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EXPERT REVIEW

From Serendipity to Mitochondria-Targeted Nanocarriers

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ABSTRACT This review illustrates how a random observation at the laboratory bench has helped pave the way towards the development of organelle-targeted pharmaceutical nanocarriers. A fortuitous discovery in the mid 1990s involving the self-assembly of a molecule, known to accumulate inside mitochondria, has lead to the development of subcellular nanocarriers suited for the selective delivery of biologically active molecules to mitochondria inside living mammalian cells. Applications for mitochondria-specific drug and DNA delivery are described, the current state-of-the-art of mitochondrial drug targeting technology is reviewed, and its future perspectives are discussed.

KEY WORDS apoptosis · dequalinium · DQAsomes · gene therapy · mitochondria · mitochondria-specific liposomes · mitochondria-targeted nanocarriers · STPP liposomes · triphenylphosphonium

THE SELF-ASSEMBLY OF A MITOCHONDRIAL POISON

1,1′-Decamethylene bis (4-aminoquinaldiniumchloride), referred to as dequalinium (DQA), has been used for more than 50 years as an antimicrobial agent in over-the-counter mouthwashes, lozenges, ointments, and paints (1,2). Dequalinium is a cationic bolaamphiphile composed of two quinaldinium rings linked by ten methylene groups (Fig. 1). The exclusive localization of DQA inside energized mitochondria was demonstrated experimentally 25 years ago (3–6), and mechanistic aspects of its mitochondriotropism have been discussed recently, employing the combination of a quantitative structure-activity relationship (QSAR) model with a Fick-Nernst-Planck physicochemical model (7).

Dequalinium displays a large variety of pharmacological activities, most prominent of which is its ability to selectively block K^+ channels (8). It has also been discussed to target F1-ATPase (9), calmodulin (10), and proteinase K (11). DQA has been demonstrated to synergize the *in vitro* antitumor effects of TNF against a panel of human ovarian cancer cell lines (12) and to inhibit the growth of rat colon tumor isografts (13). DQA causes deletion of mtDNA in human cervical carcinoma cells (14) as well as in yeast (15). Its potential for modifying, i.e. enhancing, oxidative stress and subsequently for increasing the production of ROS has been shown to induce cell death in human leukemia cells (16) and in Plasmodium berghei-infected erythrocytes (17). Thus, it seems rather surprising that such a “mitochondrial poison” (18), which even had been implicated in necrosis of the penis (19), could actually lead to the development of mitochondria-targeted pharmaceutical nanocarriers.

In the search for at that time putative DNA gyrase-like topoisomerase activity associated with the 35 kb apicoplast DNA in Plasmodium falciparum (20,21), a variety of compounds known to interfere with DNA metabolism (22) have been screened, and dequalinium chloride was one of them. For reasons being elusive 15 years after the fact, a concentration of 10 mM was chosen for a DQA stock solution in distilled water. To accelerate the solubilization of 10 mM DQA suspended in water, first bath, then probe sonication was applied. Very surprisingly, instead of a clear solution, the resulting 10 mM DQA stock solution appeared opaque, strongly resembling colloidal solutions of phospholipid vesicles, i.e. liposomes. A subsequent size distribution analysis revealed the presence of colloids in the

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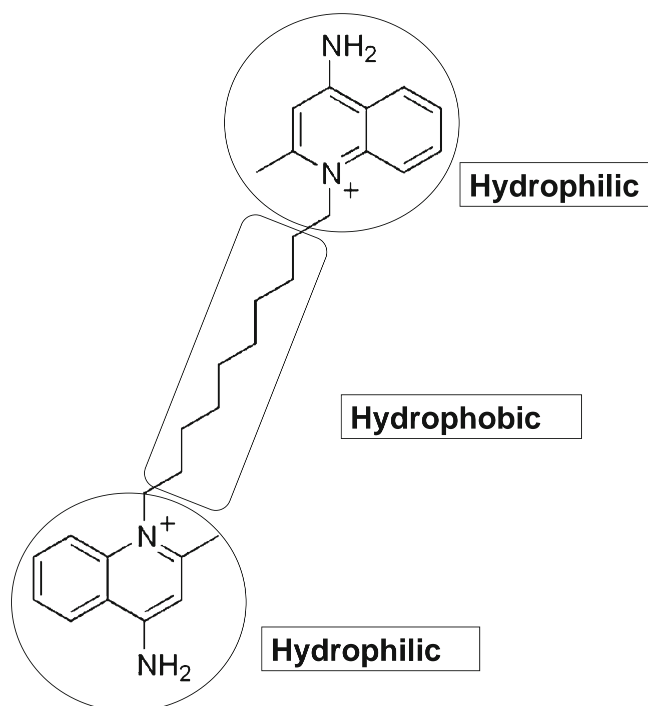

Fig. 1 Chemical structure of dequalinium chloride with overlaid shapes depicting the molecule's amphiphilic character.

size range between 70 and 700 nm, while Freeze Fracture electron microscopic images showed both convex and concave fracture faces (23). Apparently, under the experimental conditions used, dequalinium chloride could self-assemble into liposome-like vesicles, which were termed DQAsomes (DeQAlinium-based lipoSOMES) at that time (23).

DQA, as a bola amphiphile, is different from the well-characterized bipolar lipids derived from Archaea, as it has only one instead of two hydrophobic chains linking the hydrophilic head groups. Symmetric amphiphilic archaeal lipids are known to form stable monolayer membranes, with the lipids either assuming a U-shaped or stretched conformation (24–26). However, the self-assembly behavior of "single-chain" bola lipids, like DQA, had not been characterized yet at that time. Therefore, the self-association properties of DQA were studied in more detail. Monte Carlo computer simulations applied to a system of DQA-like single-chain bola-form amphiphiles, in a coarse-grained model, revealed the formation of vesicles, micelles, cylinders, disks, and planar aggregates as well as the possible co-existence of horseshoe and stretched conformation, all depending on lipid concentration and temperature used in that model (27,28).

Combining all data obtained from photon correlation spectroscopy, electron microscopy, freeze fracture electron microscopy, and Monte Carlo computer simulations, a hypothetical structure of DQAsomes, as shown in Fig. 2, can be assumed. It should be noted here that over the last ten years, the study of the self-assembly of bipolar

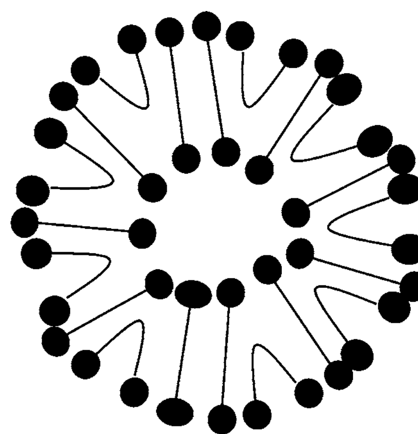

Fig. 2 Hypothetical scheme illustrating the self-assembly of dequalinium cations into liposome-like vesicles, including possible horseshoe and stretched conformations of the bolaamphiphile.

amphiphiles has re-gained significant interest. A large variety of symmetrical and asymmetrical bola amphiphiles has been synthesized, and the study of their self-organizing and packing properties led to the generation of new insights into the biophysics of membranes as well as to the creation of new nanostructures, such as lipid nanotubes (29–34).

The accidental discovery of the vesicle-forming capacity of dequalinium chloride in the mid-1990s raised several questions. Dequalinium has been shown to accumulate in mitochondria following the exposure of CV-1 type cells to 0.019 mM of dequalinium chloride (3), while DQAsomes have been formed during the sonication of a 10 mM DQA suspension. Have these investigators observed the mitochondrial accumulation of dequalinium monomers or, without being aware of it, of vesicles, i.e. DQAsomes? The same question applies to a series of studies conducted during the early 1990s, in which dequalinium chloride at concentrations below 0.01 mM was tested for its anticarcinoma activity in xenograft models (12,18,35). How stable are DQAsomes upon dilution? Do DQAsomes form at dequalinium concentrations far below 10 mM? Quite disappointingly, dequalinium's Critical Vesicle Concentration (CVC) was found to lie between 7 and 3 mM. Within hours following a 1:10 dilution, a 10 mM DQAsome colloidal solution did not show any light scattering signal anymore, and though DQAsomes formed at 7 mM, any attempts to make DQAsomes at 3 and 1 mM failed (V. Weissig, 1997, unpublished).

It was found later that the vesicle stability can be significantly increased by modifying the structure of DQA (36). The substitution of dequalinium's methyl group by an aliphatic ring system, though increasing the hydrophobicity of the heterocycle, confers unexpected superior vesicle-forming properties to this bolaamphiphile. Vesicles made from such "cyclohexyl derivative" have, in contrast to vesicles made from dequalinium, a very narrow size

distribution around 169 nm, which hardly changes at all, even after storage at room temperature for over 5 months. Upon 1:10 dilution, these vesicles do not show any change in size distribution for more than 96 h. From this "structure/vesicle-forming/relationship study," it was concluded that bulky aliphatic residues attached to the quinaldinium heterocycle seem to favor self-association of the planar ring system. It was then speculated that the bulky group sterically prevents the free rotation of the hydrophilic head of the amphiphile around the hydrophobic CH2— axis, thus contributing to improved intermolecular interactions between the amphiphilic monomers (36).

MITOCHONDRIA-TARGETED DNA DELIVERY

Returning to the fluorescence microscopic images published 1987 in PNAS (3), it is evident that these authors must have observed the mitochondrial accumulation of dequalinium molecules and not that of DQAsomes. Was the unplanned discovery of the self-assembly behavior of dequalinium therefore in the end without any practical relevance? Despite the apparently low stability of DQAsomes following dilution, an intriguing potential and very unique application emerged.

Since the first formulation of cationic liposomes in 1987 based on newly synthesized cationic lipids (37), the early 1990s saw a boom in the synthesis of hundreds of cationic lipids as well as cationic polymers and their widespread use as non-viral transfection vectors.

Preformed cationic liposomes were mixed with anionic plasmid DNA, leading to so-called lipoplexes; the mixing of cationic polymer results in the formation of so-called polyplexes. Both, lipo- and polyplexes are still under intense investigation as nuclear-targeted DNA delivery systems as alternative to viral transfrection vectors (38–42). During the 1990s, the assembly of lipid-DNA complexes has been intensively investigated, and several models have been proposed, which are comprehensively summarized in (43). Within each of these models, self-assembly of the lipid component into larger structures has been recognized as a necessary prerequisite for the complexation of DNA with cationic lipids. Upon binding and condensing the DNA to their surface, cationic liposomes lose their vesicle character and form DNA-lipid aggregates of varying shape and size.

Would DQAsomes behave like cationic liposomes? Could DQAsomes be used to bind and condense pDNA, resulting in dequalinium-DNA complexes similar to cationic lipid-DNA complexes? The low vesicle stability of "empty" DQAsomes shouldn't matter once the dequalinium-DNA complex has been formed. The vesicle nature, as known from cation liposomes, is apparently needed only for the process of complex formation starting with DNA condensation at the cationic surface. The ability of DQAsomes to bind and condense pDNA was assessed utilizing SYBR™ Green I, a nucleic acid-specific dye, the fluorescence intensity of which is proportional to its binding to DNA. It was found that the DNA binding capacity of DQAsomes is comparable to that of lipofectin, a commercially available cationic liposome preparation. Moreover, vesicles made from the above-mentioned cyclohexyl derivative of dequalinium proved to be even superior to lipofectin when compared based on the amount of DNA bound per cationic molecule (36). Electron microscopic images of DQAsome-DNA complexes, i.e. DQAplexes (27), strikingly resembled the "spaghetti-and-meatball" shapes observed earlier with lipoplexes (44). It was further demonstrated that DQAplexes containing DNA at a molar ratio of DQA/DNA 6:1 completely protect the DNA against DNAse activity (45), and as shown earlier for lipoplexes (46) the addition of negatively charged lipids also releases intact DNA from the DQAsome-DNA complex (45).

Considering that DQAsomes are composed entirely of molecules known to accumulate in mitochondria, the proposition of using DQAsomes as a mitochondria-targeted transfection vector seemed evident (47). However, the need for delivering pDNA to mitochondria, i.e. the need for transfecting mitochondria with transgene DNA, was understood about 15 years ago only by investigators directly involved in mitochondrial research, with hardly any of them working in the area of pharmaceutical drug and DNA delivery. On the other hand, at the same time, investigators engaged in gene therapeutic research seemingly have been unfamiliar with the need for the development of a mitochondrial transfection vector. For example, even in 2004, it was still challenging to appropriately tag an abstract about mitochondrial DNA delivery (48) submitted to the Annual Meeting of the American Society for Gene Therapy considering the absence of the term "mitochondria" in the meeting's official key word index (49).

The existence of human diseases caused by mutations or deletions in mitochondrial DNA was revealed for the first time in 1988 with the publication of two landmark papers, one in *Science* (50) and one in *Nature* (51). The *Science* paper reported the association of a mitochondrial DNA mutation with Leber's hereditary optic neuropathy, and the *Nature* paper identified mitochondrial DNA deletions in patients suffering from spontaneous mitochondrial encephalomyopathies. During the first half of the 1990s, the number of diseases identified as having their roots in mitochondrial DNA defects has started to climb significantly. As of today, more than 250 pathogenic mtDNA mutations have been identified (52) which cause more than 350 distinct mitochondrial disorders (53), with the majority displaying either neurodegenerative or neuromuscular symptoms.

Apparently due to the lack of any mitochondria-targeted DNA delivery system during the 1990s, only one strategy towards the correction of mitochondrial DNA defects was pursued which aimed at redirecting mitochondrial gene expression to the nucleus. Taking advantage of the availability of nuclear-targeted transfection vectors, this approach involves the delivery of a recoded wild-type copy of the defective mitochondrial gene into the nucleus and following nuclear transcription and cytosolic expression, the gene product would then be imported into the mitochondrial matrix via the mitochondrial protein import machinery. This approach has become known as "allotopic expression" (54,55) and has also been named "indirect mitochondrial gene therapy" (47,56). Though successful allotopic expression of mtDNA-encoded polypeptides in mammalian cells has been reported (57,58), limitations of this approach, most importantly among them the applicability to all 37 mitochondrial genes, have been discussed early on (47,56,59,60). Recently, even issues related to potential misinterpretations of successful allotopic expression experiments in mammalian cells have been raised (61,62).

Though not addressing the complexities of intramitochondrial gene transcription and translation, let alone any issues related to intramitochondrial DNA recombination, the proposal made in 2001 (47,56) to develop DQAsomes as mitochondria-targeted transfection vectors for the delivery of wild-type mitochondrial genes directly to mitochondria harboring the corresponding mutated gene ("direct mitochondrial gene therapy") has opened an alternative to allotopic expression. It should be remembered that despite the development of hundreds of viral and non-viral nuclear-targeted transfection systems over the last 25 years, permanent cures of monogenetic diseases like Cystic Fibrosis still remain elusive. The hurdles to overcome when aiming at the replacement or repair of mutated genes located at any of the 23 chromosomes still appear as insurmountable. All progress made towards gene therapeutic treatments of diseases like cancer are linked to the temporary expression of episomal DNA. Moreover, it shall be dared to speculate that the replacement of an only 16.5 kb large DNA circle with a cloned wild-type copy appears much less challenging than any similar approach towards gene replacement in the nucleus. The entire mouse mitochondrial genome has already been cloned (63–65), and the first report of cloning the entire human mitochondrial genome appears elsewhere in this theme issue (66). Moreover, even the entire synthesis of the mitochondrial DNA circle appears feasible (67). Last but not least, as discussed in more detail below, both mitochondrial membranes do not seem as impermeable for nucleic acids as previously widely thought (68).

DQAsomes have been shown to meet all criteria for a mitochondria-targeted DNA delivery system. Foremost, DQAsome are able to stably bind pDNA and to protect it from nuclease digestion (45). Until five years ago, utilizing the mitochondrial protein import machinery was the only imaginable strategy for importing DNA into the mitochondrial matrix; it was also demonstrated that the presence of a Mitochondrial Leader Sequence (MLS) peptide chain does not interfere with binding of the DNA to and its release from DQAsomes (69). Employing different experimental systems involving liposomes mimicking the composition of cytosolic and mitochondrial membranes (70) and energized isolated mitochondria (71), it was shown that DQAsome/DNA complexes selectively release the DNA upon contact with mitochondrial but not cytosolic membranes. Employing a novel protocol for selectively staining free pDNA in the cytosol of living cells, it was further demonstrated that DQAplexes appear able to escape from endosomes without losing their pDNA load. The complex then translocates to the site of mitochondria at which a portion of the pDNA is released from its DQAsomal carrier. Free pDNA could not be detected anywhere else inside the cytosol of cells transfected with DQAplexes, demonstrating the target selectivity of DQAsome-mediated DNA delivery to mitochondria (72).

The belief that mitochondrial leader sequence peptides conjugated to the DNA would be essential for any mitochondrial import of transgene DNA was put into question in 2006, when a landmark paper (68) reported the capacity of mammalian mitochondria to import naked DNA while preserving its functionality. Following their discovery of this so-called natural competence of mammalian mitochondria for DNA import, the authors concluded that mitochondria *in vivo* might be able to import DNA once it has been delivered to the organelle. It was hypothesized that any delivery device just must be able to cross the plasma membrane and then target mitochondria where the cargo needs to be released (68). DQAsomes have been identified by these authors as an ideal candidate that satisfies most of their criteria (68). The natural competence of mitochondria for DNA import was confirmed one year later in a paper proposing the use of cellular internalization of isolated exogenous mitochondria as a potential tool for studying mitochondrial genetics in living mammalian cells (73).

The unique properties of DQAsomes for the delivery of DNA to mitochondria were emphasized in a recent review comparing DQAsomes with known viral and non-viral transfection vectors currently being explored for nuclear-cytosolic transfection (74). Following an examination of all available data, it was concluded that in contrast to DQAsomes, cationic liposomes and various viral carriers are incapable to serve as vectors for mitochondria-specific DNA delivery, mainly since viral vectors are nucleus-bound by virtue of their nature, and cationic liposome-DNA complexes seem to release their DNA cargo into the cytosol

during and after endosomal escape (74). A most recent review over current approaches for modifying the mitochondrial genome in mammalian cells (75) further highlighted the potential utility of DQAsomes for the delivery of functional recombinant mtDNA constructs to mitochondria. DQAsome-mtDNA complexes with and without a drug resistance gene have been hypothesized being able to serve as effective mediators for mtDNA transfer into the mitochondrial network of wild-type tissue culture cells and mtDNA-less rho(0) cells, respectively (75). Finally, the first study demonstrating the unique capability of DQAplexes to specifically transfect mitochondria appears elsewhere in this theme issue (76). Using immunofluorescence and a combination of immunohistochemical and molecular-based techniques, it is shown that DQAsomes are capable of delivering an artificial mini-mitochondrial genome into mitochondria of a variety of mammalian cells including a mouse macrophage cell line. Despite low transfection efficiencies ranging between 1 and 5%, the mini-genome's transcription into mRNA and subsequent translation into GFP protein inside the mitochondrial compartments could be established. Undoubtedly, further optimization of DQAsome-based mitochondrial transfection is highly warranted in order to provide flexible and rapid means to manipulate the mitochondrial genome in living mammalian cells and to eventually pave the way towards direct mitochondrial gene therapy.

MITOCHONDRIA-TARGETED DELIVERY OF LOW-MOLECULAR-WEIGHT COMPOUNDS?

Concomitant with the development of DQAsomes as a mitochondria-specific DNA delivery system, the question arose whether there might be any potential benefit of using DQAsomes for the delivery of small molecules. Considering the spreading of the mitochondrial network throughout almost all mammalian cells, the notion that small molecules once inside the cytoplasm will eventually "bump into" mitochondria appears somewhat plausible. Subcellular, i.e. organelle-specific, drug delivery not even having been in its infancy yet a decade ago didn't help much either to overcome this faulty notion. With the exception of wide-ranging investigations of all the intracellular barriers DNA constructs have to surmount on their way into the nucleus (77), not much attention has been paid to intracellular factors controlling the diffusion of internalized low-molecular weight compounds (78).

A large portion of the intracellular space is filled with organelles, all of which possess distinctive lipid-bilayer compositions, membrane-linked proteins, electrical charges / potentials, as well as intraluminal pH values (78,79). Obviously, these organelle-specific properties play an important if not decisive role for the intracellular distribution of any small drug molecule which has entered the cell. Utilizing published and his own experimental data, Horobin has created a QSAR model which allows predicting the cellular uptake, intracellular distribution, and potential intracellular sites of accumulation for low-molecular-weight fluorescent dyes (80). In this paper, Horobin makes evident that the overall size, size of the aromatic system, amphipathic character, presence of planar aromatic rings, electric charge, and acid/base properties all control the intracellular fate of small molecules (80). More recently, Horobin's QSAR approach was applied in combination with a Fick-Nernst-Planck physicochemical model to a set of more than 100 molecules know to accumulate at or in mitochondria allowing to specify in detail the physicochemistry of so-called "mitochondriotropics" (7). In conclusion, it has become evident that a random, statistical interaction of low-molecular-weight molecules with mitochondria can hardly be expected. Pharmaceuticals will affect mitochondria only if the molecule's physico-chemical properties "allow" it to interact with the mitochondrial network or when the drug has been transported to mitochondrial sites by a mitochondria-targeted drug carrier system.

TARGETING CANCER CELL MITOCHONDRIA

DQAsomes have been proposed as a mitochondria-targeted colloidal drug carrier system at a time during which the central role of mitochondria for apoptosis was just being revealed (81,82) and studies unraveling the association between cancer and mitochondria-mediated apoptosis were being launched. Fig. 3 shows the number of publications having both terms "cancer" and "apoptosis" in their abstract increased almost exponentially around 1998, i.e. the same year DQAsomes were introduced (23). Though

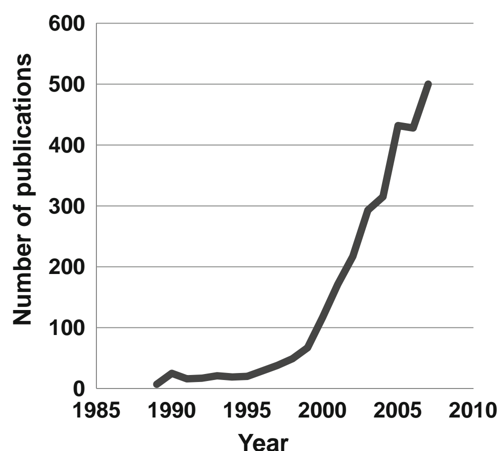

Fig. 3 Number of publications listed on PubMed having both terms "cancer" and "mitochondria" in their abstract.

nowadays mitochondria are regarded as prime targets for cancer therapy (83–85), they were considered as novel cancer targets for the first time also around 1998 (86,87). Therefore, despite the above-discussed stability problems, it appeared tempting to explore DQAsomes for the mitochondria-targeted delivery of drugs known to have mitochondrial target sites. Arguably not the best choice in hindsight, paclitaxel was chosen as a model drug based on a study published in 2000 (88) which showed for the first time that in addition to being an antimicrotubule agent, paclitaxel also acts directly on mitochondria, causing the release of cytochrome C and subsequently triggering apoptosis.

The preparation of DQAsomes in the presence of paclitaxel resulted in a surprisingly stable colloidal solution of the drug with an average size distribution between 400 and 600 nm (89). Though empty DQAsomes are spherical vesicles of similar size, the vesicle character of colloidal paclitaxel incorporated into DQAsomes could not be demonstrated. Cryo-electron and transmission electron microscopic images showed with considerable reproducibility rod-like-shaped structures approximately 400 nm in length (89). Considering the reproducible stoichiometric composition of 2 mole dequalinium per 1 mole paclitaxel, it actually seems likely that the obtained colloidal solution of DQAsomal paclitaxel contains crystal-like solid nanoparticles instead of vesicles with encapsulated paclitaxel. Interestingly, electron microscopic images of colloidal solutions obtained when preparing DQAsomes in the presence of another drug, etoposide (VP-16), show the same rod-like structures (V. Weissig, 2005, unpublished). Attempts to exactly determine the nature of these DQAsomal drug preparations have not been made so far. Nevertheless, the formulation of paclitaxel in DQAsomes increases the solubility of the drug in comparison to free paclitaxel by a factor of roughly 3,000, thereby presenting a possible alternative to Cremophor-based formulations of the highly insoluble paclitaxel. A series of studies was then undertaken to test the hypothesis whether DQAsomal preparations of paclitaxel could increase the drug's efficiency in triggering apoptosis by directly acting on mitochondria. First, fluorescence microscopic images of cells incubated with free fluorescently labeled paclitaxel and DQAsomal formulations thereof display a strikingly different intracellular drug disposition. While the free drug randomly diffuses throughout the cytosol, fluorescently labeled paclitaxel formulated with DQAsomes does colocalize with mitochondria (90). Second, two apoptosis-specific assays based on changes of the nuclear morphology and subsequent DNA laddering showed that DQAsomal paclitaxel in contrast to all controls triggers cell death via apoptosis in cancer cells (90). Third, in a tumor growth inhibition study with DQAsomal paclitaxel in nude mice bearing human colon carcinoma cells, DQAsomal paclitaxel inhibited the tumor growth by about 50% in comparison to the free drug, which at the concentration used didn't have any impact on tumor growth (89). In summary, these studies demonstrate that the subcellular distribution of drugs can be altered by the use of an organelle-specific nanocarrier system with the result of a changed (improved) activity of that drug.

The rod-like structure of paclitaxel formulated with DQAsomes, their co-localization with mitochondria, and the resulting increased apoptotic activity of paclitaxel were confirmed recently in an independent study (91). These authors also succeeded in further increasing the activity of DQAsomal paclitaxel by conjugating folic acid to the surface of DQAsomes, making them specific for tumor cells over-expressing the folate receptor.

Most recently, the mitochondriotropic properties of dequalinium were exploited to make phospholipid vesicles (liposomes) mitochondria-specific (92). The authors prepared probe-sonicated vesicles from a chloroform and methanol (2:1, v/v) solution of egg phosphatidylcholine, cholesterol, polyethylene glycol-distearoylphosphatidylethanolamine and dequalinium at 58/29/4/9 molar ratio in the presence (or absence) of daunorubicin and /or amlodipine. The resulting vesicles (called "mitosomes") displayed an average size distribution around 100 nm and had a zeta potential between about −19 and −13 mV. The latter seems quite surprising considering the two-fold positive charge of dequalinium. Unfortunately, the authors also did not hypothesize any constraints a phospholipid bilayer membrane might impose on the accommodation of the bola amphilic dequalinium cation. As demonstrated already in 1998, the stable incorporation of dequalinium into liposomes made of phosphatidylcholine and/or phosphatidyserine does not seem to be possible based on a very limited ability of dequalinium to mix with phospholipids (93). However, a variety of *in vitro* and *in vivo* assays including cytochrome C release, Bid cleavage, and caspase 8 and 3 activation suggest an enhanced pro-apoptotic activity of daunorubicin / amlodipine incorporated into dequalinium-containing liposomes (92), and the authors concluded in their 2010 paper that "mitochondria-specifically targeting drug-loaded liposomes would provide a new strategy for treating resistant cancers" (92). The proof-of-concept for this new strategy involving the targeting of drug-loaded liposomes to mitochondria was actually provided a few years earlier. The well-established mitochondriotropic triphenylphosphonium cation (7,94,95) has been hydrophobized by reacting triphenylphosphine with stearylbromide, yielding stearyl-triphenylphosphonium bromide (STPP) (96). Anchoring hydrophilic molecules to/into phospholipid membranes via modifying them with fatty acid (or phospholipid) residues is a well-known principle used by nature (97–99) and has subsequently been applied

widely in Liposome Technology for the functionalization of liposomes with surface-bound ligands (100–103). The proper incorporation of STPP into the liposomal membrane was confirmed via ^{31}P-NMR and zeta potential measurements (96). Quantitative confocal fluorescence microscopy was used to prove the colocalization of STPP liposomes with mitochondria in comparison to control liposomes containing DOTAP (104), a cationic lipid used for nuclear-targeted DNA delivery (105–109) (see Fig. 4).

Though 1.5 mol% DOTAP and 1.5 mol% STPP-containing liposomes possess the same zeta potential of $+30\pm12$ mV and consequently bind to the surface of cells to the same extent as shown by flow cytometry, their intracellular distribution proved to be strikingly different. While STPP liposomes almost exclusively colocalized with mitochondria, no such mitochondrial localization of DOTAP liposomes could be observed (104).

At this point the question arises whether actually whole and intact liposomes are able to enter the cytosol and deliver any cargo irrespective of its solubility selectively to mitochondria. The answer to this question has to be most likely not. For example, attempts to show the colocalization of mitochondria with liposomes containing both a lipid and an aqueous fluorescence marker have failed (V. Weissig, unpublished). However, in all experiments (as referenced above and to be referenced below), the fluorophore was covalently linked to a phospholipid, the mitochondrial targeting moiety (TPP) linked to steric acid, and the liposomal cargo (ceramide and sclareol, see below) was highly hydrophobic. All three components are "held together" by being incorporated in a liposomal bilayer membrane; none of these components are able to exist in solution as a monomer at measurable concentrations. It shall be speculated that though not whole liposomes but at least liposomal membrane fragments survive cellular uptake and endosomal escape and reconstitute into vesicles possibly by including endosomal membrane lipids while still accommodating, i.e. "holding together," the fluorescence marker, targeting moiety, and hydrophobic drug. If this hypothesis holds true, then mitochondriotropic STPP liposomes should be able to significantly enhance the pro-apoptotic activity of hydrophobic molecules which are known to trigger apoptosis by directly acting on mitochondria.

Two papers, published in 2008 and 2009, respectively, confirm this hypothesis without a doubt. In the first paper (104), ceramide, a sphingolipid signaling molecule, was chosen as a model drug. Ceramide, a lipid being able to form large stable pores in membranes, has been implied to play a role for the mitochondrial membrane channel formation during apoptosis (110). Ceramide concentrations are also known to increase prior to the induction of apoptosis by chemotherapeutic drugs (111), and, most interestingly, anti-apoptotic Bcl-2 family proteins seem to be able to disassemble such ceramide channels (112), Hence, it was hypothesized that the selective delivery of ceramide to mitochondria in STPP liposomes would increase the mitochondrial ceramide level, which in turn should favor the induction of apoptosis via channel formation in the mitochondrial outer membrane. Indeed, only ceramide incorporated into mitochondria-targeted STPP liposomes was found to trigger apoptosis as revealed by DNA ladder formation, while ceramide at the same concentration either in free form or incorporated into non-mitochondria-targeted DOTAP liposomes entirely failed to cause any detectable laddering of chromosomal DNA (104). For tumor growth-inhibition studies in mice, STPP liposomes were modified with 3 mol% PEG5000-PE; a safe injectable dose was determined, and it was verified that the cationic targeting ligand does not alter in any appreciable way the biodistribution of STPP liposomes in comparison to charge-neutral PEG-liposomes. Upon tumor formation, mice were treated either with buffer, empty STPP liposomes, or 6 mg/kg ceramide incorporated into STPP liposomes. After 12 days, 50% of the animals in both control groups developed severe necrotic morbidity, while no such morbidity was observed even after 18 days in the drug treated group. Statistical analysis after 12 days revealed a significant tumor growth inhibition in the group treated with STPP liposomal ceramide (104). Remarkably, the dose of ceramide used in this study was 6 mg/kg, six times less than the effective dose used in a previous study in which ceramide was incorporated into plain, i.e. non-mitochondria-targeted, liposomes (113).

In the second study, published in 2009, STPP liposomes were used to deliver sclareol to tumor cell mitochondria *in vitro* (114). Sclareol (labd-14-ene-8,13 diol) is a labdane diterpene isolated from the plant *Salvia sclarea*, which has been shown to induce apoptosis possibly via both the mitochondrial and death receptor pathways (115–117). Interestingly, formulations of sclareol into non-mitochondria-targeted plain liposomes were found to reduce the compound's cytotoxicity (118). Subsequently, it was hypothesized that the targeted delivery of sclareol to mitochondria would increase its proapoptotic and cytotoxic activity, and a series of apoptotic assays confirmed just that (114). The authors concluded that "mitochondria-targeted, sclareol-loaded liposomes represent a significantly optimized formulation of sclareol for subsequent *in vivo* evaluation" (114).

CONCLUSION

DQAsomes and STPP liposomes present prototypes of mitochondria-targeted nanocarriers, the capacity of which for the selective delivery of biologically active molecules to

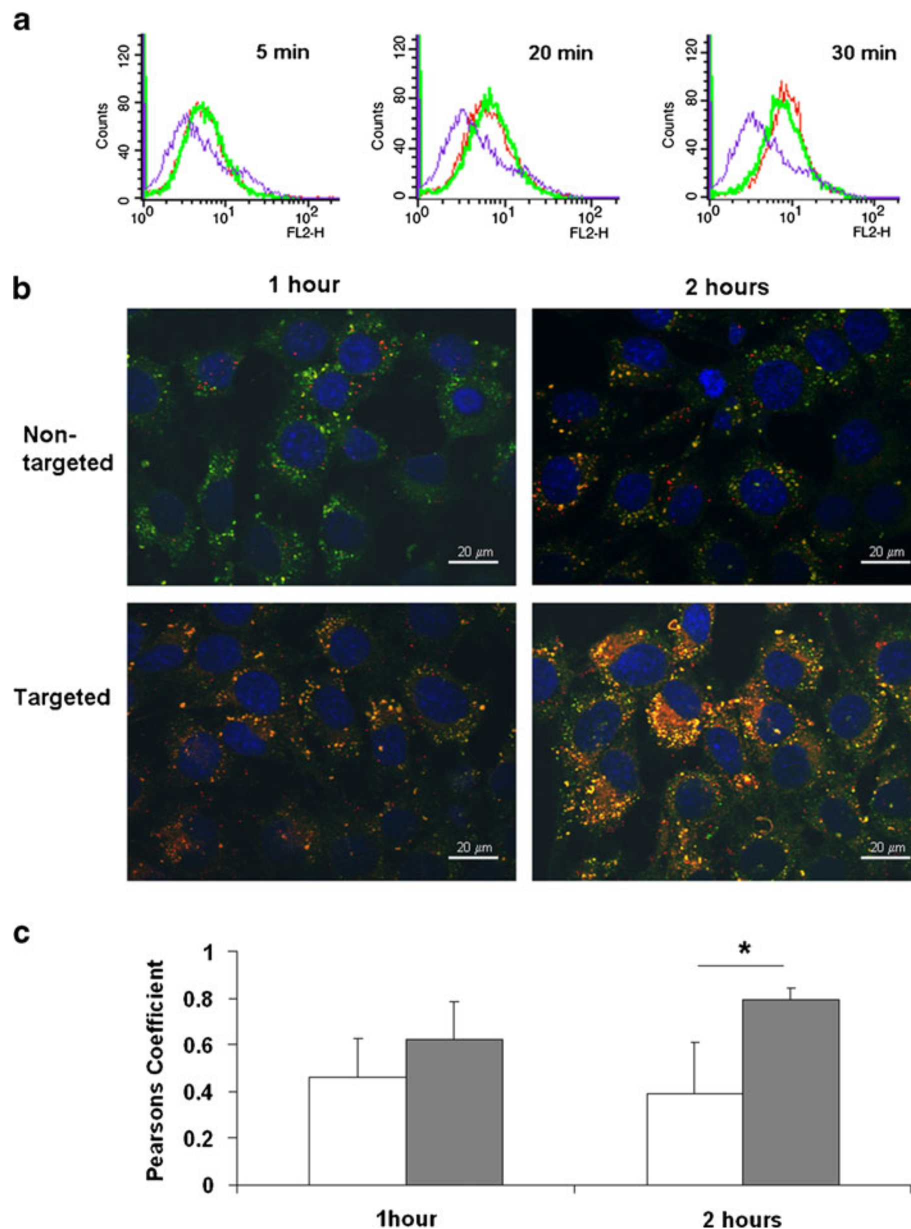

Fig. 4 Interaction of liposome formulations with cells. (**a**) Flow cytometric analysis of nanocarrier binding to 4T1 mouse mammary carcinoma cells. Nanocarriers prepared with rhodamine-labeled PE and either 1.5 mol % DOTAP or 1.5 mol % STPP were incubated with 4T1 cells for 5, 20, or 30 min, and the amount of cell-associated fluorescence after washing was measured by flow cytometry. Purple line shows unstained cells, red line shows nontargeted (DOTAP) nanocarriers, green line shows (STPP) nanocarriers. (**b**) Overlaid multichannel confocal fluorescence micrographs. Red channel (EX 548 nm, EM 719 nm): Rhodamine-labeled PE. Green channel (EX 505 nm, EM 530 nm): MitoFluor Green stained mitochondria. Blue channel (EX 385 nm, EM 470 nm): Hoechst 33342 stained nuclei. Yellow: colocalization of red and green fluorescence. (**c**) Analysis of fluorescence colocalization. Pearson coefficient (standard deviation $n=6$) for colocalization of rhodamine fluorescence with Mitofluor green fluorescence obtained with ImageJ. Open bars indicate nontargeted nanocarrier, shaded bars indicate targeted nanocarrier. * indicates a P value of <0.005. Reprinted with permission from S.V. Boddapati *et al*., Organelle-targeted nanocarriers: Specific delivery of liposomal ceramide to mitochondria enhances its cytotoxicity *in vitro* and *in vivo*. Nano Lett. 8, 2559 (2008).

mitochondria in living mammalian cells has been demonstrated. In particular, formulating experimental and clinically approved anticancer drugs into mitochondria-specific drug carrier systems has been shown to significantly improve cytotoxic and pro-apoptotic drug activity. Significantly more work is warranted to move from the provided proof-of-concept to future clinical applications. Moreover, it shall be predicted that the launched merger of pharmaceutical nanotechnology with mitochondrial medicine will eventually lead to the development of a large variety of mitochondria-specific nanotools for accessing, probing, and manipulating mitochondria under physiological and pathological conditions.

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