EXPERT OPINION

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Delivery to mitochondria: a narrower approach for broader therapeutics

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Introduction: Research has revealed a relationship between mitochondrial dysfunction and diseases such as diabetes, ischemia–reperfusion injury, cancer and many more. As a result, mitochondria have gained attention as a target organelle for the treatment of many diseases. Successful delivery of the drug molecule to the mitochondria could be achieved by keeping in mind the normal intracellular trafficking fate of molecules in cell as well as through the mitochondria and exploring the new possibilities to reach the target in an efficient manner.

Areas covered: This review covers important areas such as structure and physiology of mitochondria, mitochondrial genome and its role in the diseases led by mitochondrial dysfunction, generation of reactive oxygen species and its disbalance in pathophysiological conditions and apoptosis. Further, the review focuses on various human mitochondrial diseases, particularly cancer, and strategies and methods of targeting drug and genetic materials to mitochondria. Novel nanotechnology-based carriers for mitochondria delivery are discussed with an attempt of providing readers with a current and future prospective of mitochondrial therapeutics.

Expert opinion: Numerous investigators have attempted to establish a mitochondrial drug delivery system; still, many hurdles yet remain to be overcome before mitochondrial medicine reaches clinical applications. We need to develop a delivery system to encapsulate drugs, proteins and genes that would be practically viable for scale-up and strategies to target and regulate drug release to the cytosol after endosomal escape, and thereafter to deliver the released drug to the mitochondria. Current innovations in the nanotechnology could be effectively utilized with mitochondrial medicine for designing optimal nanoparticle drug delivery system for mitochondrial diseases on clinical setting.

Keywords: anticancer drug delivery, intracellular delivery, mitochondrial diseases, mitochondrial medicine, mitochondriotropic, nano-technology, triphenyl phosphonium ion

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1. Introduction

Mitochondrion, an organelle of the cell, is of 1 μm in diameter and of variable length, which is hypothesized to be originated by endosymbiosis. After their first recognition in 1840, their naming in 1889 and their structural analysis in 1950s, mitochondria have incurred a plethora of importance because of their inherent life-sustaining properties that have opened the gate for a new era of mitochondrial medicine. Mitochondria are an energy-harnessing factory of the cell, which play a crucial role in the normal functioning of the cell and hence on the functioning of the body. Collective research of many organizations has revealed that mitochondrial dysfunction contributes to a spectrum of human diseases broadly, including neurodegenerative disease, neuromuscular disease, diabetes, ischemia–reperfusion injury

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Article highlights.

- Mitochondria have gained attention as a target organelle for the treatment of diseases such as neurodegenerative disease, diabetes, ischemia–reperfusion injury and cancer.
- Mitochondrial delivery is possible by exploiting the inherent traits of the mitochondria, which include high membrane potential (approximately -200 mV) and protein import machinery of mitochondria.
- The lipophilic cationic molecules, such as triphenyl phosphonium (TPP) cation, have a tendency to accumulate inside the mitochondrial membrane responding to mitochondrial membrane potential.
- Approach for mitochondrial delivery involves the use of TPP cations, mitochondrial-penetrating peptides, cationic bola lipid and so on.
- Major hurdle in the delivery of therapeutic molecules is the scarcity of the carrier system, which could be able to load various molecules including genes and proteins and also should possess cell-specific targeting and internalization capability with further strength to regulate intracellular trafficking of the load from endosome to cytosol and thereafter to mitochondria.
- Recently, MITO-Porters, a device including ligands for specific receptors, pH-sensitive fusogenic peptides (DOPE) for endosomal escape, mitochondriotropic residues for enhanced mitochondrial delivery, are reported.
- Evidence suggests that the increased reactive oxygen species stress in cancer cells has a pivotal role in the acquisition of the hallmarks of cancer such as immortalization and transformation including chemoresistance.
- The strategy of targeting anticancer drug to mitochondria using mitochondriotropic agent, stearyl triphenyl phosphonium cationic in liposome, is also reported.
- A synthetic strategy for attaching triphenyl-phoshonioalkylthiosulfate ligands to the surface of AuNPs, nanodelivery system for CoQ10 using ABC miktoarm star polymers, is another system attempted for mitochondrial delivery of drugs.
- Merger of pharmaceutical nanotechnology with mitochondrial medicine, which would eventually lead to the development of a large variety of mitochondria-specific nanotools, is warranted.

This box summarizes key points contained in the article.

and cancer [1]. Although mitochondrial dysfunction and mtDNA mutation seem to be in senescence, there are many other factors including environmental toxins that predispose detrimental effect to mitochondria. Mitochondrial importance cannot be ignored since it has key role in apoptosis and necrosis, or in regulation of cancer. Complete knowledge of pharmacological response of mitochondria for a drug molecule and implementation of these strategies along with pharmaceutical approaches could be used for the treatment of diseases associated with mitochondrial dysfunction. Mitochondrial dysfunction could be cured by delivering gene or drug to the mitochondria. Desired effect of any targeted drug or gene delivery can be achieved only if bioactive molecule is delivered to the destined organ and/or cell type and also if it targeted the correct location within the cell [2]. Mitochondrial therapy is a tedious task that can be achieved only when a delivery system is attached to a cell-specific homing ligand along with a mitochondria-selective drug delivery port to make it both cell and mitochondria specific. This present review looks over the role of mitochondria in healthy and diseased cells. It also focuses on possibilities of present mitochondrial therapy in regard to future trends of more selective targeting approaches, which can be used up to make mitochondrial medicine a practical reality.

2. Physiology and structure of mitochondria

Mitochondria are surrounded by two membranes (**Figure 1**), the outer membrane (OM), which is highly permeable, and the relatively impermeable inner mitochondrial membrane (IMM) that restricts the entry of the polar molecules that lack their specific transporters. In between the two membranes, there is an intermembrane space containing large number of specialized proteins. To increase the surface area of inner mitochondrial membrane for the accommodation of thousands of enzyme complexes (required for the generation of ATP), nature has folded IIM into numerous cristae; moreover, impermeable nature of the membrane helps in imbalanced distribution of protons between matrix and cytosol that develop a driven force for the synthesis of ATP. The subunit complexes (I – V) of oxidative phosphorylation system required for ATP synthesis are embedded in the cristae membrane. Enclosed within the inner membrane is the mitochondrial matrix, a site where citric acid cycle, fatty acid oxidation and the urea cycle take place. Matrix contains mitochondrial DNA (mtDNA), ribosomes and enzymes involved in various cycles [3].

Permeability characteristics of both the membranes vary a lot as the outer membrane is considered to be permeable to small molecules (< 5 kDa), which get diffused through the pores in the membrane formed by the membrane-spanning proteins called porins, whereas for the larger molecules such as proteins, outer mitochondrial membrane is equipped with the protein import machinery. In comparison with the outer membrane, inner membrane is more proteinaceous and contains an unusual anionic phospholipid – cardiolipin [4]. Under normal conditions, cardiolipin acts as an anchor for binding pro-apoptotic cytochrome c to the inner mitochondrial membrane. Inner membrane is also equipped with specific transport systems for delivering specific compounds to the matrix. This advanced barrier function of IMM is so because it shields the matrix, a place where ATP synthesis takes place. Transport proteins in the inner membrane include ATP/ADP carriers (AAC) and metabolite transporter. It also possesses voltage-dependent anion channel (VDAC), adenine nucleotide translocase (ANT), respiratory chain complex and ATP synthase.

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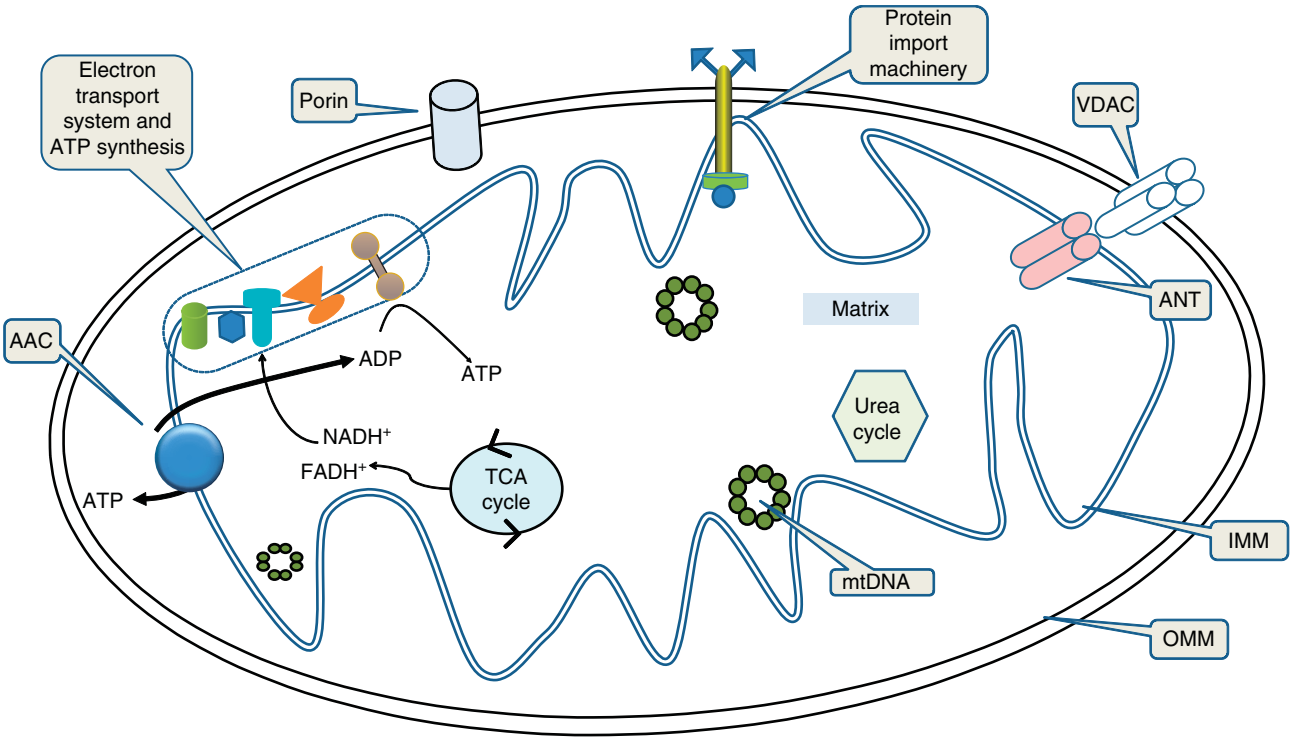

Figure 1. Structure of mitochondria demonstrating various transport systems involved in mitochondrial membranes.

2.1 Mitochondrial genome and its importance

It was in the year 1963 that N.M.K Nass and S. Nass first detected the presence of DNA in mitochondria [5], and soon after this, mitochondrial DNA sequencing of humans was achieved by Anderson *et al.* [6]. The study revealed that mtDNA is a circular double-stranded 16.6-Kb genome and mainly comprises coding sequences. The circular mitochondrial DNA (mtDNA) genome contains 37 genes of which 13 genes encode protein subunits of respiratory complexes I, III, IV and V, whereas protein subunit of complex II is solely encoded by nuclear genes [6]. Proteins encoded by mitochondrial DNA are essential for the assembly and functioning of the electron transport chain, whereas transcription of these genes is dependent on the factor (RNA and DNA polymerase) encoded by nucleus [7]. Although mitochondrial DNA is largely dependent on nuclear-encoded factors, still it possesses some autonomy. Replication of mitochondrial DNA is independent of cell cycle. The fact that mitochondrial DNA is relatively small but an abundant DNA species tends it more susceptible to mutation in comparison to nuclear DNA. Since 1980, it has become evident that qualitative and quantitative changes in mtDNA play an important role in a large number of diseases.

2.2 Role of mitochondria in living cell

Mitochondria have a unique role to play in cell. Intense research has revealed that mitochondria are involved in the generation of reactive oxygen species (ROS), in the regulation of apoptosis, in electron transport system and in ATP synthesis.

2.3 Mitochondria and reactive oxygen species

Respiratory chain releases water as end product through the four-electron reduction of the molecular oxygen by cytochrome oxidase (complex IV), out of which minor amount of oxygen undergoes monoelectric reduction to generate O_2^- (primary ROS generated by mitochondria). The primary generation of ROS occurs mainly at complex III due to proton cycling among ubiquinone, cytochromes b and c1, and iron–sulfur proteins [8], although complex I may also contribute to this process. In the presence of mitochondrial superoxide dismutase (Mn-DSO), O_2^- can be converted to H_2O_2, which can then diffuse out of mitochondria into the cytoplasm through aquaporins present in inner mitochondrial membrane [9,10]. H_2O_2 present in the cytosol of the cell undergoes Fenton reaction in the presence of excess of transition metals (such as Fe^{2+}) to form highly reactive hydroxyl radical (OH^-), which causes damage to cell components. Along with its conversion to different peroxidated products, H_2O_2 also acts as a signaling molecule in the cytosol, which regulates cell cycle, stress response, energy metabolism and redox balance [11,12]. Even in the mitochondria, H_2O_2 is found to be associated with the activation of the mild uncoupling pathways, which plays key role in the regulation of the ROS generation [13]. Mitochondrial generated O_2^- can also react with nitric oxide to form the highly reactive peroxy nitrite anion ($ONOO^-$). The level of ROS in cell is found to be associated with the release of cytochrome c from mitochondria [14]. As it has been reported that acyl moieties

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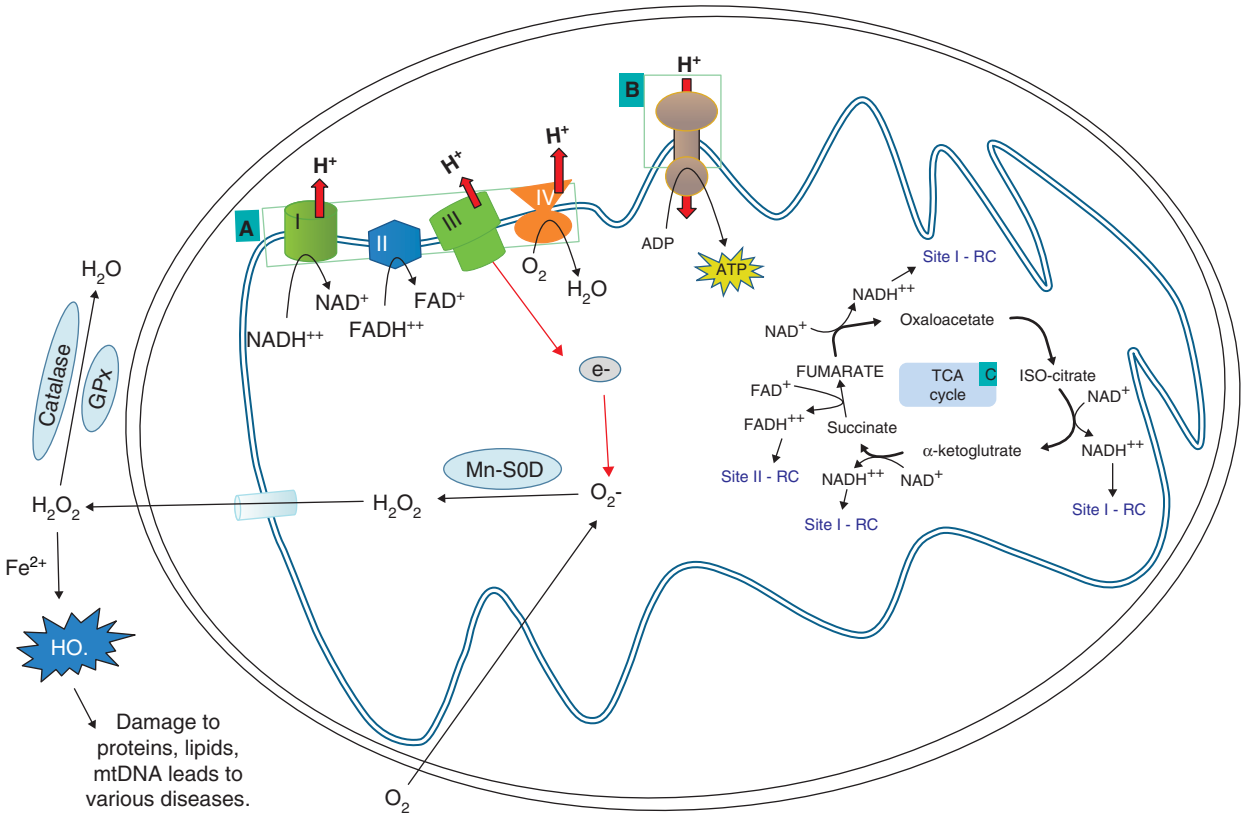

Figure 2. The mitochondrial electron transfer system (A) makes use of free energy released through the oxidation of $NADH^{++}$ transferred from TCA cycle (C) and builds electrochemical proton gradient. This proton concentration gradient drives back the protons to mitochondrial matrix through ATP synthase complex (B) resulting in ATP generation. Free radical generated from electron transport chain (through complex III) causing oxidative damage to mtDNA, proteins and lipids and hence imparting various diseases.

of cardiolipin are susceptible to peroxidation by ROS, in conditions involving excess of ROS level in the mitochondria, cytochrome c bound to cardiolipin can function as an oxidase and induces extensive peroxidation of cardiolipin in the mitochondrial membrane leading to the release of pro-apoptotic factors [15].

To tackle the increased level of ROS, nature has equipped cells with scavenging systems such as superoxide dismutase (MnSOD and CuZnSOD), glutathione peroxidase and phospholipid hydroperoxides. These systems decompose or neutralize ROS or their peroxidated products. Scavenging systems consist primarily of mitochondrial (manganese-containing) and cytosolic (copper–zinc containing) superoxide dismutases respectively), glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase [16-18]. Increased ROS generation and accumulation in cell causes mtDNA mutations [19], lipid peroxidation [20], protein oxidation [21] and damages to the cell and the whole organism [22-24] as depicted in **Figure 2**. Mitochondrial DNA (mtDNA) mutations are associated with many degenerative diseases, including Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis [25,26]. So for this reason, different ROS-based therapeutic can be exploited to achieve clinical benefits. Antioxidant supplements to scavenge free radicals or some drugs to increase free radicals have been used to achieve therapeutic benefits.

2.4 Mitochondria role in electron transfer system and ATP synthesis

Oxidative phosphorylation in mitochondria generates 80 – 90% of ATP in major mammalian tissues. The process involves passing of electron along the series of carrier molecules called electron transport chain. The mitochondrial respiratory chain is located in the inner mitochondrial membrane and is composed of enzymes and coenzymes that transport reducing equivalents – hydrogen atoms or electrons – from respiratory substrates to molecular oxygen in accordance with the redox potential of the components of the respiratory chain. The most important mitochondrial function is the synthesis of ATP from ADP and phosphate. Therefore, dysfunction of ATP production is associated with large number of mitochondrial diseases. For example, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF) and

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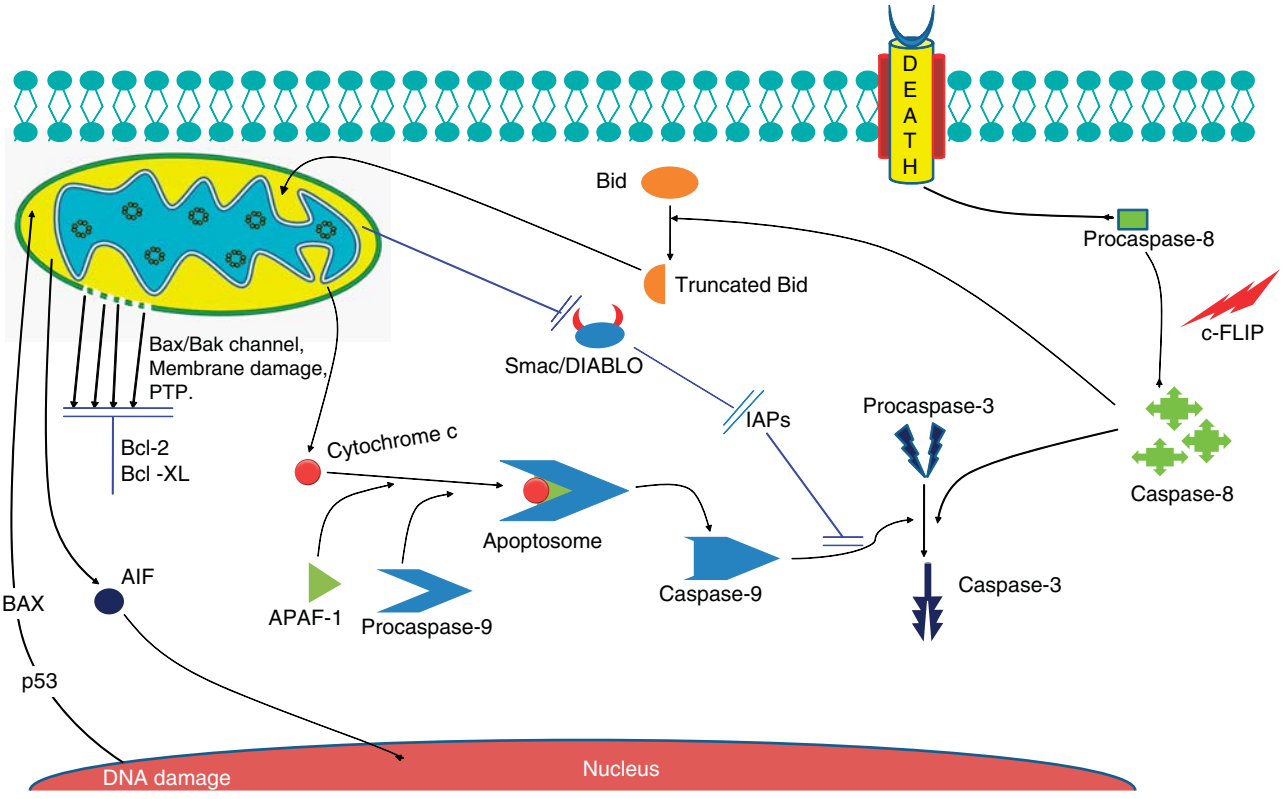

Figure 3. Figure demonstrating two semi-interdependent routes of apoptosis, one through mitochondria and the other through ligand binding to death receptors present on the surface of the cell. Apoptogenic factors such as cytochrome c (cyt c) and apoptosis-inducing factor (AIF) are secreted by mitochondria in response to the large number of cell death stimuli. Caspase-9 is stimulated as cyt c binds to apoptotic protease-activating factor-1 (Apaf-1), whereas AIF degrades nuclear DNA independent of caspase.

chronic progressive external ophthalmoplegia (CPEO) have been attributed to defects in the respiratory chain [27].

2.5 Role of mitochondria in cell death

Mitochondria act not only as power house but also as an arsenal. Tissue maintains homeostasis by the removal of superfluous, damaged and ectopic cells (nearly 10 billion of our cells dies per day) and countering of those cells with new cells arising through mitosis. The state of homeostasis is crucial in maintaining normal physiology of body. For example, in case of cancer, cell attains immortality. So initiation of the cell death becomes a major objective in cancer therapeutics, whereas hampering of cell death is desirable in neurodegenerative diseases [28]. The removal or, we can say, the death of the cell takes place by two pathways: one is 'necrosis' (rapid uncontrolled cell lysis showing inflammation) and the other is apoptosis (a programmed cell death). Apoptosis refers to programmed cell death, which proceeds with membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation ceased by rapid engulfment of corpses by neighboring cells. Apoptosis mediates through two semi-interdependent routes, which converge at the level of caspase-3 activation as presented in **Figure 3**. One includes initiation by ligand binding to death receptors present on the surface of the cell [29] and another one is through route involving activation of mitochondria [30]. The route involving death-receptor pathway is triggered by members of the death receptor super family (such as CD95 and tumor necrosis factor receptor I). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death-inducing signaling complex. This complex recruits multiple procaspase-8 molecules, resulting in caspase-8 activation, which further activates caspase-3. Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP.

Whereas in route involving mitochondrial pathway, there is an early trigger of the apoptotic pathway causing an increase in the permeability of the outer membrane, and hence release of apoptogenic factors, such as cytochrome c (cyt c) [31]. Research has revealed that combination of cyt c and other proteins forms adaptor proteins (Apaf-1), which further leads to activation of caspase-9 in cytosol. Along with these series of events in cytosol begins the activation of intracellular proteases of the caspase family [32], which eventually results in

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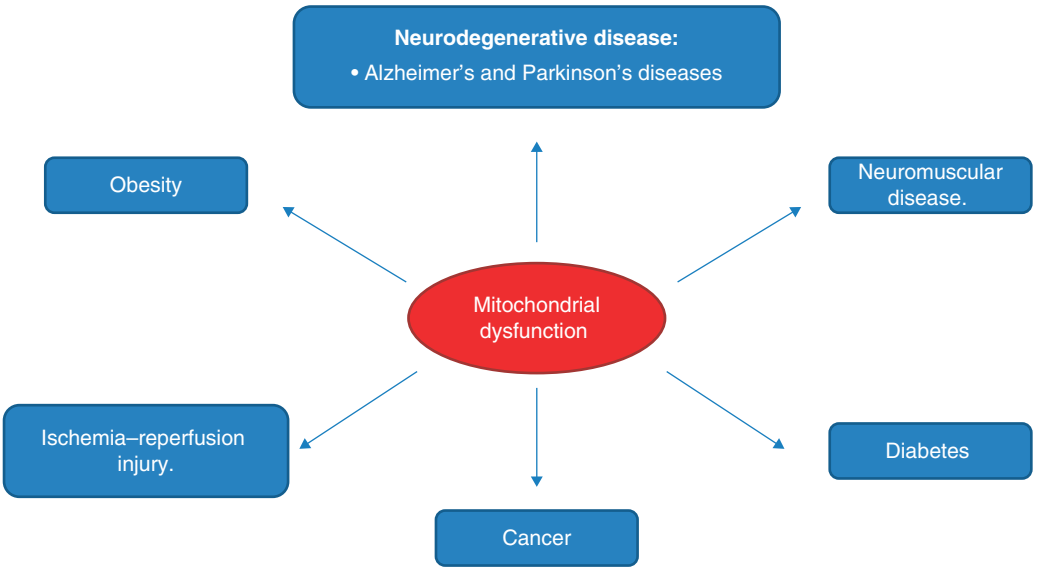

Figure 4. Mitochondria-associated disorders.

partial self-digestion of the cell. Caspase-3 activated at the confluence of both pathways was found to be antagonized by IAP proteins, which themselves get antagonized by Smac/DIABLO protein released from mitochondria. Bcl-2 family of proteins plays a profound role in the release of apoptogenic factors from mitochondria. As most of the members of the Bcl-2 family spend their life tenure as being remained attached to the intracellular membranes, some of its members (including Bax, Bad, Bim and Bid) possess tendency to shuttle between cytosol and organelles [33]. These freely roaming proteins get activated by the pro-apoptotic signals and start competing with anti-apoptotic Bcl-2 family proteins at the surface of mitochondria. Their success determines the fate of cell as if they win they trigger release of apoptogenic factors from mitochondria to the cells cytosol, which further initiates sequential death of the cell. As this dramatic play of biology has not been elucidated to its fullest, there might be many other pathways that govern this self-killing nature of the cells.

3. Mitochondria role in human diseases

As mentioned earlier, mitochondria are power house of the cell and reside in large numbers in cytoplasm where their principal function is of synthesizing ATP by oxidative phosphorylation. Metabolic dysfunction behavior of mitochondria consequently leads to the disease state of that cell and/or of tissue (**Figure** 4). Diseases originated from mitochondrial dysfunction are Friedreich's ataxia [34], diabetes [35], Parkinson's diseases [36], Huntington's diseases [37], disorders associated with mitochondrial DNA mutations [16,38], improper apoptosis in cancer and neurodegenerative diseases [39] and pathophysiology of ageing [24,40].

3.1 Mitochondria and ischemia–reperfusion

Reduced or complete block of blood flow to tissue and/or organ with a rapid loss of oxygen supply to cells is the condition marked to ischemia [41]. Due to this loss of oxygen, oxidative phosphorylation is impeded with reduction in the ATP molecules in the cell and simultaneous increases in ADP molecules and Pi energy packets. At this point of time, lactate formation by anaerobic glycolysis takes place in cells, which leads to a decrease in cytosolic pH. The Na^+/K^+ ATPase system, which is governed by the level of ATP molecules, gets blocked with decrease in the ATP molecules and causes imbalance in the ion concentration. This imbalance results in high sodium (Na^+) ion intracellular concentration and high extracellular potassium (K^+) ion concentration. Similarly various ion channels are activated leading to an increase in both cytosolic and mitochondrial calcium (Ca^{2+}) ion concentration, which results in the onset of MPT [42]. If mitochondrial calcium ion concentration is low, cell injury in ischemic condition is prevented by acidosis of the cell; however, conditions for the opening of the mitochondrial pores exit (high P; decrease ATP and ADP). In reperfusion, oxygenated blood flow to the ischemic tissue is restored, suggesting the restoration of mitochondrial respiratory chain, mitochondrial potential and the recovery of both cytosolic and mitochondrial Ca^{+2} ion to a normal level. A sudden increase in oxygen influx leads to the burst of ROS, which deplete mitochondrial pyridine nucleotide and glutathione due to oxidative stress [43]. All the above condition favors the opening of mitochondrial membrane pore with swelling, loss of membrane potential and subsequently the inhibition of mitochondrial functions [44,45]. This causes cell death by any of the two mechanisms, either through apoptosis (higher continuous ATP production) or necrosis

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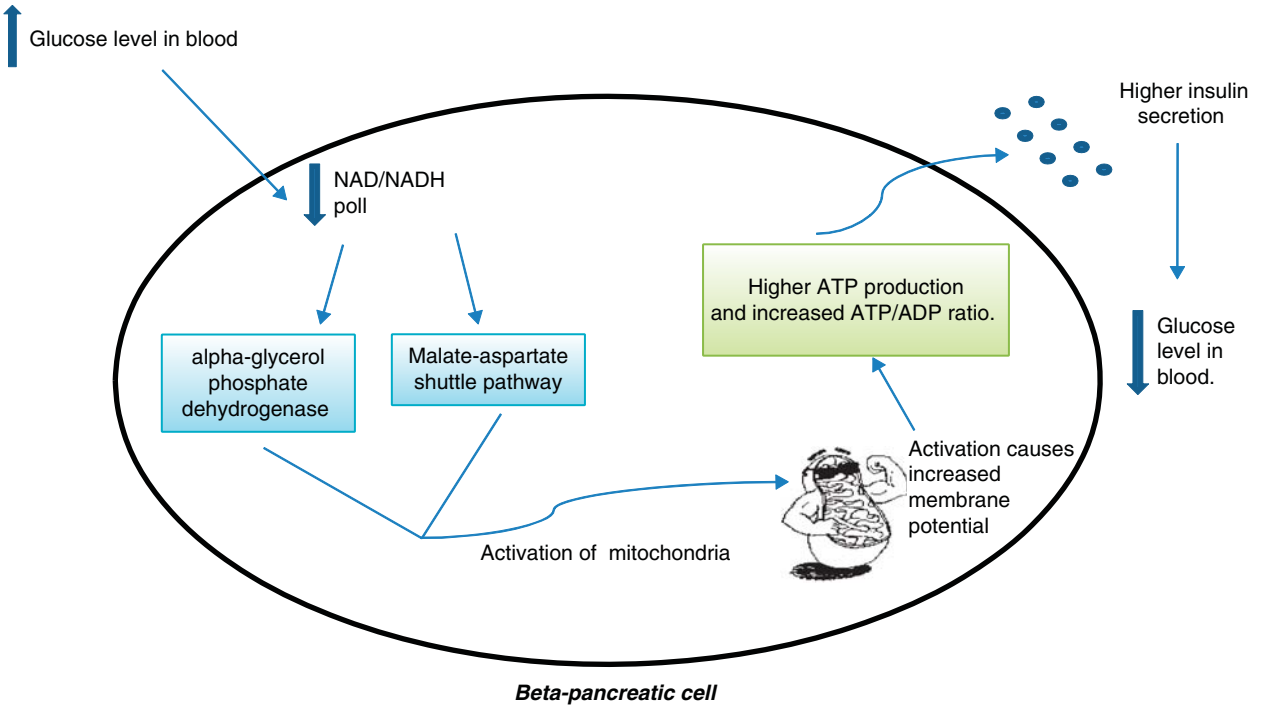

Figure 5. Role of mitochondria in control of diabetes.

(rapid ATP loss) depending on the ability of the cell to maintain ATP synthesis [46,47]. Presence of high ATP and ADP supply also prevents the cell injury caused by Ca^{+2} ion overloading, if sufficient pyridine nucleotides are maintained in reduced state.

3.2 Mitochondria and diabetes

Diabetes is a disease marked with an increase in the blood glucose level, and mitochondria has been investigated to play a vital role in the regulation of blood glucose level especially in the case of type 2 diabetes [48]. Mitochondrion stimulates secretion of insulin from pancreatic β cells. Morphological alteration in the mitochondria is exhibited in diabetes patients [49]. These alterations include reduced mitochondrial membrane potential, decreased ATP production, high density of swollen mitochondria and insulin secretion. As glucose level in the blood plasma increases, a sudden reduction in NAD/NADH pool takes place. To tackle this problem, mitochondria are activated through two routes: one is by enzyme alpha-glycerophosphate dehydrogenase and another via malate-aspartate shuttle pathway. Mitochondrial activation results in increased membrane potential due to which more calcium uptake following stimulation of NADH reduction via citric acid cycle occurs, which ultimately increases production of ATP molecules (**Figure 5**). This activated generation of more ATP molecules increases cytosolic ATP/ADP ratio by which pancreatic β cells secrete insulin in higher concentrations [50].

3.3 Mitochondria and mutation-associated disorder

Mitochondrial DNA is more prone to mutation in comparison with nuclear DNA as it possesses limited capacity to repair (lacks DNA polymerase γ). Additionally, mtDNA lacks protective histones, which renders it to be almost 500 times more sensitive to mutation than nuclear DNA [51]. Mutation-associated disorders can be divided into two classes: i) disruption of oxidative phosphorylation function by mutation mtDNA or nuclear gene; ii) disruption of non-respiratory function resulting from nuclear gene mutations. Mutation in mitochondrial DNA is a major cause for large number of inherited human disorders [52,53]. Genes encoding for components of mitochondrial respiratory chain and F0 – F1 ATP synthase are present in mtDNA, so high-energy-requiring tissues such as muscular, neuronal, cardiac, renal and endocrine system are typically more prone to be affected than other body tissues [54,55]. Studies revealed that mutation in mtDNA can cause increase in mitochondrial oxidative stress or mtDNA damage, which further leads to an increase in mitochondrial radical production [56]. Diseases associated with genetic mutations of mtDNA are Leber's hereditary optic neuropathy (LHON) [57], neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP), mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [58], myoclonic epilepsy with ragged-red fibers (MERRF) [59], and maternally inherited cardiomyopathy (CM), chronic progressive external ophthalmoplegia (CPEO) and Kearns–Sayre syndrome (KSS). Moreover, some mutation in the nuclear gene also impairs

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functioning of mitochondria and leads to diseases such as Friedreich's ataxia, Wilson's disease and human deafness dystonia syndrome [60,61].

3.4 Mitochondria and neurodegenerative disorders

Physiological functions of the neurons are predominantly influenced by the morphological dynamics of the mitochondria. The most common neurodegenerative disorders Alzheimer's disease and Parkinson's disease are now found to be associated with the morphological defect of mitochondria. Mitochondrial-linked neurodegenerative disorders result from the increase in ROS levels and from the mutations taking place in the fission/fusion genes. Mitochondrial respiratory chain is a major source of free radical formation in cell. These free radical causes damage to lipids, proteins, DNA and many respiratory chain proteins, especially iron–sulfur proteins, and further generates neurodegenerative disorders [16,40,53]. Increase in mitochondrial free radical level along with the calcium overloading leads to mitochondrial permeability transition (MPT) [62]. Relationship between Parkinson's disease and mitochondria was first found to be associated with deficiency in the activity of complex I in the peripheral tissues of patients [54]. Both environmental and genetic factors are responsible for the Parkinson's disease by their interaction with mitochondrial function. Environmental toxins such as MPTP and anonnacin inhibit complex I. Genetic causes such as over-expression of α-synuclein inhibit mitochondrial activity [63]; mutations of DJ-1 protein, which is found to be associated with antioxidant character at the outer membrane of mitochondria under conditions of oxidative stress [64], and mutation of *PINK1* genes are found to be associated with Parkinson's disease. Amyloid (Aβ) has a potency for inhibiting oxidative phosphorylation in mitochondria and mutations in the genes for amyloid precursor protein or presenilin 1 and 2 are associated with Alzheimer's disease (AD). Moreover, Aβ is found to interact directly with Aβ-binding alcohol dehydrogenase (ABAD), a mitochondrial enzyme, and causes its inhibition. ABAD is upregulated in AD neurons and its co-expression with amyloid precursor protein (APP) exacerbates Aβ-induced free radical-mediated cell damage and death [65]. Friedreich's ataxia is caused by the deficiency of Frataxin, a mitochondrial protein involved in heme biosynthesis and in the construction of iron–sulfur proteins, such as those that play a critical role in oxidative phosphorylation, as well as in aconitase, an enzyme involved in both the Krebs cycle and the regulation of iron homeostasis. Relationship between mitochondria and neurodegenerative disorders has been exploited to such an extent where we can attempt to develop therapies that can ameliorate disease progression. Some therapies for mitochondria-linked neurodegenerative diseases include delivery of antioxidants such as coenzyme Q10, vitamin E and idebenone.

4. Strategies to target mitochondria

Mitochondrion as target for both cytoprotective and cytotoxic therapies has been recognized by the community of drug delivery. Along with our advancement in knowledge about mitochondria, delivery strategies have nurtured for delivering of both small drug molecules and macro molecules to and into mitochondria. Different research groups have tackled the problems of mitochondrial delivery by exploiting the inherent traits of the mitochondria, which include high membrane potential (approximately -200 mV) and protein import machinery of mitochondria.

4.1 Mitochondrial targeting involving mitochondriotropics

Lipophilic phosphonium cations were first used to investigate mitochondrial biology in late 1960s. This work hardened the base for the decade of long assumption of the existence of large membrane potential across the mitochondrial inner membrane [66]. This large membrane potential across the mitochondrial inner membrane forms the driving force for the accumulation of the lipophilic phosphonium cations without the assistance of any ionophores. The lipophilic cationic molecules, which have tendency to accumulate inside the mitochondrial membrane responding to mitochondrial membrane potential, are termed as mitochondriotropics. In contrast to the hydrophilic cations, lipophilic cations are more permeable to biological membranes as they need far lower activation energy to pass through hydrophobic core of biological membrane. The contributors for the lower activation energy are the decrease in the repulsive Born energy due to the large ionic radii of lipophilic cations and the attractive hydrophobic effect due to the extensive hydrophobic surface of the molecules [67]. The uptake of lipophilic cations into mitochondria increases 10-fold for every 61.5 mV of membrane potential at 37°C leading to 100- to 500-fold accumulation, and uptake into cells is also driven by the plasma membrane potential (30 – 60 mV, negative inside) [68]. There are two approaches through which these lipophilic cations can be used for drug delivery to mitochondria. These approaches are as follows.

4.2 Mitoconjugate-mediated mitochondrial delivery

Mitoconjugates are drug molecules that are covalently attached to mitochondriotropic moieties for their selective delivery to the mitochondria. From the last decade or more, Murphy and his research group have been engaged in exploring the potential of mitochondriotropic conjugated bioactive molecules for their selective delivery to the mitochondria. A wide range of antioxidants could be targeted to mitochondria by conjugation to the triphenyl phosphonium (TPP) moiety, and those that have been employed to date include TPP-conjugated derivatives of ubiquinone [69], tocopherol [70], lipoic acid [71], spin traps [72] and the peroxidase mimetic Ebselen [73].

4.3 Targeting involving mitochondrial protein transport system

Studies have revealed that most of the mitochondria protein import machineries were created de novo and were installed

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into the first eukaryote, which supports the idea that all eukaryotes are descendant of a single ancestor species [74]. With continuous efforts, researchers have probed out a much more detailed picture of how the plethora of nuclear-encoded proteins are targeted to the mitochondria and sorted to the various mitochondrial subcompartments. Majority of proteins required by mitochondria are encoded by nuclear DNA. These proteins are transported to mitochondria in a form of precursor proteins that are rendered resistant to aggregation and misfolding by the cytosolic chaperone proteins [75]. Precursor proteins are the unfolded proteins that are endowed with the signals for targeting to the surface of the mitochondria and for transport and sorting to the various mitochondrial subcompartments. In order to become functional proteins, these precursors have to be recognized by receptors, threaded through pores in the membranes of the mitochondria, proteolytically processed, folded and often inserted into membranes and assembled with cofactors and other proteins to macromolecular complexes [76]. Transport of these proteins across the mitochondrial membrane is mediated through the cooperation of the protein translocator complex present at both inner and outer mitochondrial membranes. The proteins that are destined for the intermembrane space, inner membrane or matrix contain a mitochondrial-targeting signal (MTS) attached to the precursor proteins. Two classes of MTS are identified: one is amino-terminal MTS (N-MTS) and internal MTS (INT-MTS) with their carrying potential for matrix and outer membrane/inner membrane proteins. Typically, an N-MTS consists of approximately 20 – 30 amino acids. Although there is no sequence identity shared among N-MTSs, they all form amphiphilic α-helices that are enriched in basic, hydroxylated and hydrophobic residues [77].

Application of MTS has been proved in the mitochondrial delivery of pDNA and gene therapy with restriction enzymes [78]. Despite of all this, MTS-based mitochondrial targeting suffers a demerit of being limited by the size of cargo and could not be used to correct the defects in the mitochondrial protein import machinery [77]. These conjugates are reported to be imported into mitochondria through the outer and inner membranes via the protein import machinery. So this MTS-mediated delivery approach cannot be used for the mitochondrial malignancies associated with the defect of mitochondrial protein import machinery.

4.4 Mitochondrial targeting using cell-permeable peptides and Mitochondria-penetrating peptides

Another approach for mitochondrial delivery that is independent of mitochondrial protein import machinery involves the use of cell-permeable peptides (CPPs). The TAT peptide is a protein transduction domain (PTD or CPPs) obtained from the immunodeficiency virus-1. This peptide has been used to deliver oligonucleotides, peptides, full-length proteins and even 200-nm liposomes [79]. Irrespective of all these mitochondrial-targeting technologies for mitochondrial gene therapy, there are still some problems that should be addressed. Studies revealed that modified DNA or RNA may pass through the mitochondrial membrane but has difficulty entering the plasma membrane. Therefore, another strategy is required for the delivery of DNA or RNA to the mitochondrial site from extracellular space. Shokolenko *et al.* evolved with a novel approach involving mitochondrial delivery of exonuclease III protein (Exo III) via the combination of PTD and MTS, where PTD works as a cytoplasmic delivery device and MTS guides its intracellular trafficking to mitochondria. They reported the effective delivery of the protein to the mitochondrial matrix, where it decreased repair of mtDNA and rendered cells more susceptible to the lethal effects of oxidative stress [80].

Delivery properties of the CPPs are further investigated to advance their delivery profiles. Some critical features or chemical modifications inspired from the survey of naturally derived sequences were designed to increase the cellular internalization of these molecules. Some highly cationic CPPs have been shown to respond to membrane potential; the details of the mode of cellular internalization and membrane permeation remain elusive. However, this finding begs a question: could CPPs be engineered with the proper chemical attributes for transport into mitochondria? The mitochondria penetrating peptides (MPPs) would have many advantages over other mitochondriotropics, including biocompatibility and straightforward synthesis, which would facilitate modification with therapeutic cargos [81]. The arginine-based peptide oligomers indicated that high levels of cellular uptake delivered through the inclusion of cationic residues [82]. However, the inner membrane of the mitochondria is much more hydrophobic than the plasma membrane, which necessitates preservation of a high degree of lipophilicity in order to allow partitioning of the peptides through the lipid bilayer. Thus, the combination of cationic and hydrophobic residues that would provide electrostatic driving force for uptake through the energized plasma and mitochondrial membranes, while preserving the lipophilic character that would facilitate passage through the latter, is required. The MPPs produced by the addition of lysine (K) and arginine (R) can provide positive charge, whereas phenylalanine (F) and cyclohexylalanine (FX) residues can be used to impart lipophilicity (Horton KL, 2008). His studies with amino acids, including unnatural residues displaying diphenyl, naphthyl or hexyl functionalities, fluorinated F (FF), methylated tyrosine (YMe) and tyrosine (Y) residues, revealed that the compounds that possessed log P values higher than -1.7 exhibited strong mitochondrial localization, while those with log P values lower than -2.0 were essentially excluded from mitochondria and instead appeared in the nuclei and cytoplasm of HeLa cells. These results inferred the need for a critical lipophilicity threshold where mitochondrial entry is permitted and cationic compounds with a +3 charge must possess sufficient lipophilicity to access mitochondria.

These MPPs represent effective mitochondrial transporters that are well suited to traverse an organellar membrane that is

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difficult to penetrate. Modifications, such as exchanging the amide backbone for a peptoid [82], carbamate [83], β-amide [84] or alternative chemical scaffold [85], resulted in enhanced proteolytic stability and even stronger cellular uptake. Furthermore, the overall chirality of the molecules was examined in an effort to increase proteolytic stability and cellular uptake for the CPPs [86]. Recently, MPPs have been used to deliver the DNA alkylating drug chlorambucil (Cbl). Interestingly, MPP–Cbl conjugates were shown to evade cellular resistance mechanisms typically used by cancer cells to diminish drug toxicity [87]. Further, to enhance the use of these peptides as drug delivery vectors, the addition of cationic character and hydrophobicity into a single amino acid residue was studied [88].

4.5 Mitochondrial gene delivery and therapy

Mitochondrion contains around 1000 proteins, of which only 13 are encoded by mitochondrial genome and play a crucial role in oxidative phosphorylation. As mtDNA is free of exons, it possesses a much higher information density than nuclear DNA and is considered to be more susceptible to mutation than nuclear DNA (> 20-fold). This sensitivity results in a high frequency of mitochondrial diseases [53,89]. Majority of diseases related to mitochondria originates from incorporation of either mutated or damaged protein into complexes of electron transport chain [90-92]. These mutations in the mtDNA typically lead to brain and skeletal muscle manifestations; therefore, they are often referred to as mitochondrial encephalomyopathies [93,94]. Presently, there are hundreds of point mutations, deletions or rearrangements in mtDNA that are found to be associated with diseases. In addition, mitochondrial diseases can also arise from defective nuclear-encoded proteins [95]. Ideally, mtDNA should exist in homoplasmy or non-mutated one. But in reality, cell possesses both mutant and wild-type mtDNA, which is termed as heteroplasmy. Moreover, the state of heteroplasmy accrues with the progression of age and results in mitochondrial disease once the percentage of mutated mtDNA exceeds a certain threshold to become clinically apparent [89,96].

4.6 Genetic approaches for mitochondrial therapeutics

Analyzing the alterations in the mtDNA in association to mitochondrial diseases has led us to a point where mitochondrial gene therapy seems to be a plausible way out. The various mitochondrial gene therapy approaches involve delivery of wild-type mtDNA into the mitochondrial matrix, or selective inhibition of the mutant mtDNA replication or conduction of DNA repair assay with RNA/DNA oligonucleotides. Delivery of wild-type mtDNA to mitochondrial matrix of diseased cell could suppress mitochondrial diseases by decreasing mutated DNA level. The therapeutic approach involving selective inhibition of the mutant mtDNA replication allows the propagation of only wild-type DNA with further amelioration of mitochondrial functioning [97].

There are plenty of therapeutic approaches involving large number of molecules that need to be delivered to mitochondria of desired cell. But major hurdle in their delivery lies in the scarcity of the carrier system that could be able to load various molecules including genes and proteins. Moreover, the carrier system opted should possess cell-specific targeting and internalization capability with further strength to regulate intracellular trafficking of the load from endosome to cytosol and thereafter to mitochondria [98]. To date, DNA has been introduced into isolated mitochondria by covalently linking the mitochondria targeting signal peptide (MTS) to either ODN, double-stranded DNA or PNA [99-101] conjugated the ornithine carbamoyl transferase leader sequence to double-stranded DNA of 7 or 322 bp in size. The leader sequence conjugated DNAs were imported to the mitochondria regardless of the size, suggesting that comparatively large DNA with a size of more than 300 bp could be transported to mitochondrial matrix by attaching leader sequence. The mitochondrial leader sequence was also conjugated to peptide nucleic acids (PNAs) [102]. PNA is a synthetic polynucleotide, in which each nucleotide is linked via peptide bond. PNAs form strong double strands with complementary DNA and RNA molecules in a sequence-specific manner. Therefore, PNA can be applied to antisense gene therapy.

Gene therapy approach involving inhibition of mutated mtDNA replication was tried by Taylor *et al.* [97]. The authors validated this approach by synthesizing PNAs complementary to human mtDNA templates containing a deletion breakpoint or single-base mutation to cause disease. They showed that the antigenomic PNAs specifically inhibited replication of mutant, but not wild-type mtDNA templates using an *in vitro* replication run-off assay. They concluded that their antigenomic PNA therapy could help patients with heteroplasmic mtDNA disorders.

Del Gaizo and Payne [103] have constructed a TAT–mitochondrial leader sequence peptide–green fluorescent protein (TAT–MLS–GFP) fusion protein and TAT–GFP protein. They reported that both TAT fusion proteins rapidly enter cultured cells and transduce into mitochondrial matrix by mechanism that neither involve the mitochondrial membrane potential nor the protein import machinery. Moreover, they found that presence of MLS peptide between TAT and GFP allowed intra-mitochondrial protein processing leading to removal of TAT from GFP. As these TAT peptides penetrate the mitochondrial membrane in non-selective manner, there is a possibility of their redistribution to the cytosol after its accumulation in mitochondria. To overcome this, the intra-mitochondrial cleavage of TAT from GFP is crucial for rendering the GFP to remain inside the organelle. Whereas GFP–TAT was able to transduce out of the mitochondrial matrix in order to maintain equilibrium of TAT on both sides of membrane. Therefore, insertion of degradable linker between CPPs and cargo appears generally as a valuable strategy for the irreversible CPP-mediated transport across membranes.

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Despite all of this, the MTS-mediated mitochondria delivery approaches suffer from the limitation by the size of cargo. So to overcome these challenges, alternative strategies for the delivery of macromolecules to mitochondria are under progress [104]. More recently, cationic bola-lipid vesicles, DQAsomes, were studied for the delivery of pDNA to mitochondria [105]. Detailed potential of DQAsomes in drug delivery to mitochondria is discussed further in the later part of this section.

Yamada and Harashima [77] evolved with a new strategy termed as 'programmed packing,' which involves the novel gene delivery system – the multifunctional envelope-type nano device (MEND). They proposed MEND for selective mitochondrial delivery as MITO-Porter. These devices contrive a way to deliver pDNA by overcoming the nucleic acid barriers of intracellular delivery [106]. They are equipped with various functional devices that guide their delivery to specific intracellular organelle of a specific cell type. These devices include ligands for specific receptors, pH-sensitive fusogenic peptides (DOPE) for endosomal escape and mitochondriotropic residues for enhanced mitochondrial delivery. Ideally, MEND consists of condensed DNA core and a lipid envelope structure equipped with various functional devices. The DNA condensed in the lipid envelope not only increases loading efficiency but also provides protection of DNA from DNAase. Further, lipid envelope provides with an opportunity to control topology of the carrier system, which decides intracellular fate of carrier system. Comparative study of R8-MEND with adenoviruses in cervical cancer Hela cells and in a human lung epithelial carcinoma cell lines A549 was done to evaluate their transfection efficiency and cytotoxicity profile. The R8-MEND was found to be equipotent with adenovirus in terms of its transfection efficiency with 1×10^5 particles per cell. Moreover, R8-MEND showed no significant toxicity, while higher doses of the adenovirus produced significant cytotoxicity [107].

5. Mitochondrial drug delivery and therapy

Mitochondrial drug delivery can be divided into two subclasses. First involves delivery of drug in the form of drug conjugate with the mitochondriotropic delivery porter and second involves carrier-mediated drug delivery to the mitochondria. Despite intensive search, there is dearth of viable cell-selective mitochondrial drug delivery system. A comprehensive list of drugs investigated for mitochondrial delivery is compiled in **Table 1**. But the carrier-mediated approach has shown some potential of developing a carrier system with a predetermined trafficking fate, which can deliver drug to mitochondria by crossing two barriers. First barrier involves the efficient targeting and internalization of the carrier by specific cell types and second involves sophisticated regulation of intracellular trafficking from endosome to cytosol and thereafter to the mitochondria [98].

5.1 Antioxidant delivery to mitochondria

Mitochondria power house of cell also generates ROS, which mediate many reactions such as lipid peroxidation and protein oxidation, and at last damages the cell and the whole organism [22-24]. So delivery of antioxidants such as vitamin E and ubiquinone can provide ways to protect cell against oxidative damage. Murphy and Smith [108] were the first to report for the selective delivery of antioxidant vitamin E. They achieved selective delivery by exploiting the highly negatively charged potential state of the mitochondria through covalently coupled conjugate of vitamin E with the lipophilic (TPP) cation [70]. In comparison with the native vitamin E, TPP-modified vitamin E was found to be efficiently accumulated in mitochondrial matrix by crossing the lipid bilayer. Soon after this, antioxidant potential of ubiquinone was exploited by delivering it selectively to mitochondria using TPP conjugation approach [69,109]. Mito-Q, ubiquinone-conjugated TPP, was found to revert back to its active ubiquinol form by undergoing reduction by the respiratory chain and thus shielding mitochondria from oxidative damage. Antioxidant activity of ubiquinone was found to be recycled after it detoxifies ROS. Recently Murphy and Smith [108] investigated the role of mitochondrial oxidative stress during development of hypertension in the stroke-prone spontaneously hypertensive rat. Studies include treatment of hypertensive rat by administering $MitoQ_{10}$ in drinking water for 8 weeks. Administration of the mitochondria-targeted antioxidant $MitoQ_{10}$ protects against the development of hypertension, improves endothelial function and reduces cardiac hypertrophy in young stroke-prone spontaneously hypertensive rats. So they concluded that $MitoQ_{10}$ provides a novel approach to attenuate mitochondrial-specific oxidative damage with the potential to become a new therapeutic intervention in human cardiovascular disease [110].

5.2 Delivery of selective electron scavenger to mitochondria

Maintenance of ROS homeostasis is crucial for normal cell growth and survival. Cells control ROS levels by balancing ROS generation with their elimination by ROS-scavenging systems such as superoxide dismutases (SOD1, SOD2 and SOD3), glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin and catalase. But sometimes cells fail to maintain ROS level and undergo detrimental effect of ROS. So in those cases, external administration of ROS-scavenging agents to the cell in need provides ways to prevent cell from ROS-linked damage. One of the novel mitochondrial-targeting electron and ROS-scavenging agents is XJB-5-131 [111]. This scavenging agent is equipped with both mitochondrial-targeting portion and payload portion having stable nitroxide radicals, which get converted to hydroxylamines by accepting electron. These nitroxide radicals have superoxide dismutase mimetic activity [112]. Targeting portion of XJB-5-131 is the one which is a fragment of gramicidin, a bacterial membrane-active cyclopeptide antibiotic. So it can be

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Table 1A. Drugs investigated for mitochondrial delivery.

Drug molecule	Description	Ref.
Violacein	Poly (D, L-lactide-co-glycolide) nanoparticles acting on human leukemic cells. Found to trigger cell death by apoptosis	[165]
ClPhIQ Acid	ClPhIQ Acid-labeled dendrimers have potential for targeting mitochondria	[166]
Silicon phthalocyanine 4 (Pc4)	Enhanced photodynamic efficacy toward melanoma cells by encapsulation of Pc4 in silica nanoparticles. More localization in mitochondria	[153]
PAMAM Dendrimers	Nanosized polyamidoamine (PAMAM) dendrimer-induced apoptosis mediated by mitochondrial dysfunction	[167]
Ubiquinone	Mitochondria-targeted antioxidant MitoQ10 improves endothelial function and attenuates cardiac hypertrophy	[110]
Mesochlorin e_6 (Mce_6)	Novel HPMA copolymer-based delivery systems that employ TPP ions as mitochondriotropic agents for combined tumor and mitochondrial targeting	[154]
Paclitaxel	Mitochondria-specific nanocarrier system (DQAsomes) prepared from the amphiphilic quinolinium derivative dequalinium chloride to deliver paclitaxel to mitochondria in cells	[150]
Poly (amidoamine) (PAMAM) dendrimer	Polyamidoamino (PAMAM) dendrimer of generation 3.5 (G3.5)-specific interactions with rat liver mitochondria	[168]
Mildronate	Mildronate protected mitochondria from AZT-induced damage predominantly at the level of complex I, mainly by reducing hydrogen peroxide generation	[169]
Plastoquinone (SkQ1)	SkQ1, a conjugate of a lipophilic decyl triphenyl phosphonium cation with an antioxidant moiety of a plastoquinone	[170]
Carboxy-proxyl nitroxide (Mito-CP)	Mito-CP, a covalently coupled carboxy-proxyl nitroxide to a triphenyl phosphonium cation prevented mitochondrial dysfunction, reduced superoxide production in SOD1(G93A) astrocytes and restored motor neuron survival	[171][172]
Oligomycin	Mitochondria-targeting drug oligomycin blocked P-glycoprotein activity and triggered apoptosis in doxorubicin-resistant HepG2 cells	[173]
MitoQ and MitoVit. E	Demonstration that mitochondria-targeted antioxidants prevent cell death that arises in response to endogenous oxidative damage	[172]
CuZn-superoxide dismutase	Liposome-entrapped superoxide dismutase reduces cerebral infarction in cerebral ischemia in rats	[174]

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Table 1B. Mito protein/macromolecular delivery to mitochondria.

Macromolecule	Description	Ref.
Green fluorescent proteins	Selective targeting of green fluorescent nanodiamond conjugates to mitochondria in HeLa cells	[175]
Recombinant VDAC and Bak proteins	Proteoliposomes containing the voltage-dependent anion channel (VDAC) and pro-apoptotic Bak for cancer protein therapy	[176]
MLS-conjugated plasmid DNA and oligonucleotides	DQAsome (self-assembling mitochondriotropic bola-amphiphile dequalinium chloride)-based delivery of oligonucleotides and mitochondrial leader sequence (MLS)-conjugated plasmid DNA to the mitochondria of mammalian cells	[177]
Plasmid DNA	DQAsome–DNA complexes (DQAplexes) selectively release pDNA when in contact with mitochondria-like membranes	[99]
Green fluorescent protein	TAT–mitochondrial leader sequence peptide–green fluorescent fusion protein transduce into mitochondrial matrix by mechanism independent of mitochondrial membrane potential and protein import machinery	[103]

interpreted from this that the targeting portion of XJB-5-131- has potency to target mitochondria.

5.3 Selective mitochondrial drug delivery to cancer cell

Mitochondria's importance in the regulation of apoptosis has lured scientists to explore the potential of mitochondria-based drug therapies to cure cancer. In cancer, therapeutic precision in drug delivery to specific site or cell type is of prime importance, as nonspecific delivery of anticancer drug could lead to many severe life-threatening side effects that could make patient's life more miserable. Mitochondrion as target for cytotoxic therapy has been recognized by the community of drug delivery. The mechanism of cytotoxic therapy involves impairing ROS stress beyond threshold for cell kill.

5.3.1 ROS stress in cancer: roles and mechanisms

Compared with normal cells, malignant cells seem to function with higher levels of endogenous oxidative stress in culture and *in vivo* [113,114]. Moreover, the levels of ROS-scavenging enzymes such as SOD, glutathione peroxidase and peroxiredoxin have been shown to be significantly altered in malignant cells and in primary cancer tissues, suggesting aberrant regulation of redox homeostasis and stress adaptation in cancer cells.

Although the precise pathways leading to ROS stress in cancer cells remain unclear, several intrinsic and extrinsic mechanisms are thought to cause oxidative stress during cancer development and disease progression. Activation of oncogenes, aberrant metabolism, mitochondrial dysfunction and loss of functional p53 are intrinsic factors known to cause increased ROS production in cancer cells [115,116]. The expression of genes that are associated with tumor transformation, such as Ras, Bcr-Abl and c-Myc, was found to induce ROS production [117]. For instance, in H-Rasv12-transformed NIH3T3 fibroblast cells, a large amount of superoxide was generated through the activation of the membrane-associated ROS-producing enzyme NADPH oxidase (NOX) [115]. This increase in ROS is required for the tumorigenic function of Ras [118]. Interestingly, besides the direct activation of ROS-producing enzymes, a recent study showed that Ras oncogenic signaling also suppresses the antioxidant molecule sestrin 1 (SeSN1) [119], resulting in an increase in ROS levels. Besides oncogenic transformation, mitochondrial DNA (mtDNA) mutations have also been shown to be correlated with increased ROS levels in certain types of cancer cells, including those in solid tumors and leukemia [120]. Several protein components of the electron transport chain are encoded by mtDNA. Thus, mutations of mtDNA are likely to cause impairments in electron transfer, leading to leakage of electrons and the generation of superoxide, which can subsequently be converted to other types of ROS.

5.3.2 Cancer redox biology: a biological basis for therapeutic selectivity

ROS might function as a double-edged sword. A moderate increase in ROS may promote cell proliferation and survival.

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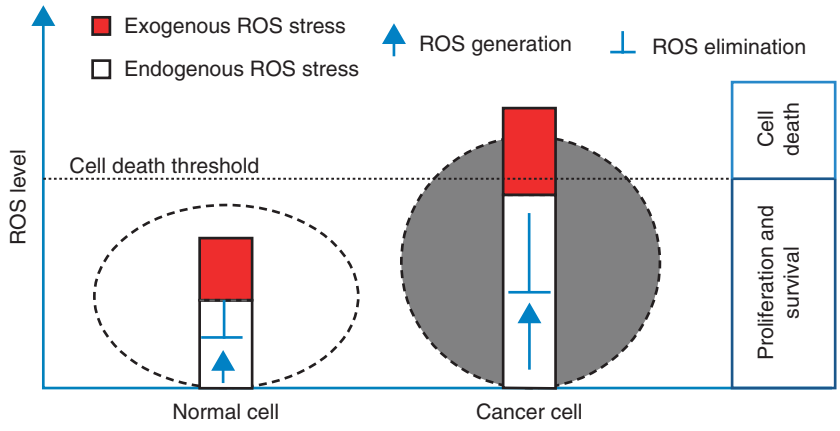

Figure 6. ROS levels in normal and cancer cell.
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However, when the increase in ROS reaches a certain level (the toxic threshold), it may overwhelm the antioxidant capacity of the cell and trigger cell death. Under physiological conditions, normal cells maintain redox homeostasis with a low level of basal ROS by controlling the balance between ROS generation (pro-oxidants) and elimination (antioxidant capacity). Normal cells can tolerate a certain level of exogenous ROS stress owing to their 'reserve' antioxidant capacity, which can be mobilized to prevent the ROS level from reaching the cell death threshold (horizontal dotted line in figure). In cancer cells, the increase in ROS generation from metabolic abnormalities and oncogenic signaling may trigger a redox adaptation response, leading to an upregulation of antioxidant capacity and a shift of redox dynamics with high ROS generation and elimination to maintain the ROS levels below the toxic threshold. As such, cancer cells would be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant system. A further increase in ROS stress in cancer cells (red bar) using exogenous ROS-modulating agents is likely to cause elevation of ROS above the threshold level, leading to cell death. This might constitute a biochemical basis to design therapeutic strategies to selectively kill cancer cells using ROS-mediated mechanisms.

Compelling evidence suggests that the increased ROS stress in cancer cells has a pivotal role in the acquisition of the hallmarks of cancer such as immortalization and transformation [117]; cell proliferation [121] and mitogenic signaling [115]; cell survival and disruption of cell death signaling [122]; epithelial–mesenchymal transition and metastasis [123,124]; angiogenesis [125]; and chemoresistance [126,127].

5.3.3 *Targeting redox alterations in cancer*

Therapeutic selectivity is essential in cancer treatment. As cancer cells have elevated ROS generation and are under increased intrinsic oxidative stress, it is conceivable that these malignant cells would be more dependent on antioxidants for cell survival and, therefore, more vulnerable to further oxidative insults induced by ROS-generating agents or by compounds that abrogate the key antioxidant systems in cells. The idea of inducing preferential cancer cell death by an ROS-mediated mechanism based on different redox states in normal and malignant cells (**Figure 6**) was proposed a decade ago [128], but its feasibility has only recently gained momentum. Although ROS-generating agents have been found to be effective in many cases, low clinical response and resistance to those agents were also reported. Elevation of certain transcription factors, antioxidants and survival signals as a result of redox adaptation probably explains the drug-resistant phenotype [129]. To develop effective therapeutic agents, which are selective and able to overcome drug resistance, it will be extremely important to understand the pros and cons of different redox-modulating strategies and to reconcile the concepts of intrinsic oxidative stress in cancer cells and their redox adaptation.

Cellular redox homeostasis is maintained by the balance between ROS generation and elimination. Exogenous agents that increase ROS generation or decrease antioxidant capacity will shift the redox balance and result in an overall increase in the level of ROS, which when above a cellular tolerability threshold may induce cell death. Approaches to further increase oxidative stress and kill cancer cells are summarized in **Figure 7**.

To meet this challenge, different research groups have demonstrated the delivery of anticancer molecules using carrier-based approaches. Majority of these carrier systems reared from the field of nanotechnology, an arena where small is considered to be more powerful. These nanotechnology-based approaches include vesicular systems such as DQA-somes, mitochondriotropic liposomes and many other surface-modified liposomes for achieving cell-selective mitochondriotropic delivery. Other mitochondria-targeting carrier systems include solid nanoparticles, quantum dots and dendrimers. The mitochondria-targeting potential of all these

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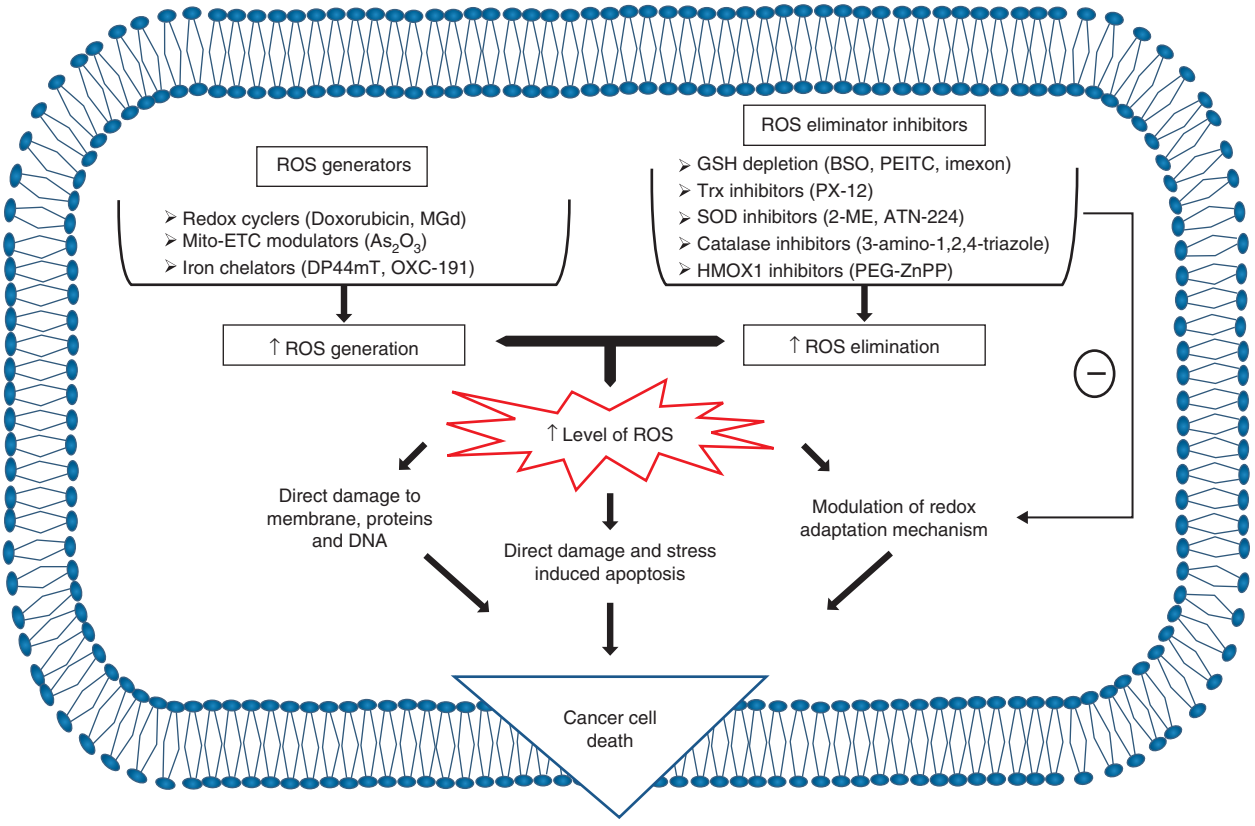

Figure 7. Approaches to kill cancer cell by manipulating cellular redox homeostasis.

systems relies on the integration of some other mitochondriotropic residue in or out of the carrier systems. Other targeting mechanisms include the use of antibodies or the mitochondrial leader sequence conjugates.

6. Mitochondriotropic nanotechnology

Research in the field of nanotechnology has provided us the way in the form of nanovesicles and nanoparticles that have the potential of probing or manipulating mitochondrial function. Their affinity toward mitochondria comes from the presence of mitochondriotropic moieties that reside in these novel carrier systems (**Figure 8**). These mitochondriotropic moieties include rhodamine, methyl-TPP and dequalinium chloride [105]. Nanovesicles for mitochondrial targeting include DQAsomes (DeQuAlinium-based liposome-like vesicles) [99] and phospholipid vesicles with surface modification with TPP cations [130]. These systems have proved their efficacy in delivering both drug and DNA to the mitochondria. These systems are under further investigation for mitochondrial gene therapy and for anticancer chemotherapy.

6.1 Liposome-based drug delivery to mitochondria

Liposomes have gained a huge respect as a drug carrier system as it can entrap both lipophilic and hydrophilic drugs. Moreover, its surface can be easily modified with ligands and other membrane-fusing agents to achieve a predetermined delivery fate of the drug delivery. Another attempt of mitochondrial delivery was made with sclareol, a labdane-type diterpenes. It possesses a growth-inhibiting and cytotoxic activity against a variety of human cancer cell lines and has been reported to induce cell-cycle arrest and apoptosis, while downregulating the expression of the proto-oncogene, c-myc [131]. A significant improvement in the apoptotic and cytotoxic action of sclareol, a poorly soluble potential anticancer drug, was also achieved by its intracellular delivery using mitochondriotropic liposomes [132].

Liposomal systems for drug delivery to mitochondria also include octaarginine (R8)-modified liposomes and cell-targeting liposomes that are equipped with a pH-dependent fusogenic peptide [133,134]. Tf-GALA-LP is formed for the delivery of functional peptides, such as mastoparan (MP) encapsulated liposomes [135]. It was also concluded that topology of GALA is critical for it fusogenic function; better endosomal escape was reported when GALA peptide was exposed onto the liposomal surface [133]. Desired liposomal system for selective cell as well as mitochondrial targeting shall possess ligand for specific cell detection, moiety for endosomal escape and intra cell trafficking moiety that could carry system from cytoplasm to mitochondria [136].

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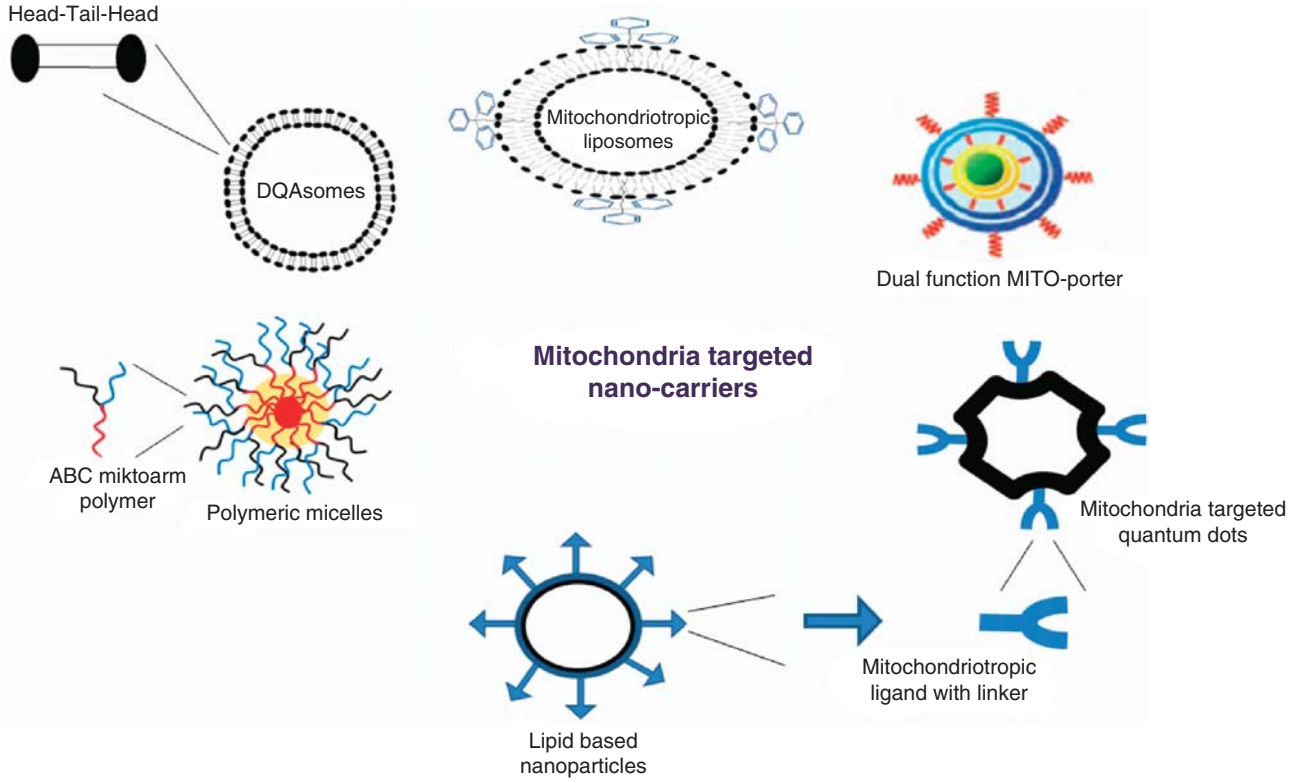

Figure 8. Nanocarriers having potential for mitochondrial targeting.

To achieve this, a new packaging concept has been evolved known as 'programmed packaging.' This includes the novel non-viral gene delivery systems – MEND, which is flexible enough to be modified to meet the requirement of drug delivery in a variety of different mitochondrial diseases [106,137]. To date, MEND has been found efficient in carrying pDNA, functional nucleic acid, proteins and other substances.

Most recently, Boddapati *et al.* have demonstrated that liposomes can be rendered mitochondria specific via surface modification with mitochondriotropic residues. One of these surface modification approaches involves the incorporation of mitochondriotropic residue: triphenylphosphine to the liposomal bilayer [130] using TPP. This lipophilic TPP cation provides a delocalized positive charge to the liposomal bilayer, which reduces the free energy change for liposome movement from aqueous to hydrophobic environment in response to the mitochondrial membrane potential. In order to attach mitochondriotropic TPP cations to the liposomal surface, SUV liposomes were prepared in the presence of alkylated TPP cations like stearyl TPP (STPP), as schematically shown in **Figure 9**. Following their comprehensive physico-chemical characterization, they incubated NBD-PE-labeled STTP-bearing liposomes with human breast cancer cells and studied their intracellular distribution using confocal fluorescence microscopy. Strikingly, it was found that at least partially intact phospholipid vesicles must have been accumulated at the site of mitochondria.

Boddapati *et al.* investigated mitochondrial delivery of the apoptosis-inducing agent, ceramide, using STPP liposomes, and the report showed that improved animal survival and the retardation of tumor growth rate were found by intracellular delivery of ceramide using the mitochondria-targeted liposomes [138]. Exogenous ceramide is known to induce the formation of ceramide channels in mitochondria, releasing cytochrome c and hence leading to apoptosis [139], which makes ceramide the model drug for specific delivery to mitochondria.

Folic acid- and TPP-appended mitocancerotropic liposomes were recently reported to permit the cancer cell-specific mitochondrial delivery of drug [140]. The cytotoxicity, ROS production and cell uptake of doxorubicin-loaded liposomes were evaluated in FR (+) KB cells and found to be increased considerably with FA-MTLs in comparison with folic acid-appended, mitochondria-targeted and non-targeted liposomes. The confocal microscopy confirmed the delivery of DOX to the mitochondria of cancer cell by TPP-appended liposomes and also showed higher ROS production and cytotoxicity in comparison with folic acid-appended and non-targeted liposomes. Most importantly, mitocancerotropic liposomes showed superior activity over mitochondria-targeted liposomes, which confirm the synergistic effect imparted by the presence of dual ligands – folic acid and TPP – on the enhancement of cellular and mitochondrial delivery of doxorubicin in KB cells [140].

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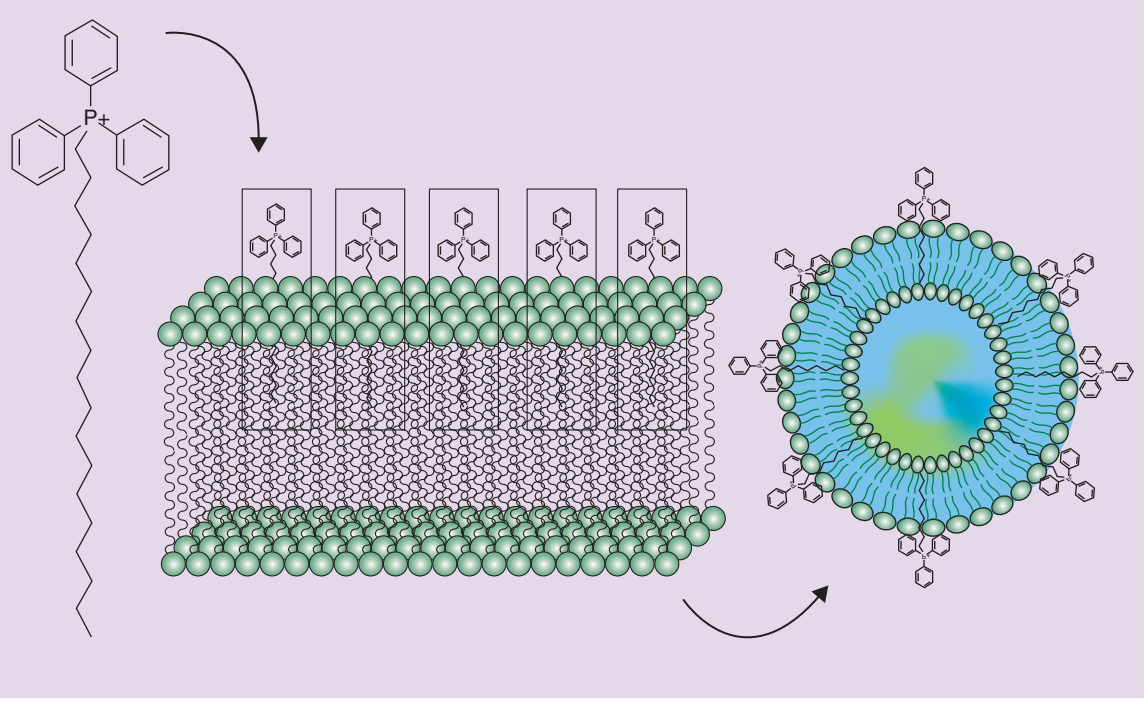

Figure 9. Liposomes are rendered mitochondria specific by attaching triphenyl phosphonium (TPP) moiety to the liposomal surface.

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6.2 DQAsomes

A fortuitous discovery in the mid-1990s involving the self-assembly of a molecule, known to accumulate inside mitochondria, has led to the development of subcellular nanocarriers suited for the selective delivery of drugs to mitochondria. Among the known mitochondriotropics, dequalinium appeared to be a molecule that has a potential to self-associate into larger vesicle-like structures (**Figure 10**), due to their molecular shape [141]. Dequalinium is a dicationic compound resembling 'bola'-form electrolytes, that is, it is a symmetrical molecule with two charge centers separated at a relatively large distance. Such symmetric bola-like structures are well known from archaeal lipids, which usually consist of two glycerol backbones connected by two hydrophobic chains [142]. Generally, it has been shown that these symmetric bipolar archaeal lipids can self-associate into mechanically very stable monolayer membranes. The most striking structural difference between dequalinium and archaeal lipids lies in the number of bridging hydrophobic chains between the polar head groups. Contrary to the common archaeal lipids, in dequalinium, there is only one hydrocarbon chain that connects the two cationic hydrophilic head groups. Therefore, Weissig named this type of bola lipids 'single-chain bola-amphiphile' [143]. Mitochondria can be differentiated from other cell organelles on the basis of their negative membrane potential and this difference in potential was first explored by Weissig *et al.* [144] for the development of novel drug and gene delivery system: DQAsomes. It is a unique mitochondria-targeted drug carrier formed of a dicationic compound 'dequalinium,' which accumulates at and inside mitochondria of living cell in response to the mitochondrial membrane potential. The molecules are symmetrical in nature with two charge centers separated at a large distance, which make them resemble the 'bola'-form electrolyte. The self vesicle-forming nature of these compounds was reviewed by Weissig *et al.* [145]. Before migrating to mitochondria in response to the mitochondrial membrane potential, however, endocytosed DQAsomes have to be released from endosomes into the cytosol. From studies about the intracellular fate of cationic liposome/DNA complexes (lipoplexes), it is known that cationic lipids exert a destabilizing effect on endosomal membranes leading to the release of at least a fraction of the lipoplex from early endosomes [146,147]. DQAsomes had been reported for the selective delivery of pDNA to mitochondria by protecting it from nuclease digestion and mediate its cellular uptake most likely via non-specific endocytosis [148]. Using membrane-mimicking liposomal membranes and isolated rat liver mitochondria, Weissig *et al.* have further shown that DQAsome/pDNA complexes become destabilized upon contact with mitochondrial but not at cell plasma membranes [144]. DQAsomes possess a wide delivery potential, which extends from drugs to DNA delivery. The hydrophobic anti-microtubule agent, paclitaxel, was the first drug to be encapsulated in DQAsomes. Its encapsulation in the DQAsomes resulted in an increase in the solubility by a factor of about 3000 [149], which further increase the subcellular, that is, mitochondrial, bioavailability of the drug. The transmission electron

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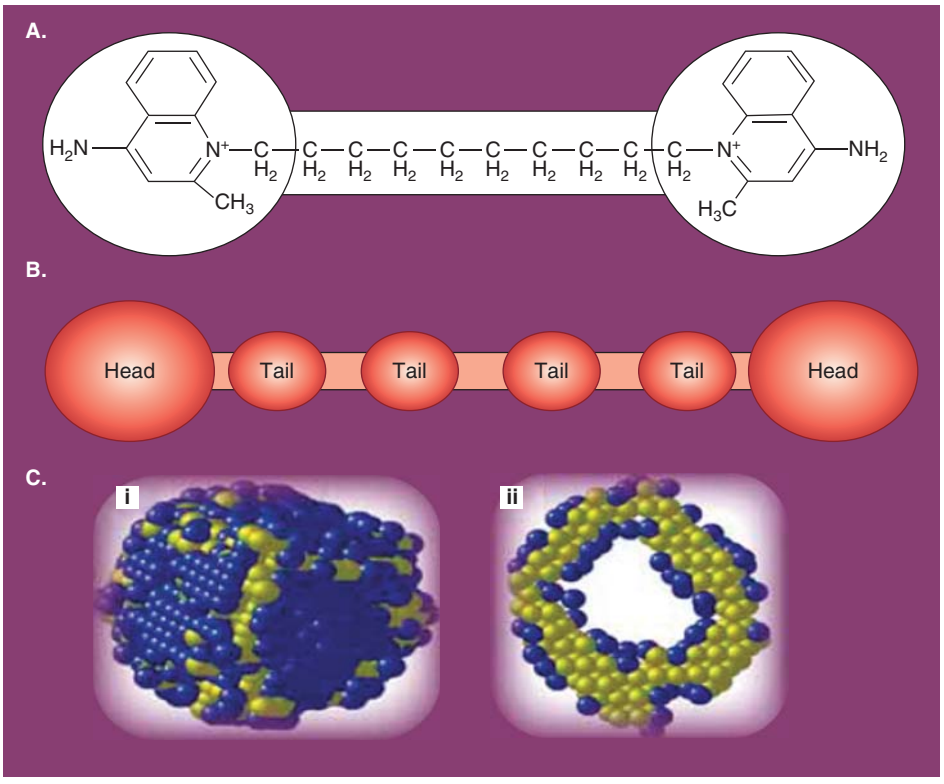

Figure 10. A. Chemical structure of dequalinium. **B.** Dequalinium after coarse graining. **C.** Snapshot from Monte Carlo computer simulation (i) whole vesicles (ii) cross section.

micrograph of these paclitaxel-loaded DQAsomes confirms the worm- or rod-like structure of these vesicles. Loading of paclitaxel in DQAsomes has shown an increase in the therapeutic potential of paclitaxel. In *in vitro* cancer cell lines, DQAsomes have revealed an increase in the subcellular, that is, mitochondrial, bioavailability of the drug. In tumor inhibition studies, paclitaxel-loaded DQAsomes have shown an inhibition of up to 50% [150]. In another study, surface of DQAsomes encapsulated with paclitaxel were modified with tumor cell-specific ligand folic acid. An increase in cytotoxic activity of paclitaxel-encapsulated FA-PEG-DQAsomes on Hela cells has been reported.

6.3 Mitochondria targeted nanoparticles

6.3.1 Solid nanoparticles for mitochondrial targeting

Salnikov and collogues probed out the permeability of mitochondrial outer membrane using calibrated Au nanoparticles [151]. They incubated rat permeabilized ventricular cells and isolated cardiac mitochondria under quasiphysiological ionic condition and during permeability transition with 3- and 6-nm AuNPs, respectively. They found that, while outer membrane was impermeable to 6-nm AuNPs in the absence of permeability transition, the smaller 3-nm AuNPs were able to enter mitochondria, even in the presence of cyclosporine A, which is known to prevent MPT. Additional incubation of isolated mitochondria with 3-nm particle in the presence of voltage-dependent anion channel (VDAC) inhibitors strongly suggests the VDACs to be the port for the entry for the 3-nm particles. Most interestingly, they found that the density of the 3-nm AuNPs in isolated mitochondria to be approximately 20 times higher than that observed in mitochondria within permeabilized whole cells. The reduced uptake of 3-nm AuNPs by mitochondria within whole cells relative to isolated organelles is as expected as there were no mitochondria-specific targeting ligands on the surface of these polyvinylpyrrolidine-coated AuNPs and these AuNPs are not mitochondriotropic per se.

A synthetic strategy for attaching mitochondriotropic ligands to the surface of AuNPs was recently introduced by Ju-Nam and colleagues [152] using triphenyl-phoshonioalkylthiosulfate, a member of a whole group of newly synthesized zwitterions, and potassium tetrachloroaurate. They were able to isolate 5- to 10-nm-sized AuNPs with surface-attached triphenylphosphonium residues.

Zhao *et al.* encapsulated phthalocyanine 4 (Pc4) in 25- to 30-nm silica nanoparticles. Pc4 is currently tested as a photosensitizer for cancer photodynamic therapy. They found that more Pc4-encapsulated silica nanoparticles (Pc4SNP) than free Pc4 were localized in the mitochondria [153]. This increased the efficacy of the Pc4 and will lead to even better early diagnosis and targeted treatment of melanoma in the future. Another attempt toward the achievement of combined

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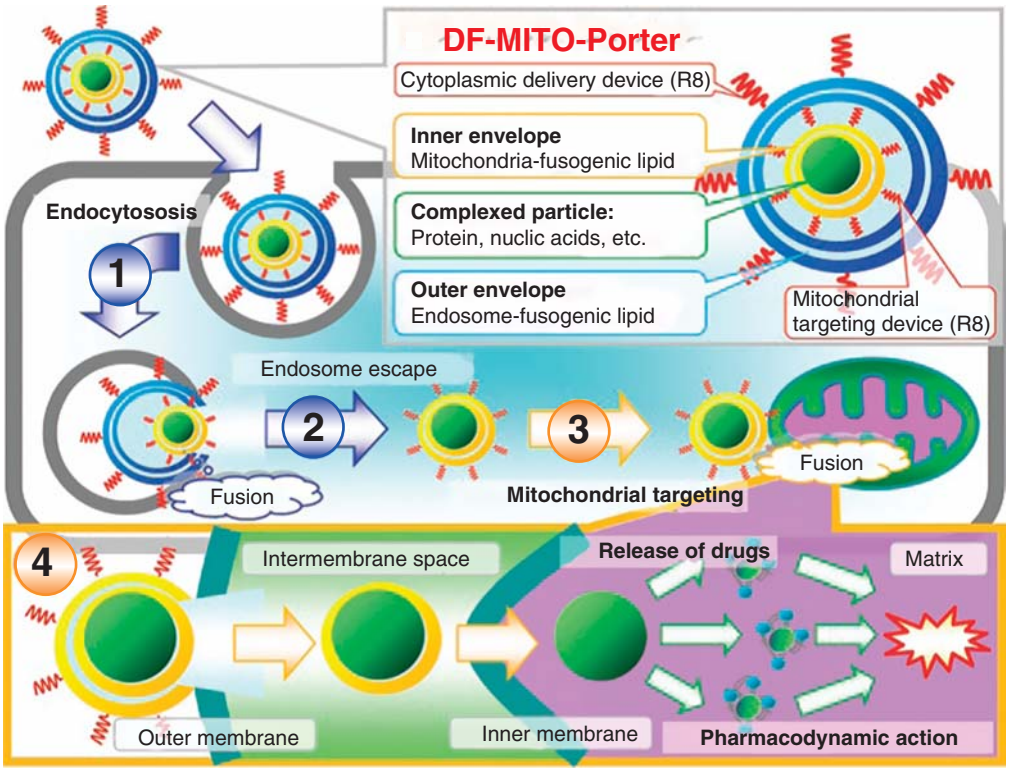

Figure 11. Schematic diagram illustrating mitochondrial macromolecule delivery via a series of membrane fusions using dual function (DF)-MITO-Porter. Complexed particles of cargos are coated with mitochondria-fusogenic lipid envelope (inner) and endosome-fusogenic lipid envelope (outer). Octaarginine (R8) functions as a cell-uptake device in the outer envelope and as a mitochondrial-targeting device in the inner envelope.
Reproduced with the permission of Nature Publishing Group from [157].

tumor and mitochondrial-targeting delivery system was taken by Cuchelkar *et al.* [154]. They developed a novel strategy to target the mitochondria in cancer cells by designing an HPMA copolymer-bound conjugate that incorporates lysine as a linker to connect TPP and drug. The TPP–lysine–drug construct was subsequently attached to the HPMA copolymer backbone via a degradable disulfide bond. The strategy is expected to combine tumor targeting due to incorporation in HPMA copolymer-based drug delivery systems, with degradation of the disulfide bond on internalization and subsequent release and mitochondrial targeting of the TPP–lysine–drug construct [154]. They further incorporated the photosensitizer mesochlorin e_6 (Mce_6) to the HPMA copolymer and found enhanced cytotoxicity of mitochondria targeted of HPMA copolymer-bound Mce_6 in comparison with non-targeted HPMA copolymer–Mce_6 conjugates.

Futaki *et al.* [155] and Nakase *et al.* [156] reported a nano device, dual-function MITO-Porter (DF-MITO-Porter), for mitochondrial delivery system based on the concept of both high-density R8-modified liposomes and the conventional MITO-Porter. A schematic diagram of DF-MITO-Porter as illustrated by Yamada Y *et al.* [157] (2011) is given in **Figure 11**. The critical structural elements of the DF-MITO-Porter include a drug-loaded particle that is coated with two mitochondria-fusogenic inner membranes and two endosome-fusogenic outer membranes. Modification of the outer envelope surface with a high density of R8 greatly promotes internalization of the carriers into cells. The endosomal escape of the internalized carrier takes place via membrane fusion, by virtue of the outer endosome-fusogenic lipid membranes. The carrier then binds to mitochondria via R8 and fuses with the mitochondrial membrane. Recent studies reported that transcriptional activator has the ability to deliver a certain cargo to mitochondria [103,158] and also showed that modification of the liposome surface with R8 significantly enhances binding to mitochondria, regardless of the lipid composition.

6.3.2 *Quantum dots for mitochondrial targeting*

Luminescent semiconductor nanocrystals (quantum dots) have emerged in recent years as a new generation of fluorescent biosensors. Considering their photophysical properties, such as quantum yields, broad absorption spectra with narrow photoluminescent emission and expected resistance to both photoleaching and chemical degradation, quantum dots seem to be far superior to many of the currently used organic dyes and protein-based fluorophores. Medda *et al.* [159] were able to visualize the 3D entanglement of the microtubular and mitochondrial networks with unprecedented details,

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using two-color 4Pi microscopy and antibody-bearing quantum dots. Following the conjugation of the mitochondria-targeting signal peptide of cytochrome c oxidase VIII subunit to ZnS-coated CdSe nanocrystals, they were able to visualize the movement of these conjugates to the mitochondria within living cells [160]. Paunesku *et al.* (2003) and colleagues have shown that excited semiconductor TiO_2 nanoparticles cause DNA damage and ultimately scission following transfer of electropositive holes into the DNA [161]. Provided such nanoparticles are able to first enter the mitochondrial matrix of living cells and second to selectively bind to mtDNA circles bearing a specific mutation, a novel approach toward the elimination of mutated mitochondrial DNA could be developed [162].

6.3.3 *Polymeric micelles for mitochondrial targeting*

Polymeric micelles consist of a core shell architecture: the core with the inner hydrophobic part of amphiphilic copolymer, which can encapsulate poorly water-soluble drugs and control their release, and the outer shell or corona is generally hydrophilic, which provides aqueous solubility and prevents the recognition of micelles by reticuloendothelial system. Although polymeric micelles have been extensively studied for biomedical applications, most of the research has been focused on utilizing linear block copolymers [163]. Amphiphilic miktoarm star copolymers have gained considerable interest recently due to their unique aggregated morphologies in bulk and self-assembly behavior in solution. Miktoarm polymers are branched macromolecules with linear polymeric chains emanating from a common central core, and these polymeric arms can vary in chemical identity and/or molecular weight. The composition of both the core and arms can be fine-tuned based on the desired application.

Recently, mitochondria-targeting nanodelivery system for CoQ10 using ABC miktoarm star polymers were constructed using 'click' chemistry [164]. They have developed an easy and efficient way of constructing multifunctional miktoarm polymer-based nanocarriers for the delivery of CoQ10, using a combination of click chemistry with ring-opening polymerization. CoQ10 micelles efficiently delivered drug to mitochondria and also exerted beneficial antioxidant effects in insulted neural cells.

7. Conclusion

As mitochondria play a vital role in disorders such as diabetes, ischemia–reperfusion injury, cancer and so on, targeting the carrier system containing gene or drug to mitochondria could be a new approach to treat diseases in a more efficient manner.

8. Expert opinion

Mitochondria were first considered as the site of cellular energy production, but recently it was recognized as the organelle that regulates apoptosis. Currently, the relationships between mitochondrial dysfunction and intractable diseases are being investigated. The number of diseases identified as having their roots in mitochondrial DNA defects has increased significantly and, as of today, more than 250 pathogenic mtDNA mutations have been identified. As a result, the mitochondria are considered as promising site for targeted delivery of large number of bioactive (s) to treat many disorders such as ischemia–reperfusion, obesity, diabetes and cancer.

Dequalinium (DQA), used as an antimicrobial agent in over-the-counter mouthwashes, lozenges, ointments and paints, was demonstrated experimentally to show exclusive localization inside energized mitochondria. Serendipitous discovery of self-assembling capacity of dequalinium chloride gave rise to a new class of liposomes termed DQAsomes, and the use of DQAsomes to cargo therapeutic agents to mitochondria was investigated. Use of STPP cation-appended liposomes to deliver drugs to tumor cell mitochondria, *in vitro*, opened up a new chapter in designing mitochondriotropic liposomes. Later, the development of dual-function MITO (DF-MITO)-Porter for intracellular trafficking made revolutions in mitochondrial delivery.

Another significant achievement was the development of MPPs that are able to enter mitochondria efficiently. These MPPs identified are not only cationic, but also lipophilic; this combination of characteristics facilitates permeation of the hydrophobic mitochondrial membrane. However, lipophilicity and charge distribution must be carefully balanced to ensure localization within mitochondria.

Even though certain breakthrough technologies have been identified to target mitochondria, there are few challenges for which proper solutions are needed to achieve efficient mitochondrial drug delivery. Two independent processes, that is, 'cytoplasmic delivery through the cell membrane' and 'mitochondrial delivery through the mitochondrial membrane,' are required to achieve this. In addition, there are certain specific problems such as the strategy of mitochondrial delivery by conjugating mitochondrial-targeting signal peptides to exogenous proteins that were restricted to small linear DNAs but failed in case of macromolecules and hydrophobic molecules, including mitochondrial DNA and proteins. Thus the development of 'mitochondrial pharmaceutics,' that is, the design and evaluation of mitochondria-specific drug carrier or delivery systems, is still lagging behind.

Hence the research in the area of mitochondrial targeting has become very potential as it would lead to find out an ultimate solution to many genetic disorders such as inherited mitochondrial diseases, neurodegenerative disorders, diabetes mellitus and cancer. Based on recent developments in mitochondrial research, increased pharmacological and pharmaceutical efforts have led to the emergence of 'mitochondrial medicine' as a whole new field of biomedical research. Targeting of biologically active molecules to mitochondria in living cells will open up avenues for manipulating mitochondrial

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functions, which may result in the selective protection, repair or eradication of cells. The ultimate goal of mitochondrial targeting should be to develop a suitable delivery device that identifies specific cell, enters the cell and releases the drug to cytosol and to preferentially concentrate in the mitochondria. In addition, the delivery device should ensure stability and integrity of the active pharmaceutical ingredient (API) during this long and challenging journey.

However, research in mitochondrial medicine is highly interdisciplinary encompassing genetic manipulations also. Genetic research initiated in the late 1980s to identify the gene responsible for various mitochondrial diseases has become the basis of diagnostic applications for mitochondrial diseases today. In spite of the huge amount of mechanism-based studies of mitochondrial diseases, effective therapies have not yet been established mainly because of the lack of an adequate delivery system. To date, numerous investigators have attempted to establish a mitochondrial drug delivery system; still, many problems detailed below remain to be overcome before a clinical application can be achieved.

1. We may need to establish a method to encapsulate various drugs, proteins, peptides and genes into a drug carrier depending on their physical characteristics to achieve success to deliver drugs to mitochondria on a clinical setting.
2. Develop strategies to target it to a specific cell.
3. These processes of intracellular trafficking and mitochondrial targeting should regulate drug release to the cytosol after endosomal escape and thereafter to deliver the released drug to the mitochondria.
4. Innovations in the nanotechnology of intracellular trafficking are the ultimate requirements of designing optimal drug delivery system for medical therapies for mitochondrial diseases. This warrants for merger of pharmaceutical nanotechnology with mitochondrial medicine, which would eventually lead to the development of a large variety of mitochondria-specific nanotools for accessing, probing and manipulating mitochondria under physiological and pathological conditions.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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secretion from pancreatic β cells. The electrons of the glycolysis-derived reduced form of nicotinamide adenine dinucleotide (NADH) are transferred to mitochondria through the NADH shuttle system. By abolishing the NADH shuttle function, glucose-induced increases in NADH auto-fluorescence, mitochondrial membrane potential and adenosine triphosphate content were reduced and glucose-induced insulin secretion was abrogated.

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