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Targeted drug delivery to mammalian mitochondria in living cells

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Mitochondrial dysfunction causes or contributes to a large number of human disorders including neuromuscular and neurodegenerative diseases, diabetes, ischaemia–reperfusion injury and cancer. Increasing efforts are being made towards mitochondria-directed pharmacological intervention, leading to the emergence of 'mitochondrial medicine' as a new field of biomedical research. The identification of new molecular mitochondrial drug targets in combination with the development of methods for selectively delivering biologically active molecules to the site of mitochondria will eventually launch new therapies for the treatment of mitochondria-related diseases, based either on the selective protection, repair or eradication of cells. This review discusses the need for the development of mitochondria-specific drug and DNA delivery systems, and evaluates the currently employed strategies for mitochondrial drug targeting, including some of their potential therapeutic applications.

Keywords: delocalised cations, drug targeting, gene therapy, intracellular drug delivery, mitochondria, mitochondrial diseases, mitochondrial medicine, mitochondrial transfection vector, mitochondriotropic molecules

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1. Introduction

Mitochondria are essential for the cell's energy metabolism (ATP synthesis by oxidative phosphorylation) and for the regulation of programmed cell death (apoptosis). In addition, mitochondria play a crucial role in a variety of catabolic and anabolic cellular pathways, including the modulation of intracellular calcium concentrations. The mitochondrial respiratory chain is a source for the formation of damaging reactive oxygen species (ROS), which in turn may exert devastating effects on all mitochondrial components including the mitochondrial genome (mtDNA). Correspondingly, mitochondrial dysfunction causes, or at least contributes to, a large number of human disorders including neuromuscular and neurodegenerative diseases, diabetes, ischaemia–reperfusion injury and cancer. Increasing efforts are being made towards mitochondria-directed pharmacological intervention, leading to the emergence of 'mitochondrial medicine' as a new field of biomedical research [1-3]. The identification of new molecular mitochondrial drug targets in combination with the development of methods for selectively delivering biologically active molecules to the site of mitochondria will potentially launch new therapeutic approaches for the treatment of mitochondria-related diseases, based either on the selective protection, repair or eradication of cells. This review discusses the need for the development of mitochondria-specific drug and DNA delivery systems, and it describes the currently employed strategies for mitochondrial drug targeting, including some future potential therapeutic applications.

2. Mitochondria as cell organelles

The number of mitochondria per single cell largely depends on the cell's energy demand. Metabolically active organs such as liver, brain, cardiac and skeletal muscle tissues may contain up to several thousand mitochondria per cell, whereas the number of mitochondria in cells with a lower energy demand is reduced to a few dozen of these organelles. Each mitochondrion is composed of two membranes creating two separate compartments: the internal matrix and the narrow intermembrane space. The outer membrane is freely permeable to molecules < 5 kDa; the inner membrane, in contrast, is highly impermeable and characterised by a high content of membrane proteins as well as a unique lipid composition. The mitochondrial inner membrane hosts mostly proteins that are components of the respiratory chain (oxidative phosphorylation; OXPHOS), including a variety of transport proteins. The impermeability of the inner membrane is required for creating an imbalance in the distribution of protons between the mitochondrial matrix and the cytosol, which in turn is the driving force for the synthesis of ATP. In order to increase its total surface area, the inner mitochondrial membrane is convoluted into cristae. Recent analysis of mitochondrial morphology by electron microscope tomography [4,5] has provided new and detailed insight into the morphology of the inner mitochondrial membrane (reviewed in [6]), which is now seen to be composed of two or more topologically continuous, but distinct domains.

3. Mitochondria as an emerging pharmacological target

The number of excellent review papers discussing molecular targets either localised in the outer or inner mitochondrial membranes, the inner membrane space or the mitochondrial matrix has grown significantly over the last couple of years, and recently > 14 groups of potential mitochondrial drugs or mitochondrial drug targets have been summarised [7]. Therefore, only a brief overview of the three most widely recognised areas in which mitochondria-specific drug targeting may provide for future effective therapies shall be given, followed by a discussion of some mitochondrial pharmacological targets, which so far and in this author's opinion have drawn lesser attention in this field.

The vast majority of the recently published reviews centre around drug targets related to the crucial role mitochondria play during apoptosis [8-19]. At the core of all efforts described in these papers lies the idea that the development of new cytotoxic drugs that target components of the mitochondrial apoptotic machinery will provide a new strategy to induce apoptosis in tumour cells. The mitochondrial permeability transition pore complex (mPTPC), a multiprotein ensemble formed at the contact site between inner and outer mitochondrial membrane, has evolved so far as a key pharmacological target [10,20].

A second area of high interest is the protection of mitochondria from oxidative stress [21-23]. About 0.1% of the electrons passing through the respiratory chain complexes 'escape' from the OXPHOS pathway and react with oxygen to form superoxide $O_2^{\cdot(-)}$, which then produces hydrogen peroxide (H_2O_2) by dismutation [24]. Hydrogen peroxide in turn may cause nonspecific oxidative damage to mitochondrial lipids, proteins and DNA, and may also give rise to other damaging ROS. Under normal conditions, ROS and their peroxidation products are neutralised by a natural defence system consisting of several superoxide dismutases and peroxidases. However, under conditions of an impaired antioxidant defence system and/or under conditions of an increased ROS formation (e.g., in ischaemia–reperfusion, upon the action of xenobiotics, during inflammation and under ionising irradiation) ROS can accumulate, which in turn may lead to severe damage to the cell and the whole organism (reviewed in [25]). In conclusion, it has been hypothesised that the selective prevention of mitochondrial oxidative damage may be an effective therapeutic approach for a wide range of human diseases [26].

A third area of interest is disease caused by mutated mitochondrial DNA [27-31]. Efforts are being directed at the mitochondria-targeted delivery of therapeutic DNA and RNA such as antisense oligonucleotides, ribozymes, plasmid DNA (pDNA) expressing mitochondrial encoded genes, as well as wild-type mtDNA, in order to provide the basis for treatment of mitochondrial DNA diseases (most recently and comprehensively reviewed in [32]).

Besides these three widely discussed areas of mitochondria-targeted pharmacological intervention (i.e., defective apoptosis, ROS-caused oxidative damage and mtDNA diseases) other pathways, which under certain pathological conditions could benefit from mitochondria-specific drug targeting, are the intracellular Ca^{2+} homeostasis as well as the catabolism of glucose and fatty acids.

In recent years evidence has been accumulated that under conditions of elevated cytosolic Ca^{2+} concentrations mitochondria can as a Ca^{2+} 'sink', thereby contributing to cytosolic Ca^{2+} homeostasis [33]. Under ischaemic conditions, however, mitochondrial calcium overload is well established as a cause of damage to this organelle, and, therefore, the regulation of calcium during ischaemia–reperfusion is being considered as therapeutic beneficial [34]. The clinical application of conventional calcium channel blockers to reduce reperfusion injury and myocardial infarction, however, has been disappointing so far [34], which raises the question of the *in vivo* accessibility of mitochondrial calcium channels to these drugs. Several pathways for mitochondrial Ca^{2+} uptake have been described including a calcium uniporter, a so-called rapid uptake mode, a Na^+-independent Ca^{2+} efflux, and a Na^+-dependent Ca^{2+} efflux [35]. However, the molecular nature of the complex array of mitochondrial Ca^{2+} transporters is still largely unsolved [36]. Recently, a mitochondrial calcium uniporter identified as a highly selective ion channel has been determined as being localised in the inner mitochondrial membrane [37].

A metabolic shift from fatty acid oxidation to glucose and lactate oxidation in the experimental settings of ischaemia–reperfusion and haemorrhagic shock has been shown to improve cardiac metabolic efficiency and contractile function; that is, the inhibition of β-oxidation and stimulation of glucose oxidation can potentially protect the ischaemic heart [34,38]. Correspondingly, the specific activation of the pyruvate dehydrogenase complex, localised in the mitochondrial matrix, is being considered as an ideal therapeutic strategy to alter myocardial metabolism during resuscitation and reperfusion [34]. Other potential target enzymes involved in glucose and fatty acid oxidation, and against which potent inhibitors have been identified [38], are carnitine palmitoyltransferase-2, γ-butyrobetaine hydroxylase, several thiolases and butyryl-CoA dehydrogenase, all of which are enzymes localised inside the mitochondrial matrix. Subsequently, any drug intended to interact with these targets has to cross, besides the cell and the outer mitochondrial membrane, the highly impermeable mitochondrial inner membrane. It therefore appears as reasonable to hypothesise that drugs such as ranolazine, perhexiline, MET-88, trimetazidine, hypoglycin [38] and perhaps even dichloroacetate [38] might benefit from a drug delivery system that selectively transports the drug to mitochondria and facilitates its import into the mitochondrial matrix.

4. The need for mitochondrially targeted drug and DNA delivery systems

Due to the abundance of mitochondria in almost all cells, the perception that once inside the cell the drug or DNA molecule will eventually interact with components of the mitochondrial membrane and, depending on its physicochemical properties, perhaps diffuse into the matrix, appears plausible. Indeed there is no doubt that random (i.e., statistical) collision may lead to an interaction of drug molecules with mitochondria. However, although barriers to drug delivery between the site of administration and the target tissue are well defined and recognised, the intracellular barriers which prevent an even distribution of all drug molecules throughout the cell and which the drug has to overcome in order to reach its subcellular target site are often overlooked [39].

The individual factors that slow down diffusion or even prevent free diffusion of solutes in the cytosol are first the fluid-phase viscosity of the cytoplasm, second collisional interactions due to macromolecular crowding, and third binding to intracellular components [40,41]. 'Binding' in this context should include both specific molecular interactions, such as substrate– or inhibitor–enzyme binding (e.g., cyclosporin A–cyclophilins), as well as the trapping of weakly basic drugs inside acidic lysosomes.

Whereas the fluid-phase viscosity, which is defined as the viscosity sensed by a small probe that does not interact with any cellular components [42], appears to be only of theoretical value (for the pharmaceutical scientist), the impact on cytoplasmic solute diffusion of the free factors combined can be measured and is expressed as the translational diffusion coefficient. Verkman *et al.* [40] have used spot photobleaching to measure the translational diffusion of fluorescein-labelled double-stranded DNA fragments of different sizes after microinjection into the cytoplasm of HeLa cells. Although the ratio between the relative diffusion coefficients in water (D_w) and in cytoplasm (D_{cyto}) was about unity for small oligonucleotides, D_{cyto}/D_w progressively decreased to 0.19, 0.067 and 0.032 for DNA fragments of 100, 250 and 500 bp, respectively. The diffusion rate was dramatically reduced as DNA size increased to > 1 kb (660 kDa), and was immeasurably slow for DNAs of ≥ 3 kb [40]. Although reduced diffusional DNA mobility in the cytosol could be caused by a combination of both binding and crowding effects, the authors make molecular crowding and collisional interactions responsible for slowing the diffusion of larger DNA because binding effects should be relatively independent on DNA size [40].

The implications of such size-dependent limited cytoplasmic DNA mobility for mitochondria-targeted DNA delivery are evident. Small oligonucleotides, once delivered into the cytosol, will eventually be able to interact randomly with mitochondria. It will be reviewed below that, for example, oligonucleotides and peptide nucleic acids (PNAs) can be concentrated inside mitochondria provided the oligonucleotides or PNAs have been conjugated to a mitochondrial leader sequence peptide, which facilitates the DNA import via the mitochondrial protein import pathway. For the movement of larger pDNA across the cytosol to the site of mitochondria, however, an efficient packaging and transport system is needed. Intriguingly, limited diffusional mobility of pDNA in the cytosol has been linked to the need for the evolvement of a capsid-based mechanism for the delivery of viral DNA across the cytoplasm to the nucleus [40]. For illustration, following the fusion of herpes simplex virus with the plasma membrane, the incoming capsids bind to microtubules and use dynein to propel them from the cell periphery to the nucleus [43]. The imitation of such viral 'capsid-based' DNA transport should serve as a model for both the delivery of pDNA to mitochondria and for the nuclear-targeted pDNA delivery.

Verkman's group also determined the cytosolic translational diffusion for small solutes using a carboxyfluorescein derivative [44] and fluorescence-labelled dextrans and Ficolls as model compounds [41]. Although the translational diffusion of large solutes in cytoplasm was found to be slowed down three- to fourfold relative to their diffusion in water, it was found that in contrast to pDNA the degree of slowing did not depend on molecular size up to 30 nm gyration radius (plasmid DNA commonly used for gene therapeutic purposes has a hydrodynamic diameter of about 100 nm [45]). The authors calculated that a large macromolecule of about 500 kD would have a diffusion coefficient of ~ 2.5×10^{-8} cm^2/s in cytoplasm. In the absence of significant binding to cytoplasmic components, the diffusion transit time to move across a 10 μm cell would then be only about 7 s and it was concluded that cytoplasm therefore does not seem to be too crowded to seriously

impede the motion of solutes [41]. This conclusion is based, as stated by the authors, on the absence of binding interactions between solute and cell components. However, in contrast to dextran, Ficoll and DNA of different sizes, most chemotherapeutic drugs in use and under development display physicochemical characteristics (e.g., hydrophobicity and acid–base properties) making them prone to interact with cellular components, which in turn will prevent their diffusion-based even distribution throughout the cell.

Most of the space in a cell consists of organelles, which have unique intralumenal pH values, resident molecules, electrical potentials, lipid-bilayer compositions and membrane-bound proteins [39]. Such organelle-specific properties may have a significant impact on intracellular drug distribution; for example, Horobin and colleagues have studied the intracellular localisation for an enormous variety of dyes and fluorescent probes [46]. Recently, based on experimental and published data, Horobin has developed a QSAR modelling approach for predicting the cellular uptake, the intracellular distribution and the site of intracellular accumulation for fluorescent probes and dyes [47]. He demonstrates that the amphipathic character, the size of the aromatic system, the electric charge, the overall size, the presence of planar aromatic moieties, the lipophilicity and the acid–base properties all determine the intracellular disposition of these molecules [47]. Although developed using dyes and fluorescent probes, Horobin's QSAR approach should also be applicable to many other classes of xenobiotics, including pharmaceuticals.

In conclusion, it becomes apparent that following cell entry, a random (i.e., statistical) interaction of drug molecules or pDNA with mitochondria cannot be expected *per se*. The effective interaction of pharmaceuticals with, or uptake by, mitochondria will take place only when the drug has either been designed (intentionally or by accident) to meet Horobin's QSAR criteria for mitochondrial localisation or when the drug is being transported to mitochondria by an appropriate mitochondria-targeted delivery system. Of course, this consideration leaves out molecules that are not taken up by any cell organelles and which, therefore, are able to diffuse through the cytoplasm relatively freely. Such molecules will indeed eventually collide with mitochondria and may produce a biological effect. However, so will the interaction of these molecules with other possible targets inside the cell. Cyclosporin A (CsA), for example, has been shown to bind with nanomolar affinity to mitochondrial cyclophilin D, which potentially makes it an interesting anti-ischaemic drug candidate [48]. However, CsA also targets at least eight other cyclophilins inside the cell, which are likely to bind a large portion of the administered drug. Therefore, the mitochondrial concentration of CsA is difficult to predict and an effective CsA treatment may require high, even toxic concentrations to reach the mitochondrial target [17]. Consequently, CsA as a potential anti-ischaemic drug would almost certainly benefit from a mitochondria-specific drug carrier system able to increase its therapeutic index [49].

5. Mobility of drug molecules inside the mitochondrial matrix

Because many potential drug targets are located inside the mitochondrial matrix, the question has to be raised of whether any drug molecule having found its way into the matrix will also be able to move through the matrix in order to find its target. The matrix is characterised by a very high concentration of proteins, estimated to be as high as 270 – 560 mg/ml, which would correspond to 27 – 56 vol% solids [50,51]. Such a high density of enzymes and proteins makes the mitochondrial matrix the most crowded aqueous cellular compartment, and it has been suggested by several authors (reviewed in [53]) that the diffusion of small solutes such as metabolites and enzymes, might, therefore, be severely restricted. It has also been proposed that biochemical metabolites are passed between enzyme and enzyme complexes by a special type of channelling mechanism, which would be independent of any aqueous-phase diffusion process, and that mitochondrial water is in an organised state which might even change the basic physical chemistry of enzyme reactions (reviewed in [53]). In summary, it is widely believed that the mitochondrial matrix as a very crowded protein solution would significantly hinder the diffusion of small and enzyme-sized solutes.

Unexpectedly (to the investigators themselves), the first direct measurement of solute diffusion inside the mitochondrial matrix gives rise to a different view. Verkman's group has measured via spot photobleaching the diffusion of green fluorescent protein (GFP) expressed in the mitochondrial matrix of four different cell lines [53]. The quantitative analysis of their bleach data using a mathematical model of matrix diffusion gave diffusion coefficients for GFP only three- to fourfold smaller than that for GFP diffusion in water [53]. Likewise, measurement of the rotation of GFP by time-resolved anisotropy gave a rotational correlation time very similar to that of GFP in water [53]. In conclusion of their data, which in summary demonstrate a rapid and unrestricted diffusion of solutes in the mitochondrial matrix, these authors suggest that metabolite channelling may not be required to overcome diffusive barriers. They propose further that clustering of matrix enzymes in membrane-associated complexes do not occur for metabolite channelling, but serve to establish a relatively uncrowded aqueous space in which solutes can freely diffuse [53]. If Verkman's model holds true, then any low molecular weight drug having entered the mitochondrial matrix should have sufficient diffusional mobility to eventually statistically interact with its molecular target. Of course, as discussed above for mitochondrial (i.e., subcellular) targeting, any random interaction between drug and target inside the mitochondrial matrix would also require that the drug does not interact with any other matrix component besides its molecular target. Dealing with drug barriers inside the mitochondrial matrix, however, would bring the level of discussion from subcellular targeting 'down' to suborganellar targeting and should, due to the lack of available data, be postponed.

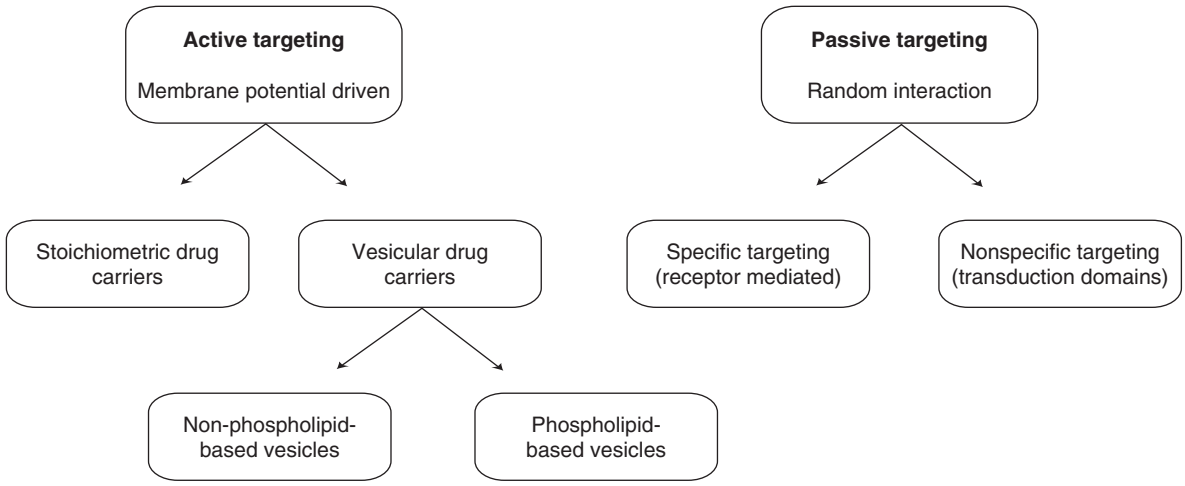


Figure 1. Schematic overview of principal strategies for the targeted delivery of bioactive molecules to and/or into mitochondria within living mammalian cells.

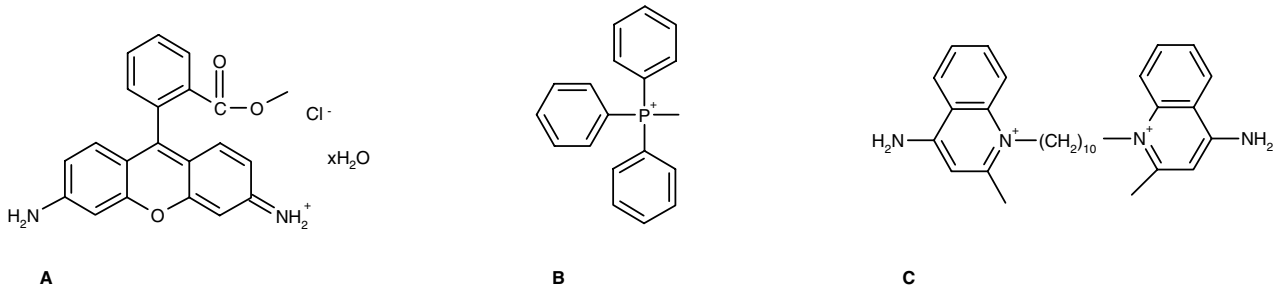
6. Strategies for the delivery of biologically active molecules to and into mitochondria in living mammalian cells

The scheme shown in **Figure 1** presents an overview of the currently pursued strategies for the mitochondria-targeted delivery of biologically active molecules independent of their final destinations, which may be targets either at the outer or inner mitochondrial membrane or inside the mitochondrial matrix.

Most of the currently made efforts [52] for transporting biologically active molecules to and into mitochondria within living mammalian cells are based on two distinct mitochondrial features: the high membrane potential across the inner mitochondrial membrane and the organelle's protein import machinery [3,54,55]. However, other approaches are emerging; for example, it has most recently been reported that titanium dioxide nanoparticles coated with mitochondrion-specific oligonucleotides were able to enter and stay inside mitochondria in a specific way, albeit the mechanism of which remains elusive [56].

The membrane potential is essential for the synthesis of ATP. Electrons derived from hydrogen (NADH, $FADH_2$) are carried along the complexes of the respiratory chain at the mitochondrial inner membrane, thereby releasing redox energy that is used to translocate protons across the inner membrane from the mitochondrial matrix into the intermembrane space. This process creates a transmembrane electrochemical gradient, which includes contributions from both a membrane potential (negative inside) and a pH difference (acidic outside). The membrane potential of mitochondria *in vitro* is 180 – 200 mV, which is the maximum a lipid bilayer can sustain while maintaining its integrity [57]. Although this potential is reduced in living cells and organisms to about 130 – 150 mV due to metabolic processes such as ATP synthesis and ion transport [3], it is by far the largest within cells. Given appropriate physicochemical properties, positively charged molecules can be attracted by mitochondria in response to the highly negative membrane potential. Although most charged molecules cannot enter the mitochondrial matrix because the inner mitochondrial membrane is impermeable to polar molecules, certain amphiphilic compounds ('mitochondriotropics') are able to cross both mitochondrial membranes leading to their accumulation in the mitochondrial matrix. Delivery systems based on the use of mitochondriotropic molecules are either stoichiometric or vesicular drug carriers, both of which 'actively' target the organelle driven by the mitochondrial membrane potential.

The mitochondrial protein import machinery transports proteins from the cytosol across the mitochondrial membranes either into the inner membrane space or into the matrix. To this end, both mitochondrial membranes contain an elaborate network of protein translocases together with a variety of chaperones and processing enzymes in the matrix and intermembrane space to mediate protein import. Although the molecular details of the mitochondrial protein import machinery are still under intense investigation, a relatively clear overall picture of this highly complex mechanism has been established [58,59]. In general, proteins synthesised at cytosolic ribosomes and transported into mitochondria, possess an amino-terminal targeting sequence (mitochondrial leader sequence [MLS] peptide). Proteins bearing a MLS peptide are recognised by translocases in the outer membrane (TOM complex), which transport the protein into the inner membrane space towards translocases of the inner membrane (TIM complex), which in turn mediate the further transport into the mitochondrial matrix. Finally, inside the mitochondrial matrix, the MLS peptide is cleaved off by a matrix-processing protease. Although the deployment of the protein import machinery for the import of oligonucleotide– and PNA–MLS peptide conjugates into the mitochondrial matrix



A B C

Figure 2. Chemical structures of commonly used typical mitochondriotropic molecules. (**A**) Rhodamine 123; (**B**) methyl-triphenylphosphonium; (**C**) dequalinium chloride.

in living cells has been described [60-62], it remains unclear whether these constructs have either been 'actively' transported to the site of mitochondria or whether mitochondrial uptake was only possible following random (i.e., statistical collision) between construct and mitochondria. Mitochondria-specific delivery systems have not been used in any of these three studies. Whereas PNA–MLS conjugates have entered cells in their free form [60], oligonucleotide–MLS peptide conjugates have been vectorised either with a cationic polymer [61] or with cationic liposomes [62] (i.e., with well-established nuclear-targeted delivery systems). Endogenous mitochondrial proteins synthesised at cytosolic ribosomes are being recognised by cytosolic protein factors (chaperones such as hsp70 and others), which keep the newly synthesised mitochondrial precursor proteins in import-competent unfolded conformations and target them in an energy-dependent manner to the outer surface of mitochondria [63,64]. As such chaperones are highly specific for mitochondrial precursor proteins, it appears unlikely that they also could play a role in a putative 'active' transport of oligonucleotide– or PNA–MLS peptide conjugates. In the context of this review (compare **Figure 1**), the use of MLS peptides for the mitochondrial import of small molecules shall, therefore, be characterised as 'passive targeting' (i.e., as based on random collision), and the mitochondrial import itself as 'specific targeting', because the MLS peptide is selectively recognised by the mitochondrial TOM complex. The same classification (i.e., 'passive-specific targeting') should also apply when (in future studies) other endogenous mitochondrial metabolite transporters, mitochondria-specific binding sites or unique protein receptor sites at the mitochondrial membranes will be utilised for the accumulation of non-mitochondriotropic biologically active molecules at or inside mitochondria within living cells. Any such specific interaction of mitochondria with non-mitochondriotropic molecules is based on random collision, which in turn is controlled by the fluid-phase viscosity of the cytoplasm by collisional interactions with and binding to intracellular components other than mitochondria.

Random collision between mitochondria and a drug entity may also be followed by nonspecific interactions leading to drug import into the mitochondrial matrix ('passive non-specific targeting', **Figure 1**). For example, small noncharged molecules displaying appropriate amphiphilic/lipophilic properties may diffuse through mitochondrial membranes, although the probability of small drug molecules entering the mitochondrial matrix is extremely low due to the high impermeability of the inner mitochondrial membrane. For overcoming the barrier the inner membrane poses to any molecule, the utilisation of protein transduction domains (PTDs), also called cell-penetrating peptides (CPPs), has recently been described [65,66], but has also most recently become an issue of controversy [67]. Before discussing CPP-based mitochondrial drug delivery, however, selected examples for membrane potential-driven mitochondrial delivery systems shall be introduced in more detail.

7. Mitochondriotropics

Figure 2 shows the chemical structure of representative mitochondriotropic molecules. The most popular among them is rhodamine 123 (**Figure 2**, compound **A**), which has been used extensively as a stain for mitochondria in living cells since its introduction in 1982 [68]. Already, in 1969, methyltriphenylphosphonium salts (**Figure 2**, compound **B**) were demonstrated to be taken up rapidly by mitochondria in living cells [69], and the mitochondrial accumulation of dequalinium chloride (**Figure 3**, compound **C**) was established during the 1980s [70].

Other examples of mitochondrial cations (structures not shown) are cyanine dyes such as *N*,*N*′-*bis*(2-ethyl-1,3-dioxolane) kryptocyanine [71] and Victoria Blue BO [72]. Mitochondriotropic molecules have two structural features in common. First, they are all amphiphilic; that is, they combine a hydrophilic charged centre with a hydrophobic core. Second, in all structures the π-electron charge density extends over at least three atoms or more instead of being limited to the internuclear region between the heteroatom and the adjacent carbon atom. This causes a distribution of the positive charge density between two or more atoms; that is, the positive charge is delocalised (delocalised cations). Both structural features are widely believed to be crucial for the accumulation

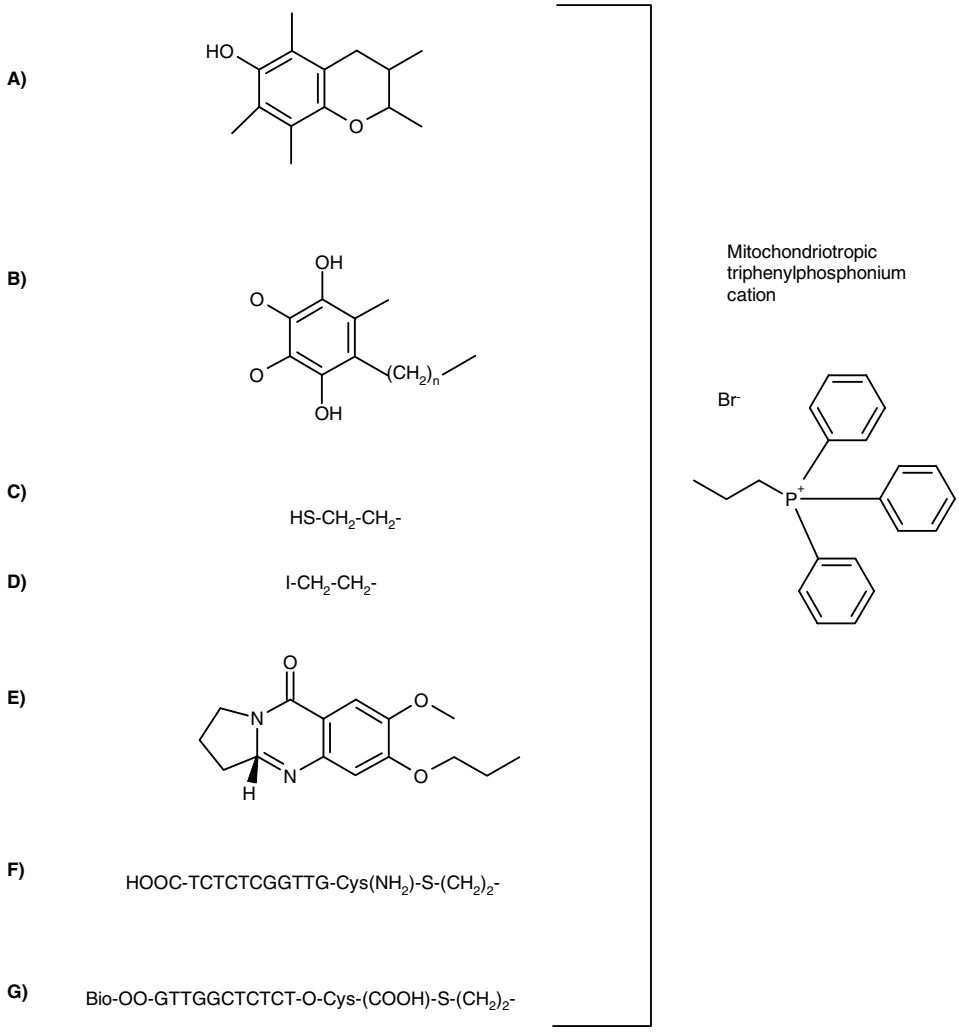


Figure 3. The mitochondriotropic triphenylphosphonium cation as a stoichiometric carrier for biologically active molecules. All compounds (**A** – **G**) on the left half of the figure are covalently linked, in a 1:1 stoichiometric ratio, to the triphenylphosphonium residue shown on the right. (**A**) Active antioxidant moiety of vitamin E. Conjugate: "Mito Vit E", TPPB, 2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol bromide [26]. (**B**) Redox-active ubiquinol. Conjugate (n = 8): "Mitoquinol", 10-(6'-ubiquinolyl)decyltriphenylphosphomium [75]. (**C**) Conjugate: Thiobutyltriphenylphosphonium bromide (TBTP) [76]. (**D**) Conjugate: Iodobutyltriphenylphosphonium bromide (IBTP) [23]. (**E**) DNA-alkylating antibiotic. Conjugate: "Mitodc-81" [74]. (**F**) Peptide nucleic acid (PNA) oligomere with sequence complementary to the human mitochondrial DNA L-chain (np 8339-8349) containing the A8344G point mutation [77]. (**G**) Biotinylated PNA oligomere, same as in (**F**) [77].

of these organic cations inside the matrix of mitochondria. Sufficient lipophilicity combined with delocalisation of their positive charge to reduce the free energy change when moving from an aqueous to a hydrophobic environment are thought to be prerequisites for their mitochondrial accumulation in response to the mitochondrial membrane potential [70].

8. Mitochondriotropics-based mitochondria-specific drug carriers

8.1 Stoichiometric carriers

Since the middle of the 1990s, a large variety of stoichiometric conjugates composed of biologically active molecules and the mitochondriotropic triphenylphosphonium (TPP) cation have been synthesised by Smith and Murphy to either probe, prevent or alleviate mitochondrial dysfunctions [23,26,55,73-76]. **Figure 3** shows some representative examples of triphenylphosphonium cations linked to (i.e., 'carrying') biologically active molecules. Conjugates (**A**) and (**B**) represent mitochondrially targeted antioxidants; conjugates (**C**) and (**D**) are mitochondrially targeted thiol reagents; conjugate (**E**) is a DNA-alkylating antibiotic rendered mitochondriotropic; and the conjugates (**F**) and (**G**) represent PNA oligomers specific for and targeted to the mitochondrial genome. In a series of extensive *in vitro* studies performed by Murphy's group, an up to several-hundred-fold intra-mitochondrial accumulation of

bioactive molecules linked to TPP in comparison with the corresponding bioactive molecules in their native form has been established (reviewed in [7]).

To test the potential of TPP as a mitochondria-specific drug carrier for *in vivo* administrations, Smith and Murphy also investigated the mode of delivery, tissue distribution and clearance of three different TPP conjugates within mice [78]. They could show that relatively high doses of TPP conjugates can be fed safely to the animals over long periods of time, resulting in steady-state distributions within heart, brain, liver and muscle. Moreover, TPP conjugates were also detectable in fetuses and neonates following oral administration to pregnant or lactating dams [78]. The intra-mitochondrial accumulation of TPP conjugates *in vivo* was demonstrated following intravenous injection of iodobutyltriphenylphosphonium bromide (IBTP) (**Figure 3**, compound **D**). IBTP is a thiol reactive derivative able to bind covalently to protein thiols via a stable thioether linkage, which survives tissue homogenisation thus making the visualisation of IBTP-labelled proteins via immunoblotting possible. The direct detection of all other TPP conjugates inside mitochondria *in vivo* is hampered by their rapid loss from mitochondria due to mitochondrial depolarisation during cell subfractionation [78].

In summary, as result of a remarkable line of work over the last decade, Murphy and co-workers have shown the feasibility of delivering by simple oral administration small molecules selectively to mitochondria in organs mostly affected by mitochondrial diseases (i.e., brain, heart and muscle). Detailed pharmacokinetic studies of TPP conjugates are ongoing [78].

8.2 Vesicular drug carriers based on mitochondriotropics

A potential, but general, drawback of the use of stoichiometric carriers is the need for covalent linkage between carrier and bioactive molecule, which may influence its biological activity. In addition, on its way to the mitochondria, the bioactive entity remains accessible to enzymatic degradation or any other nonspecific interactions with tissue or cell components. Whereas encapsulation of bioactive molecules into mitochondria-targeted colloidal vesicles would provide protection and would be without impact on the biological activity, any mitochondrial uptake of vesicular drug carriers, however, appears as highly improbable. The overall strategic goal for the development of mitochondria-specific vesicles is, therefore, the selective delivery of biologically active molecules to the outer membrane of mitochondria, while at the same time protecting the bioactive entities from premature systemic elimination, metabolism or any other interactions with tissue- and cell-specific biomolecules. Such highly selective organelle-specific targeting should significantly increase the therapeutic index of any drug intended to act on mitochondrial targets. On reaching the mitochondrial outer membrane, the carrier system has to become destabilised in order to release its drug cargo. Initial data demonstrating the feasibility of such mitochondria-specific release have been published [79-81]. Depending on the physicochemical properties of the drug, its high local concentration outside the mitochondrion should then either favour its interaction with targets localised in the outer membrane or its diffusional or transporter-mediated entry into the organelle.

8.2.1 Non-phospholipid-based vesicular drug delivery systems based on mitochondriotropics

Non-phospholipid-based mitochondriotropic vesicles described so far have been made either from dequalinium (DQA) or from one of its derivatives [82,83]. DQA (**Figure 2**, compound **C**) is a dicationic mitochondriotropic compound resembling bola-form electrolytes; that is, it is a symmetrical molecule with two charge centres separated at a relatively large distance by a single hydrophobic chain. The self-assembly behaviour of single-chain cationic bola amphiphiles such as DQA has been characterised using Monte Carlo computer simulations [84], transmission and freeze fracture electron microscopy and dynamic laser light scattering [82,83]. It was found that DQA and many of its derivatives form vesicle-like aggregates upon sonication in aqueous medium (named 'DQAsomes' when made from DQA) with diameters of about 70 – 700 nm. In a series of papers [79-81,83,85] it was demonstrated that DQAsomes meet all criteria for a mitochondria-specific DNA delivery vector [86,87]: DQAsomes bind pDNA, mediate its cellular uptake and protect it from nuclease digestion. DQAsomes are endosomolytically active and transport pDNA selectively to mitochondria in living mammalian cells. By using structural analogues of DQA, the efficiency of pDNA transport to mitochondria in living mammalian cells can be increased significantly in comparison with using DQA-based DQAsomes. It was also shown that the cytotoxicity of DQAsomes is as low as the toxicity of several lipidic transfection vectors already being used in clinical trials. Most remarkably, it was demonstrated by using artificial membrane-mimicking liposomes [80], isolated mouse liver mitochondria [81] and living mammalian cells [79] that DQAsome/pDNA complexes release the pDNA on contact with mitochondrial membranes, but not with plasma membranes. To be able to enter mitochondria, however, the pDNA has to be conjugated with mitochondrial leader sequence peptides in order to utilise the mitochondrial protein import machinery for its transport into the mitochondrial matrix. Appropriate investigations are ongoing in the author's laboratory.

Based on the successful use of DQAsomes for the delivery of pDNA towards mitochondria in mammalian cells, it has been proposed to utilise DQAsomes or related vesicles as a delivery system for the selective transport of low molecular weight drugs to mitochondria; that is, drugs that have a molecular mitochondrial target, but are not mitochondriotropic by themselves [88]. As a first step, the encapsulation of paclitaxel into DQAsomes was studied (**Figure 4** shows a cryoelectron microscopic image of paclitaxel-loaded DQAsomes) [88].

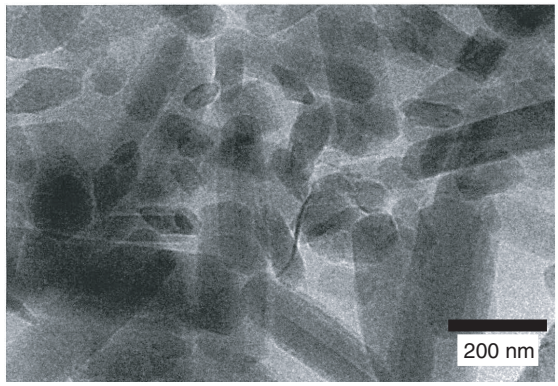


Figure 4. Cryo-electron microscopic image of paclitaxel-loaded DQAsomes (0.61 mol paclitaxel/mol dequalinium). Image taken by A. Kimpfler, Freiburg, Germany, from [88] with reprint permission granted by the Controlled Release Society for abstract 505/Annual Meeting Transactions 2003.

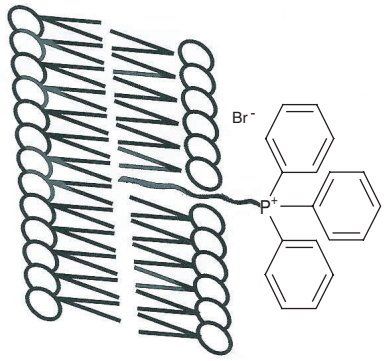


Figure 5. Schematic depiction of a mitochondriotropic triphenylphosphonium cation anchored in a liposomal phospholipid bilayer membrane via an alky residue (not drawn to molecular scale).

Paclitaxel is a potent antitubulin agent used in the treatment of malignancies [89]. It has recently been demonstrated that clinically relevant concentrations of paclitaxel target mitochondria directly and trigger apoptosis by inducing cytochrome *c* (cyt *c*) release in a permeability transition pore (PTP)-dependent manner [90]. Unfortunately, paclitaxel has a very narrow therapeutic window [91], which most likely reflects the existence of several drug targets inside the cell; thus making only a subset of the drug available for mitochondria [90]. Consequently, paclitaxel as an anticancer drug should greatly benefit from an organelle-specific delivery system.

Moreover, considering that many carcinoma cells possess, in comparison with normal cells, both an elevated mitochondrial and a higher plasma membrane potential [92-94], which causes the selective accumulation of mitochondriotropics in tumour cell mitochondria, the encapsulation of paclitaxel (and other drugs) into DQAsomes would potentially have two advantages. First, with DQAsomes being a colloidal drug delivery system, solubility problems would be overcome. Second, with DQAsomes responding to negative-inside membrane potentials, a 'double-targeting' of the drug could potentially be achieved; that is to say, on the cellular level (i.e., carcinoma cells versus normal cells) and on the subcellular level (i.e., mitochondria versus rest of the cell). Such 'double-targeting' could be the basis for new anticancer chemotherapies.

8.2.2 Phospholipid-based mitochondriotropic vesicular drug delivery systems

Colloidal vesicles composed of phospholipids ('liposomes') are one of the most versatile and most extensively studied drug delivery systems. Liposomes can encapsulate an unlimited variety of hydrophilic, amphiphilic and hydrophobic small molecules either in their aqueous inner space or in their lipid bilayer membranes. They are essentially nontoxic, nonimmunogenic and biodegradable; that is, liposomes meet all prerequisites for an ideal drug delivery system. The surface modification of liposomes with polyethylene glycol leads to prolonged circulation times in the bloodstream [95], which in turn is the basis for a variety of liposome-based drugs that have been approved by the FDA and FDA-like agencies in Europe and Asia over the last decade.

To utilise the superior drug carrier properties of liposomes for mitochondria-targeted delivery of bioactive molecules, liposomes with surface-linked mitochondriotropic residues have recently been designed (Weissig *et al.*, Patent pending). To this end, stearyltriphenylphosphonium bromide (STTP) was synthesised by replacing the methyl group in methyltriphenylphosphonium bromide (**Figure 2**, compound **B**) with a stearyl residue. When preparing liposomes in the presence of STTP according to standard procedures [96], the hydrophobic fatty acid residue 'anchors' the mitochondriotropic triphenylphosphonium cation in the phospholipid bilayer membrane (i.e., 'attaches' it covalently to the liposomal surface) (schematically shown in **Figure 5**). Such membrane anchoring via hydrophobic alkyl chains is routinely used to attach hydrophilic molecules such as enzymes, antibodies, carbohydrates and peptides to liposomal surfaces [97].

Analysing the intracellular distribution pattern of fluorescence-labelled liposomes bearing 20mol% surface-linked STTP, an identical distinct and punctuated fluorescence pattern was found, as in control cells stained only with Mitotracker, a mitochondria-specific dye (Weissig *et al.*, Patent pending). Such a comparison of staining patterns has been used by other investigators to demonstrate the localisation of labelled oligonucleotides at the site of mitochondria [62]. Considering that in the above experiments the fluorophore was covalently linked to phospholipids and not to the mitochondriotropic entity (i.e., to STTP), it was concluded that whole phospholipid vesicles seem to have accumulated at

the site of mitochondria. These first data suggest the ability of surface-linked triphenylphosphonium cations to render conventional liposomes mitochondriotropic.

9. Cell-penetrating peptides for mitochondrial import

The first description of the intracellular delivery *in vitro* and *in vivo* of biologically active proteins fused to the PTD from the human immunodeficiency virus TAT protein in 1999 [98] triggered an astonishing growth in the number of papers reporting the use of PTDs (or CPPs) for the transport of a variety of potentially therapeutic cargos, which *per se* cannot easily cross the plasma membrane, into the cytoplasm of mammalian cells (most recently reviewed in [99-102]). The mechanism underlying the translocation of CPPs across phospholipid membranes is currently under dispute. Earlier studies suggested a direct physical transfer of the peptide (and its attached cargo) through the lipid bilayer membrane, whereas newer studies are suggesting the involvement of endocytic pathways. However, even if endocytosis is involved, CPPs and their conjugates are obviously able to escape from endosomes, because their cargo molecules have been described to be biologically active, thus excluding, at least to some extent, any lysosomal degradation.

Although it has been well established that CPPs traverse phospholipid bilayer membranes, their ability to cross the mitochondrial inner membrane has come most recently under dispute.

Payne *et al.* have constructed a TAT–mitochondrial leader sequence peptide–green fluorescent protein fusion protein (TAT–MLS–GFP) as well as the related TAT–GFP protein (i.e., without including the MLS peptide) [65,66]. The authors reported that both TAT fusion proteins rapidly enter cultured cells and transduce into the mitochondrial matrix by mechanisms that neither involve the mitochondrial membrane potential nor the protein import machinery. They reported further that the presence of the MLS peptide, located between TAT and GFP, allows for intra-mitochondrial protein processing, which results in the removal of TAT from GFP. Consequently, GFP transported into the mitochondrial matrix as a GFP–MLS–TAT conjugate remains inside the organelle, whereas GFP transduced into mitochondria only as GFP–TAT does not. Supposing that CPPs cross membranes in a concentration-driven manner by 'destabilising' the phospholipid bilayer (i.e., in the absence of any endogenous biological transport mechanism), it appears as reasonable to assume that a back and forth transport of CPPs across membranes could eventually result in an equilibrium between CPPs on both sides of the membrane. Any separation of the CPP from its cargo, however, should result in its one-sited accumulation. Therefore, the insertion of degradable linker between CPP and cargo appears generally as a valuable strategy for the irreversible CPP-mediated transport across membranes.

Exposing isolated mitochondria and whole cells to a variety of low molecular weight derivatives of two CPPs (TAT and Pen), Murphy *et al.* found, in contrast to Payne's laboratory, no evidence for the ability of CPPs to cross mitochondrial membranes [67]. Most interestingly, they also reported that CPPs were unable to enter mitochondria even when conjugated to the mitochondriotropic TPP cation. To avoid any fixation-caused artifactual distribution of CPPs, these investigators have studied the intracellular localisation of TAT and a TPP–TAT conjugate in live cells. Visualisation of the conjugate in living cells was made possible by attaching a fluorophore to a C-terminal Cys residue via a maleimide-thiol linkage, which also eliminated the possibility of apparent mitochondrial localisation arising from the accumulation of partially degraded TPP–TAT fragments in mitochondria. On incubating live fibroblasts or rat basophilic leukaemia cells with TAT or TAT–TPP conjugate either alone or in tandem with mitochondria-specific or endosome-specific dyes, they found a strong colocalisation of both TAT and TAT–TPP conjugate with endosomes. The incubation time did not have any impact on the intracellular distribution; although peptide accumulation within endosomes increased over time, it was not detected in the cytoplasm at any stage [67].

Whether the apparent differences between the CPP conjugates used by both laboratories (i.e., a CPP fusion protein versus small molecular weight conjugates) or the presence or absence of a digestible linker between CPP and cargo are the cause for the quite contrary conclusions from both groups remains to be seen. From this reviewer's point of view, a direct exchange of the corresponding CPP conjugates between both laboratories followed by analysing the conjugate's intracellular distribution according to laboratory-specific protocols might provide some more insight into the cause of this controversy.

10. Expert opinion

High-throughput screening of large-scale combinatorial chemical libraries has become an increasingly major strategy for modern drug discovery. Other technologies such as microfluidics complement the screening process by collecting absorption, distribution, metabolism and excretion (ADME) data at an early stage of product development. Nevertheless, any potential drug candidate, which *per se* is unable to reach a specific subcellular target such as mitochondria, is being eliminated from the drug discovery process. Even cell-based high-throughput screening is unable to determine the potency of any molecule displaying insufficient intracellular bioavailability. Consequently, albeit still elusive considering the generally immature stage in the development of organelle-specific delivery systems, their early on inclusion into the screening process appears as desirable: a goal worth pursuing. The recent progress made in the area of mitochondria-specific drug and DNA delivery technologies seems to be a step towards achieving this goal.

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