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Towards mitochondria-specific delivery of apoptosis-inducing agents: DQAsomal incorporated paclitaxel

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DQAsomes have been developed by us as the first mitochondria-specific colloidal drug carrier system. Previously we have shown that DQAsomes meet all criteria for a mitochondria-targeted DNA delivery system. Here we describe for the first time the use of DQAsomes for encapsulating low-molecular weight compounds. As a model drug we have used paclitaxel, which has recently been shown to trigger apoptosis in tumor cells by acting directly on mitochondria. We demonstrate that paclitaxel can be incorporated into DQAsomes at an approximate ratio of 0.5 mol paclitaxel per mol dequalinium. Following an extensive physicochemical characterization of paclitaxel-loaded DQAsomes using three different electron microscopic techniques we tested in a preliminary study the ability of paclitaxel-loaded DQAsomes to inhibit the growth of human colon cancer cells in nude mice.

Key words: Dequalinium – DQAsomes – Paclitaxel – Mitochondria – Mitochondrial drug delivery – Apoptosis.

DQAsomes have been developed by us as the first mitochondria-specific colloidal drug and DNA delivery system, the design of which we have based on the intrinsic mitochondriotropism of amphiphilic cations with a delocalized charge center, i.e. on cations that accumulate at and inside mitochondria of living cells in response to the mitochondrial membrane potential (reviewed in [1,2]). For creating this system we have utilized the self-assembly behavior of dequalinium and its derivatives, which are mitochondriotropic cations resembling "bola"-form electrolytes, i.e. they are symmetrical molecules with two charge centers separated by a hydrophobic chain at a relatively large distance. We found that such "bola"-form like amphiphiles form upon sonication of aqueous suspensions cationic vesicles ("bolasomes"), which we termed "DQAsomes" when prepared from dequalinium [3].

The need for mitochondria-specific delivery systems arises from the central role mitochondria play in a multitude of metabolic pathways (reviewed in [4,5]). Mitochondria are vital for the cell's energy metabolism and for the regulation of programmed cell death. In addition, mitochondria are critically involved in the modulation of intracellular calcium concentration and the mitochondrial respiratory chain is the major source of damaging reactive oxygen species. Consequently mitochondrial dysfunction either causes or at least contributes to a large number of human diseases. Malfunctioning mitochondria are found in several adult-onset diseases including diabetes, cardiomyopathy, infertility, migraine, blindness, deafness, kidney and liver diseases and stroke. The accumulation of somatic mutations in the mitochondrial genome has been suggested as contributing to aging, age-related neurodegenerative diseases and neuromuscular diseases as well as in cancer. Consequently mitochondria are a prime target for pharmacological intervention [6].

Thus far we have focused on developing DQAsomes as a mitochondria-specific transfection vector to make mitochondrial gene therapy feasible [7]. We have established that DQAsomes meet all essential requirements for a mitochondria-specific DNA delivery system. We have shown that DQAsomes bind pDNA [3], mediate its cellular uptake and protect it from nuclease digestion [8]. DQAsomes are endosomolytically active [9], release pDNA upon contact with outer mitochondrial membranes but not with plasma membranes [10, 11] and transport pDNA selectively to mitochondria in living mammalian cells [9]. By using structural analogues of dequalinium [12], the efficiency of pDNA transport to mitochondria in living mammalian cells could be increased significantly in comparison to using dequalinium-based DQAsomes. Most recently we demonstrated that DQAsomal pDNA delivery is highly mitochondria-specific in contrast to pDNA delivery using commercially available lipidic vectors such as Lipofectin, which we found under identical experimental conditions to transport pDNA predominantly to the perinuclear area [13]. We also established that the cytotoxicity of DQAsomes is as low as the toxicity of several lipidic transfection vectors already being used in clinical trials [8].

The goal of the present study was to demonstrate the feasibility of using DQAsomes as a potential mitochondria-targeted carrier for small drug molecules, in particular of anticancer drugs known to trigger apoptosis via direct action on mitochondria. Dysregulation of the apoptotic machinery is generally accepted as an almost universal component of the transformation process of normal cells into cancer cells and a large body of experimental data demonstrates that mitochondria play a key role in the complex apoptotic mechanism [14-17]. Consequently any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells is believed to have potential therapeutic effect [18-22].

Several clinically approved anti-cancer drugs such as paclitaxel [23-25], VP-16 (etoposide) [26] and vinorelbine[25] as well as an increasing number of experimental anticancer drugs

(reviewed in[27]), such as betulinic acid, lonidamine, ceramide and CD437 have been found to act directly on mitochondria resulting in triggering apoptosis. The therapeutic potential of such anti-cancer drugs, which are known to act at or inside mitochondria, should be greatly enhanced by a drug delivery system which specifically targets mitochondria.

Moreover, since the driving force for targeting DQAsomes originates from differences in membrane potentials, this delivery system seems particularly capable of targeting mitochondria in carcinoma cells. It has been found that several carcinoma cell lines also have, in addition to an elevated mitochondrial membrane potential, a higher plasma membrane potential relative to their normal parent cell lines [28-34]. Such a difference between normal cells and human adenocarcinoma cells regarding the electrical charge of both, plasma and mitochondrial membrane, opens up fundamentally new ways for the selective targeting of cancer cells. For example, the preferential accumulation of a boron-bearing dequalinium derivative in carcinoma cells over non-transformed cells has already been demonstrated [35]. Therefore it seems reasonable to assume that DQAsomes might be able to act as a target-specific drug delivery system on both the cellular level (i.e. carcinoma cells) and the subcellular level (i.e. mitochondria).

As a model compound we have chosen paclitaxel, which is a potent anti-tubulin agent used in the treatment of malignancies [36]. Its therapeutic potential, however, is limited due to a very narrow span between the maximal tolerated dose and intolerable toxic levels. In addition, its poor aqueous solubility requires the formulation of emulsions containing Cremophor EL, an oil of considerable toxicity in itself [37]. Most recently, it has been demonstrated that clinically relevant concentrations of paclitaxel directly target mitochondria and trigger apoptosis by inducing cytochrome c release in a permeability transition pore (PTP)-dependent manner [23]. This mechanism of action is known from other pro-apoptotic, directly on mitochondria acting agents [38]. A 24-hour delay between treatment with paclitaxel [23] or with other PTP inducers [38, 39] and the release of cytochrome c in cell-free systems compared to intact cells has been explained by the existence of several drug targets inside the cell making only a subset of the drug available for mitochondria [23]. Consequently paclitaxel is a prime candidate to benefit from a mitochondria-specific drug delivery system such as DQAsomes.

I. EXPERIMENTAL

1. Materials

Dequalinium chloride was purchased from Sigma Chemical Co. and paclitaxel was obtained from Natural Pharmaceuticals, Inc.

2. Encapsulation of paclitaxel into DQAsomes

Dequalinium chloride (26 mg, 10 mM final) and paclitaxel (42.1 mg, 10 mM final) were dissolved in methanol in a round-bottom flask followed by removal of the organic solvent with a rotary evaporator. After adding 5 mM HEPES, pH 7.4, the suspension was sonicated with a probe sonicator until a clear opaque solution of mitospheres with encapsulated Taxol was obtained (usually about 1 h). To remove undissolved material, the preparation was centrifuged for 10 min at 3,000 rpm.

3. Separation of non-encapsulated paclitaxel from paclitaxel-containing DQAsomes

The solubility of paclitaxel in water at 25°C at pH 7.4 is with 0.172 mg/l (0.2 μM), extremely low, making any separation procedure of non-encapsulated paclitaxel from DQAsomes unnecessary, i.e. in an aqueous environment, only paclitaxel encapsulated in DQAsomes would stay in colloidal solution. However, for control, a paclitaxel suspension was probe sonicated under identical conditions used for the encapsulation of paclitaxel into DQAsomes, but in the complete absence of dequalinium chloride. As expected, upon centrifugation no paclitaxel was detectable in the supernatant using UV spectroscopy at 230 nm.

4. Size distribution measurements

The size distribution of DQAsomes with encapsulated paclitaxel was measured using a Coulter N+ Submicron Particle Analyzer (Beckman-Coulter, Fullerton, FL, United States).

5. Dequalinium determination

The amount of dequalinium in DQAsomes was measured using fluorescence spectroscopy (ex. 335 nm, em. 360 nm). At these wavelengths paclitaxel does not display any fluorescence and therefore does not interfere with the determination of dequalinium. For measurements, 3 μl DQAsomes were dissolved in 3 ml methanol, resulting in a concentration of dequalinium, which lies within the linear range of a previously determined standard curve (not shown).

6. Paclitaxel determination

The UV spectra of paclitaxel and dequalinium in methanol strongly overlap between 200 and 240 nm. Therefore, before measuring the amount of paclitaxel encapsulated into DQAsomes, dequalinium had to be quantitatively removed from the preparation. To this end, a Solid Phase Extraction (SPE) column (J.T. Baker Bakerbond Octadecyl 40 μm Prep LC Packing) was equilibrated with methanol and loaded with 1 ml water followed by the application of 0.02 ml DQAsomes previously dissolved in methanol/water 10/1, v/v. Dequalinium was quantitatively eluted from the column by washing with a discontinued methanol/water gradient (1 ml methanol/water (1:4, v/v), followed by 1 ml methanol/water (3:2, v/v)). Paclitaxel was eluted from the SPE column by washing with 1 ml 100% methanol and measured via UV spectroscopy at 230 nm. The lack of any absorption at 315 nm demonstrates the complete absence of dequalinium in the sample used for the determination of paclitaxel.

7. Stability testing

Over a period of several days, the paclitaxel-containing DQAsome preparation was again centrifuged (10 min, 3,000 rpm) to remove freshly formed precipitate followed by the determination of the dequalinium/paclitaxel ratio as described above.

8. Cryo-electron microscopy

After putting a drop of the sample on a Quantifoil perforated carbon film coated copper grid (Quantifoil Microtools GmbH, Jena, Germany) most of the liquid was removed with blotting

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