoQ_{10} analogues

New drugs such as quinone derivatives are under research and some have been reported to be promising. A single randomized placebo-controlled trial revealed that idebenone, a CoQ_{10} analogue, was able to maintain the vision of patients with Leber's hereditary optic neuropathy (LHON). The effect of idebenone permitted vision to be preserved for more than 2 years [146]. Other novel antioxidants that are currently being investigated in clinical trials are EPI-743 and cysteamine bitartrate, which are reported to improve cellular glutathione levels in patients with Leigh syndrome [147].

4.1.5. Cofactor therapy

Vitamin B_2 plays an important role as a cofactor for flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The administration of vitamin B_2 has been useful in improving symptoms in mitochondrial diseases caused by genetic disorders encoding FMN-dependent or FAD-dependent proteins. Improving the functions of residual enzymatic activity by using vitamin B_2 treats some mitochondrial diseases. NADH dehydrogenase flavoprotein 1 (NDUFV1), succinate dehydrogenase flavoprotein subunit (SDHA), apoptosis-inducing factor 1 mitochondria associated 1 (AIFM1) and acyl-CoA dehydrogenase family member 9 (ACAD9) would be effective [148].

4.1.6. Preservation of electron transport chain

Elamipretide, a mitochondria-targeted therapeutic and also known as SS-31 or Bendavia™ is in clinical development for the treatment of a variety of diseases caused by mitochondrial disorders and dysfunctions. Elamipretide targets the inner mitochondrial membrane where it associates with cardiolipin–the signature phospholipid of the inner mitochondrial membrane, which plays a role in a number of mitochondrial processes, including respiration and energy conversion. This elamipretide-cardiolipin association has been shown to normalize the structure of the inner mitochondrial membrane, thereby improving mitochondrial function.

In preclinical and clinical studies, elamipretide was shown to increase mitochondrial respiration, improve electron transport chain function and ATP production, and reduce the formation of pathogenic ROS levels. The functional benefit is assumed to be achieved through improving ATP production and the interruption and potential reversal of damaging oxidative stress [149]. Elamipretide is currently being investigated in late-stage clinical studies for the treatment of primary mitochondrial myopathy and Barth syndrome as well as in earlier stage clinical studies in LHON and geographic atrophy associated with dry age-related macular degeneration in USA.

4.1.7. Improvement in taurine modification

MELAS is a major mitochondrial disease, the pathogenicity of which depends on mutated transfer RNA (tRNA) $^{Leu\ (UUR)}$. Yasukawa T et al. first reported that a lack of taurine modification is one of the wobble modifications during gene translation in mitochondria, which leads to a disorder in protein synthesis [150]. High dose supplementation of taurine for MELAS patients in Japan turned out to be beneficial for the prevention of stroke-like events.

4.2. Relationships between mitochondrial diseases and mutations in mtDNA

The human mitochondrial genome contains a multicopy, circular double-strand DNA, approximately 16.6 kb in size that encodes 13 essential polypeptides of the oxidative phosphorylation subunit, 22 tRNA and 2 ribosomal RNA (rRNA) for mitochondrial protein synthesis [151,152]. This genetic material is concentrated inside the mitochondrial matrix, that is tightly enclosed by double mitochondrial membrane system separated by the intermembrane space. Mutations in the mtDNA or nuclear DNA that encode mitochondrial proteins lead to primary mitochondrial dysfunctions, as opposed to external pathological events which cause secondary mitochondrial dysfunctions [153]. Due to the chronic exposure to mitochondrial ROS, the mtDNA has a high mutation rate [1].

mtDNA is in the status of whether homoplasmy or heteroplasmy. Heteroplasmy means that the mitochondrial genome contains a mixture of both mutated and wild-type mtDNA. On the other hand, the term homoplasmy denotes the pure mitochondrial genome; 100% wild-type or 100% mutated mtDNA. Mitochondrial disorders manifest

themselves as clinical problems, when the percentage of mutated mtDNA exceeds a certain threshold [154,155]. The first findings regarding the relationship of mtDNA mutations with several diseases such as LHON, where the mutation occurs at mt11778 in ND4 where a G is converted into an A in the mtDNA, and mitochondrial myopathy were reported at the end of the 1980s by two different research groups [156,157]. One year after those reports, Moraes and co-authors strengthened the findings by identifying a decrease in the activities of four essential enzymes of the mitochondrial respiratory chain in the patients with deletions on the mtDNA [158].

A group of Japanese investigators reported that an A-to-G transition mutation at the nucleotide pair 3243 in the mitochondrial $tRNA^{Leu(UUR)}$ was found to be the primary cause of MELAS [159]. In addition, a variety of mtDNA mutations have been reported that are related to many mitochondrial diseases such as 10,158 T > C in mtDNA coding mRNA for the ND3 protein [160] (the major mutation of Leigh syndrome) [161], 1555A > G in mtDNA coding rRNA (the mutation related to hearing loss) [162–164] and 625G > A in mtDNA coding $tRNA^{Phe}$ (the mutation related to progressive hearing impairment, epilepsy and elevated lactic acid levels) [165]. A map of human mtDNA and the mutation points related to mitochondrial diseases are shown in Fig. 9.

4.3. Concept of mitochondrial gene therapy

In most cases, mitochondrial dysfunction-related diseases are still considered to be an unmet medical need. Therefore, the development of specific therapeutic strategies that focus on the actual cause of the mitochondrial dysfunction would have a tremendous medical benefit, including gene therapeutic approaches. To realize such an effective mitochondrial gene therapy, two essential tasks need to be completed, namely the construction of a functional cargo for mitochondrial gene therapy and the development of a versatile mitochondrial gene delivery system. Once again, a selective mitochondrial DDS that provides excellent protection for the carried cargo is required, due to the poor cellular uptake, cytosolic stability, and access of macromolecules to mitochondria.

Fig. 10 shows a schematic concept of mitochondrial gene therapy by the mitochondria delivery of therapeutic cargoes, including the delivery of wild-type mtDNA or artificial DNA coding therapeutic gene to the mitochondrial matrix, thus complementing normal mtDNA. Furthermore,

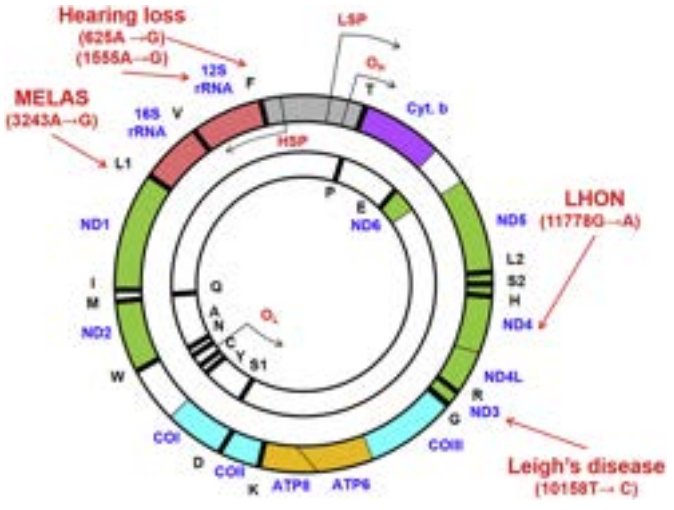

Fig. 9. Map of human mtDNA. Human mtDNA contains 13 essential polypeptides of oxidative phosphorylation subunit-encoding genes, 22 tRNAs and 2 rRNAs (12S rRNA and 16S rRNA). A single letter code is given for each tRNA-encoding gene. ND, NADH dehydrogenase coding subunits; CO, cytochrome oxidase coding subunits; ATP, F_1F_0-ATP synthase coding subunits. Point mutations corresponding to various mitochondrial diseases, such as MELAS, are also indicated.

the direct delivery of therapeutic RNA and functional protein to the mitochondrial compartment could also be a promising strategy to compensate for the inability of a defect in mtDNA related to the synthesis of a wild-type protein. These therapeutic strategies include inhibiting the replication of the mutated mtDNA, the repair of mutated mtDNA and/or the degradation of the mutated mtDNA and repair of mutated mtDNA. The site-specific correction of mtDNA mutations such as genome editing would also be an innovative strategy for treating mitochondrial genetic disorders. In the following section, we summarize several innovative efforts in mitochondrial gene therapy by delivering circular DNA, antisense RNA oligonucleotide (ASO), therapeutic RNA and related molecules to mitochondria.

4.4. Strategy for circular DNA delivery using mitochondrial nano DDS

Delivering a large amount of wild-type mtDNA to the mitochondria in damaged cells would modify the ratio between mutated and wild-type mtDNA, thus suppressing the manifestations of the mitochondrial disease. The MTS could allow these conjugates of nucleic acids access to mitochondria *via* the native mitochondrial transport machinery. DNA with lengths from 17 to 322 bp are theoretically able to pass when this method is used [166]. However, there are limitations to the size of the cargo, which suggests that the delivery of circular DNA, such as mtDNA or plasmid DNA (pDNA), would not be possible. Herein, we summarize the efforts that have been made to deliver circular DNA to mitochondria in this section (Table 6).

4.4.1. Mitochondrial delivery of pDNA using DQAsomes

Weissig and co-workers proposed the selective mitochondrial delivery of DNA conjugated with the MTS peptide using a self-assembly DQA-based cationic vesicle, namely a DQAsome [167,168]. In this strategy, the DQAsome has the function of transporting the DNA-MTS conjugate into the proximity of mitochondria *via* electrostatic interactions, as well as protecting the nucleic acid from the DNase degradation. A group from Monash University continued the application of DQAsomes in transporting DNA into the mitochondrial compartment of living cells [169]. They attempted to construct an artificial mini-mitochondrial pDNA that encoded the green fluorescence protein (GFP) that is expressed in the mitochondrial compartment (pmtGFP). The expression of pmtGFP was confirmed to be exclusively expressed in mitochondria, while no expression was detected when pDNA was transported into the nucleus. The transfection of the DQAsome-pmtGFP complex into a mouse macrophage cell line RAW 264.7 and several mammalian cell lines resulted in the expression of GFP specifically inside the mitochondria, but with relatively low transfection efficiency ranging from 1% to 5%, as judged by microscopic and flow cytometry analysis.

Attempts were also made to selectively deliver the same mitochondrial pDNA to mitochondria of HeLa cells using a surfactant-based gene transfection vector. In this strategy, a low critical micelle concentration of gemini surfactants was used to condense and protect the pmtGFP, in addition to facilitate cellular uptake *via* a combination of the endocytic pathway and direct membrane translocation followed by extensive interactions with the mitochondrial membrane [170].

To further enhance the mitochondrial targeting ability of the DQAsome-pDNA complex (DQAplex), a combination of phospholipids consisting of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and DOPE was introduced to a DQAsome system to produce a novel nanosome system, namely DQA80s [171]. The presence of DOTAP and DOPE resulted in a considerable improvement in the cellular uptake efficiency and endosomal escape ability of DQA80s, leading to selective mitochondrial ZsGreen expression. A faster endosomal escape ability of DQA80s relative to the conventional DQAsome system was observed due to the alteration of the lipid conformation to a hexagonal structure, resulting in the rapid destabilization of the endosomal membrane by altering the internal pH and ionic strength [41].

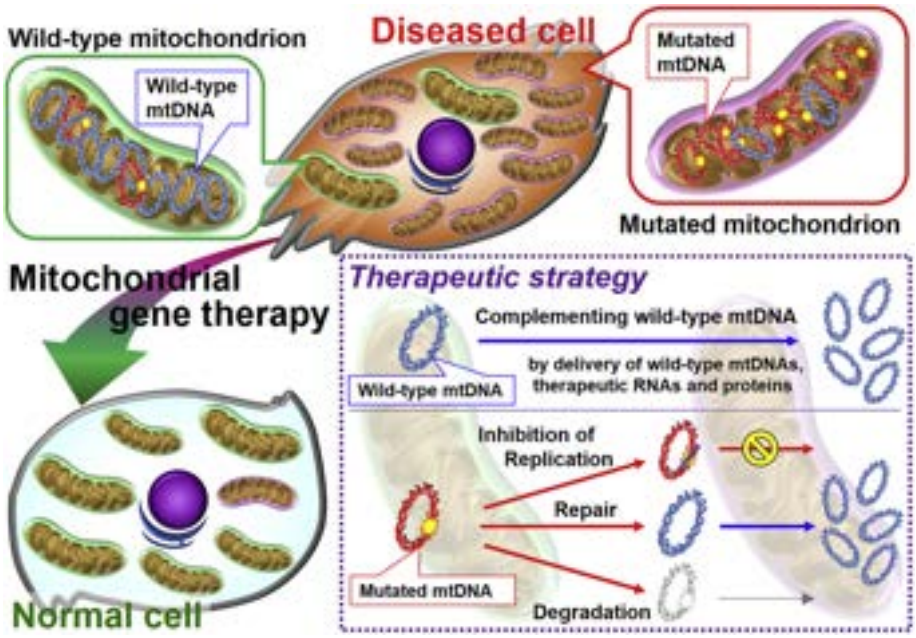

Fig. 10. Schematic concept of mitochondrial gene therapy. Strategies involving mitochondrial gene therapies include the delivery of wild-type mtDNA or artificial DNA coding the therapeutic gene to the mitochondrial matrix, delivering therapeutic RNA and a functional protein to the complementing wild-type mtDNA. Other therapeutic strategies also include inhibiting the replication of mutated mtDNA, the repair of mutated mtDNA by genome editing technology and/or degradation of the mutated mtDNA.

Another exciting effort was reported based on the utilization of a multifunctional peptide-based system for the selective mitochondrial delivery of pDNA [172]. Cationic lysine-histidine (KH) peptides were employed to promote the cellular internalization of the complex. In addition, the lysine residues have a specific role in condensing the pDNA into the core of the complex, while histidine residues induce endosomal lysis through the proton sponge effect. The MTS with a 12-residue partial sequence of yeast cytochrome *c* oxidase subunit IV was then fused with the HK peptides to mediate the mitochondrial internalization of the pDNA. A significant mitochondrial gene expression was detected at 24 h post-transfection for periods of up to 108 h with negligible toxicity, indicating its ability as a promising mitochondrial gene vector system.

4.4.2. Rhodamine-based pDNA nanoparticle for mitochondrial delivery

Rhodamine 123, a cell-permeating fluorescent dye, has been widely used to evaluate the mitochondrial membrane potential status of living cells based on membrane potential-dependent accumulation [173,174]. The combination of lipophilic characteristics and cationic properties is the main driving force for this compound for it to be extensively incorporated in the mitochondrial compartment [61], as well as to provoke cellular internalization through the negatively-charged plasma membrane. Rhodamine-based pDNA nanoparticle have been introduced as an alternative system for mitochondrial gene therapy [175–177].

A simple, economical, and rapid preparation process involving a co-precipitation method was employed to construct a mitochondrial-targeted pDNA nanoparticle system. The co-precipitation process occurs as the result of interactions between Ca^{2+} with pDNA in the presence of CO_3^{2-}, leading to a high pDNA loading efficiency (51–66%). When cellulose or gelatin was added to control the size of the nanoparticles, this improved the aqueous stability, and magnified the overall transfection efficiency. Based on microscopy observations and fluorescence intensity evaluations on isolated mitochondria, it was found that this rhodamine-based pDNA nanoparticle accumulated dominantly in the mitochondrial compartment. Moreover, an indirect functional evaluation was conducted to confirm the selective mitochondrial delivery by comparing the pDNA expression activity in the cytosol of cells transfected with the nanoparticles in the absence and presence of Rhodamine

Table 6
Strategies for the mitochondria delivery of circular DNA.

Nanocarrier/targeting system	Genetic material	Cell (s)	Evaluation method	Refs.
DQAsome	pmtGFP	RAW 264.7, OKO160, mouse fetal neuronal stem, rat fetal fibroblast, bovine fibroblast, human kidney 293	Functional evaluation by detecting the expression of GFP	[169]
Gemini Surfactant	pmtGFP	HeLa cells	Functional evaluation by detecting the expression of GFP	[170]
DQA80s (DQA-DOPE-DOTAP)	hmtZsGreen	HeLa cells & dermal fibroblast	Functional evaluation by detecting the expression of ZsGreen	[171]
Cytocox-KH	pDONR-cox2:rLuc & pDONR-cox2:gfp	HEK 293 cells	Functional evaluation by detecting the expression level of Rluc gene and GFP	[172]
Rhodamine-123	pUC19, pVAX1-LacZ, pcDNA3, pCAG-GFP, pcDNA3-myc-FLNaS2152A, & pCAG-GFP-ND1	HeLa cells, mouse N2a neuroblastoma cells, normal human dermal fibroblast	Indirect evaluation using microscopy observations and gene expression in the nucleus	[175–177]
MITO-Porter	pCMV-mtLuc	HeLa cells, G625A fibroblast obtained from a patient with mitochondrial disease	Functional evaluation by measuring luciferase activity in a combination with colocalization study	[181,182]

123. However, a direct functional evaluation is required to demonstrate the actual ability of the delivered cargo in expressing a functional protein specific to mitochondria.

4.4.3. Innovative technology for mitochondrial gene expression using MITO-Porter system

Over the last decade, our laboratory has focused on the development of a versatile mitochondrial targeting liposomal-based nanodevice, namely a MITO-Porter system. Several macromolecules, ranging from proteins to nucleic acids, have now been successfully transfected into mitochondria of living cells in order to regulate the mitochondrial genomic system, as well as to validate the application of the MITO-Porter system as a potential candidate as a mitochondrial targeting DDS [45,178–180].

More recently, we designed a mitochondrial selective DNA vector, namely pCMV-mtLuc (CGG), carrying the cytomegalovirus (CMV)-derived promoter, and the *mtLuc (CGG)* gene, which expresses NanoLuc luciferase protein in mitochondria. To further enhance transfection efficiency, we replaced the R8 moieties with a combination of the KALA peptide and a mitochondrial RNA aptamer. This MITO-Porter system demonstrated a strong mitochondrial transgene expression in fibroblast cells derived from a patient with a mitochondrial disease, as indicated by the enhanced level of mitochondrial luciferase activity [181,182].

4.5. Validation of mitochondrial RNA delivery using a mitochondrial DDS

To date, we have continued to carry out the research in attempts to establish a gene expression control system that targets mitochondria based on the use of a MITO-Porter such as “development of mitochondrial expression DNA vectors” described above [181–183]. We also are working on research related to this topic including “functional molecule delivery targeting mtDNA [184,185]” and “mitochondrial RNA delivery for gene therapy [179,180,186–188]”. Here, we summarize our efforts regarding mitochondrial RNA delivery.

4.5.1. Mitochondrial RNA knockdown

It was reported that the mitochondrial delivery of an ASO by a MITO-Porter system could cause mitochondrial RNA knockdown to regulate mitochondrial function [179,180,186]. A nano-technological combination of both a MITO-Porter system and D-arm, a mitochondrial import signal of tRNA to the matrix, was used to deliver ASO to mitochondria to achieve mitochondrial RNA knockdown. The targeted mRNA is mtDNA-encoded mRNA to express mitochondrial endogenous protein, cytochrome *c* oxidase subunit II (COX2), which is a component of the mitochondrial complex IV in the mitochondrial respiratory chain [179].

We subsequently reported on the efficient packaging of ASO in the MITO-Porter *via* a nanoparticle packaging method, which showed a 10-fold higher packaging efficiency compared to the conventional method [180]. Fig. 11 summarizes the research outcome of this study. We evaluated the antisense effect by the MITO-Porter, by quantifying the mitochondrial mRNA-levels coding COX2 at 24 h post transfection. As a result, the COX2 mRNA expression levels were depressed by the mitochondrial delivery of the ASO when the MITO-Porter was used (Fig. 11A).

We also evaluated mitochondrial membrane potential using the JC-1 dye to detect this potential (Fig. 11B). Using JC-1, in the case of non-treated cells, red colored mitochondria were observed (Fig. 11B (a)), while, when an uncoupler, FCCP, which decreases membrane potential was added, a green color in the cytosol was observed (Fig. 11B (b)). In the case of non-treated and Mock treated cells (Fig. 11B (a), (c)), red fluorescent mitochondria were observed. On the other hand, in the case of ASO, we observed a green color in the cytosol (Fig. 11B (d)). These results indicate that the MITO-Porter system is able to regulate mitochondrial gene expression and mitochondrial functions. The constructed carrier also showed a decrease in the target protein levels and ATP production. These results indicate that such a MITO-Porter has potential for use in therapies designed to regulate mitochondrial function.

4.5.2. Delivery of mRNA to cardiac mitochondria

The delivery of nucleic acids to cardiac mitochondria is anticipated to be an innovative therapy for the treatment of heart failure. H9c2 cardiac myoblasts have been used as a type of experiential *in-vitro* model of myocardium. We improved a Multifunctional Envelope-type Nano Device (MEND) as a carrier of nucleic acid in terms of mitochondrial targeting, and encapsulated an RNA expressing reporter protein in the MEND. The transfection by the MEND system showed that the particles efficiently reached the mitochondria in H9c2 cells and the direct mitochondrial transfection of exogenous RNA was detected [187].

4.5.3. Validation of mitochondrial gene therapy targeting to disease cells by mitochondrial delivery of therapeutic RNA

In a previous study, we verified a mitochondrial gene therapy by delivering nucleic acids to mitochondria of a diseased cell. The cell has a G625A heteroplasmic mutation in the $tRNA^{Phe}$ of the mtDNA [188]. As a therapeutic strategy targeting mutated mitochondria, we attempted to deliver wild-type mitochondrial pre-$tRNA^{Phe}$ (pre-WT-$tRNA^{Phe}$) using a MITO-Porter in an attempt to decrease the mutation rate of $tRNA^{Phe}$ in mitochondria.

To accomplish this, pre-WT-$tRNA^{Phe}$, prepared by *in vitro transcription*, was encapsulated in the MITO-Porter and transfected into diseased mitochondrial cells, and the resulting mutant levels were examined by amplification refractory mutation system (ARMS) - quantitative PCR. The mutation rate of $tRNA^{Phe}$ was decreased, whereas the transfection of the control sequence did not change the mutation rate. This therapeutic effect was sustained even on the 8th day after transfection. Furthermore, mitochondrial respiratory activity of the disease cells was increased after the transfection of therapeutic pre-WT-$tRNA^{Phe}$.

These results support the conclusion that the mitochondrial delivery of therapeutic nucleic acids represents a viable strategy for mitochondrial gene therapy. We are currently conducting clinical research directed toward trials to establish a viable mitochondrial gene therapeutic strategy.

4.6. Mitochondrial protein delivery for gene therapy

Several innovative strategies for transporting functional mitochondrial proteins have been reported to improve mitochondrial functions, as well as serving as an alternative to gene therapy. The combination of a protein transduction domain (PTD) with super oxide dismutase (SOD) 2 MTS, designated as a mitochondrial transduction domain (MTD), was recombined with the mitochondrial transcription factor A (TFAM) protein for selective delivery to mitochondria of LHON cytoplasmic hybrid cells. The PTD, composed of 11 arginine residues, that function as the mediator for cytosolic delivery, while SOD2 MTS facilitates entry to the mitochondrial matrix. The MTD-TFAM treatment resulted in a considerable improvement in mitochondrial functions and biogenesis, both *in vitro* and *in vivo*, probably due to the incremental increase in mitochondrial gene replication, transcription, and translation into respiratory proteins [189,190]. Due to the ability of TFAM to bind mtDNA in a non-specific manner, the application of MTD-TFAM was extended as a carrier for mtDNA, resulting in a notable improvement in mitochondrial physiology in Parkinson's disease, LHON, and Leigh's syndrome cell models [191,192].

In the current report, a biodegradable silica nanoparticle modified with cell-penetrating poly(disulfides) (CPD) and TPP was employed to deliver native proteins into mitochondria of living cells. The CPD surface modification drives the rapid cytosolic delivery of the nanoparticles, accompanied by cytosolic GSH-induced CPD depolymerization, leaving the TPP moieties on the exterior of the particles. The combination of lipophilicity and delocalized cationic properties of TPP facilitates the internalization of the particles into the interior of mitochondria and the

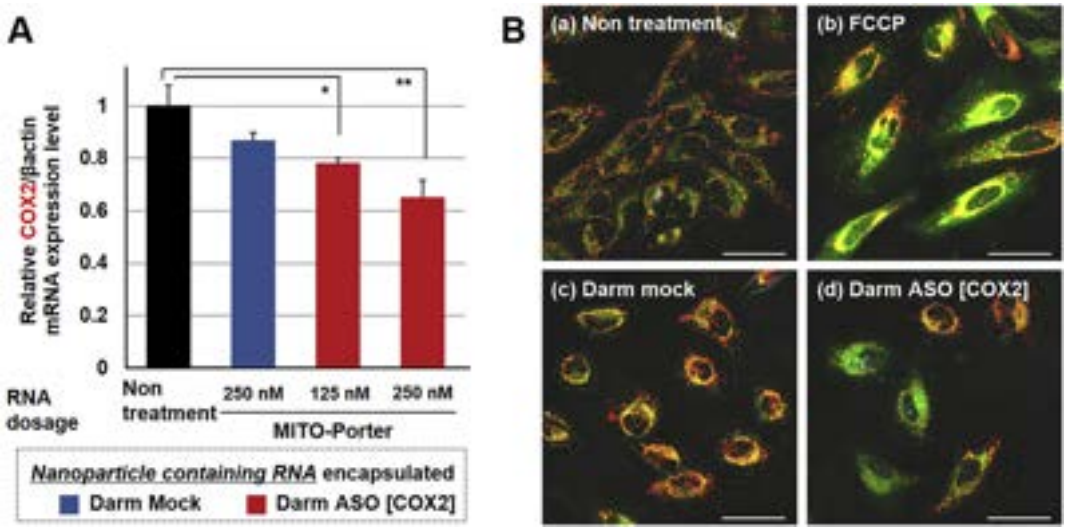

Fig. 11. Research outcome of mitochondrial ASO delivery by MITO-Porter. (A) Evaluation of mitochondrial mRNA expression levels at 24 h after transfection by the MITO-Porter system. After the transfection of Darm Mock or Darm ASO [COX2], the knockdown effect of the mitochondrial mRNA (COX2 encoded in mtDNA) was estimated by quantitative reverse transcription PCR. Bars indicate the mean with SEM ($n = 3$–7). Significant differences ($^{**}p < 0.01$, $^{*}p < 0.05$). (B) CLSM image of mitochondrial membrane potential using JC-1 at 48 h after the transfection ((a) Non-treatment, (b) FCCP, (c) Darm Mock, (d) Darm ASO [COX2]). Scale bars, 50 mm. These figures [180] are reproduced with permission from Elsevier.

release of protein catalyzed by mitochondrial GSH. Furthermore, this system was capable of selectively transporting several proteins and antibodies into the mitochondrial compartment, as indicated by microscopy and Western blot analysis [193].

In addition, some research on mitochondrial gene editing has been reported. The technology of genome editing such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats/CRISPR associated proteins (CRISPR-Cas9) systems has been found to be strong tools for gene-editing. Improvements in TALEN and ZFN technologies for mitochondrial genome editing has been proceeding. Two research groups recently reported that mitochondrial TALEN and mitochondrial ZFN succeeded differently in mitochondrial genome editing using animal models with pathogenic mitochondrial gene mutations [194,195]. Although the off-target phenomenon by genome editing would be problematic for clinical research, the precise gene editing by CRISPR-Cas9 system could be under investigation of mitochondrial genome editing.

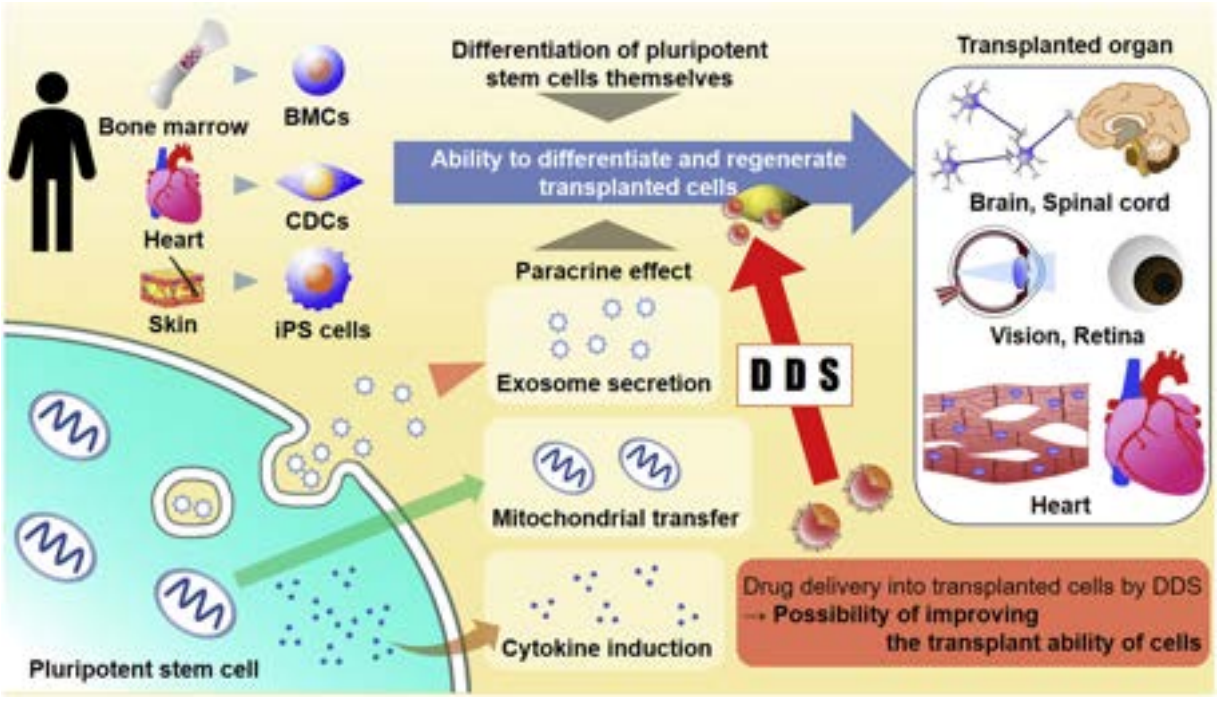

Fig. 12. Isolation and culture of pluripotent stem cells as a source of transplantation and representative organs that are their targets. In addition to the induction of stem cell differentiation, the paracrine effect is another therapeutic mechanism. A conceptual diagram showing the increase in the effect of a drug delivery system (DDS) on cell therapy for multifunctional stem cell transplantation. Transplanted cells can be strengthened by DDS technology, which has the potential to improve differentiation and paracrine effects, and is expected to improve regenerative medicine. BMCs, bone marrow-derived mononuclear cells; CDCs, cardiosphere-derived cells.

5. Cell transplantation therapy and mitochondrial activation

The use of stem cell therapy for the treatment of mitochondrial diseases has been reported [196]. Hematopoietic stem cell transplantation therapy to maintain thymidine phosphorylase activity was found to lower the levels of thymidine and deoxyuridine in patient's blood and lead to improvements in the clinical symptoms in the patients suffering from the MNGIE syndrome. Unfortunately, more than 50% therapy-related deaths have been reported [196]. Cell transplantation therapy and the relationship between the therapeutic effects and mitochondrial function are described, although it was not the focus of mitochondrial therapy

In recent years, clinical trials for cell therapy and regenerative medicine, ranging from basic research to clinical applications have been reported. It has shown efficacy at the animal level and the results in clinical trials have opened up possibilities for the future [197–199]. Basic research and clinical trials for cell therapy and regenerative therapy are being conducted using mesenchymal stem cells derived from bone marrow, umbilical cord tissue, dental pulp and cardiac muscle, embryonic stem cells (ES cells) [200], induced pluripotent stem cells (iPS cells) [201,202] as transplanted cells. Although several clinical studies indicated certain effects, the limited therapeutic efficacy of cell transplantation therapy and possible side effects such as proarrhythmia and tumorigenesis will need to be overcome for use in clinical applications [201,203,204].

The therapeutic effects of transplanted cells include not only the proliferation of stem cells themselves, but also cytokines secreted by stem cells and the mechanisms of action such as exosomes and mitochondrial transfer (Fig. 12) [205–207]. Large amounts of energy production by mitochondria are required for the division of stem cells, and it is reported that switching the energy supply from glycolysis to aerobic metabolism *via* mitochondria is essential for stem cell differentiation, improved cell function, and successful cell therapy and regenerative medicine [208,209].

Reports regarding the improvement of mitochondrial function and exosomes have attracted interest as a DDS technology (Fig. 12) [210,211]. To date, only a few basic studies dealing with the improved mitochondrial function of stem cells themselves have appeared. Cell transplantation therapy using mitochondria-activated cardiac stem cells (MITO cells) has been performed on a mouse model of doxorubicin cardiomyopathy. In this experiment, MITO cells were prepared by activating cardiac progenitor cells by delivering resveratrol, which improves mitochondrial biogenesis, to mitochondria using a MITO-Porter system. Oxidative stress and massive cell death were both significantly reduced in the MITO cell transplanted hearts, in which the expression levels of the mitochondrial oxidative phosphorylation (OXPHOS) protein and gene were also higher than that for the control group. In doxorubicin-induced cardiomyopathy, the transplantation of MITO cells, which possess activated mitochondria, showed more efficient therapeutic effects compared to conventional CPC transplantation [212,213]. Such a report will contribute to the advancement of cell therapy and regenerative medicine.

6. Conclusions

Research on mitochondria with various functions have been carried out around the world for a long time, and controlling the functions of this organelle is expected to be studied deeply in attempts to understand the life sciences and develop innovative therapeutic strategies. To achieve such an innovative research and mitochondrial therapy, a mitochondrial DDS will be required. At the beginning of the 2000s, when we started our own research to develop a mitochondrial DDS, there were only a few reports regarding mitochondrial DDS, and a technology for developing a DDS to achieve innovative therapies such as mitochondrial gene therapy was only a dream. During this past two decades, nanotechnology focused on DDS has advanced, and an innovative technology for delivering siRNA to the cytoplasm of target tissues has led to the nano medicine of nucleic acids, ONPATTRO (R). In this review, we summarized reports regarding mitochondria-targeted therapeutic strategies including antioxidant therapy, cancer therapy, mitochondrial gene therapy and cell transplantation therapy based on mitochondrial DDS. From 2020 when about 10 years have passed since the publication of our review paper on mitochondrial DDS [2], we believe that organelle-targeted DDS will open novel areas leading to scientific revolution and the development of innovative nano medicine targeting mitochondria in the next ten years. We hope that this review paper will help to accelerate the research in these fields, and we hope to be here to witness and to participate in this further research.

BW, body weight; Chol, cholesterol; i.v., intravenous; iRGD, cyclic peptide for tumor targeting; SPC, soy phosphatidylcholine; TPP, triphenylphosphonium.

Declaration of Competing Interest

The authors declare no conflict of interest.

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