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Organelle-Targeted Nanocarriers: Specific Delivery of Liposomal Ceramide to Mitochondria Enhances Its Cytotoxicity in Vitro and in Vivo

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ABSTRACT

To further increase the therapeutic activity of drugs known to act on intracellular target sites, in vivo drug delivery approaches must actively mediate the specific delivery of drug molecules to the subcellular site of action. We show here that surface modification of nanocarriers with mitochondriotropic triphenylphosphonium cations facilitates the efficient subcellular delivery of a model drug to mitochondria of mammalian cells and improves its activity in vitro and in vivo.

Specific delivery of a drug to its site of action inside cells will dramatically improve its efficacy.[1] Much effort has already been afforded to improving the tissue accumulation of drug molecules to improve efficacy and reduce side effects.[2] Despite such advances, the improvement in drug action was not always dramatic. Many drugs act on molecular targets associated with certain organelles inside mammalian cells.[3] Unfortunately, many drug delivery approaches make an assumption that just mediating cell association or even cytosolic internalization is sufficient to ensure the interaction of the drug molecule with its target by virtue of simple diffusion of the drug molecule and random interaction with organelles in the cell. Such a scenario might be likely in cases where some small molecules have sufficiently high metabolic stability allowing their eventual interaction with the target organelle. However, not all biologically active molecules fit these criteria. It is therefore important to also address the subcellular fate of the molecule in any strategy designed to potentiate the therapeutic effect. Subcellular, i.e., organelle-specific targeting has thus emerged as the new frontier in drug delivery.[4]

Most approaches to subcellular targeting have involved the chemical conjugation of targeting ligands to the drug molecule. Peptide leader sequences, cell penetrating peptides as well as organic molecules have been used successfully to direct conjugated cargo to subcellular structures like the nucleus and mitochondria.[5–7] However, this approach is limited to molecules that are amenable to being modified in such a manner while still retaining their therapeutic action. Subcellular targeted drug carrier systems that do not require the active molecule to be modified in any way can be applied to a greater variety of drug molecule classes and will prove to be a powerful alternative if they can be developed. To this end it is interesting to note the work of Savic et al. who report that "nanocontainers" prepared from block copolymer micelles distribute albeit randomly, to cell organelles.[8] The specificity of subcellular distribution does however leave much to be desired.[9] In our own earlier studies we have explored the use of amphiphilic derivatives with known and highly specific subcellular distribution to design organelle-specific pharmaceutical nanocarriers.[10] Our focus has been on the development of nanocarrier systems targeted to mitochondria. We have shown that it is possible to attach mitochondriotropic molecules to the surface of nanocarriers and that such nanocarrier systems can be loaded with drugs or DNA.[11,12]

Since the early 1990s, it has become increasingly evident that mitochondrial dysfunction contributes to a large variety of human disorders, ranging from neurodegenerative and neuromuscular diseases, obesity and diabetes to ischemia-reperfusion injury and cancer. On the basis of recent developments in pharmacological intervention aimed at mitochondria, "Mitochondrial Medicine" has emerged as a

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new field of biomedical research.[14,15] The identification of mitochondrial drug targets in combination with the development of methods for selectively delivering biologically active molecules to the site of mitochondria could allow new therapies for the treatment of mitochondria-related diseases. However, the need for mitochondria-specific drug carrier systems able to selectively transport biologically active molecules to and into mitochondria within living human cells remains unmet.[16,17] Thus, the development of a mitochondria-specific pharmaceutical carrier not only allows us to test the hypothesis that drug action can be improved by specific subcellular targeting but also meets an established need for mitochondria-specific delivery systems.

To address the need for nanocarriers targeted to mitochondria, so-called mitochondriotropic molecules that accumulate inside mitochondria in response to the high mitochondrial membrane potential represent a significant resource.[10] The mitochondriotropic triphenylphosphonium (TPP) cation has been conjugated to various biologically active molecules to facilitate a selective accumulation of these molecules in mitochondria with the intent to probe or prevent or alleviate mitochondrial dysfunction.[7,18–21] It is reasonable to imagine that a carrier system bearing several TPP residues on the surface would increasingly accumulate at the mitochondria and that biological molecules loaded in such a carrier could be rendered mitochondriotropic without the need for any chemical modification. To this end we conjugated TPP to a stearyl residue to give stearyltriphenyl phosphonium (STPP), an amphiphilic molecule that can be incorporated into lipid bilayers.[11] STPP makes possible the preparation of liposome-based nanocarriers with TPP residues on the surface.

Incorporation of a drug capable of acting on mitochondria into the STPP-modified nanocarrier should facilitate improved delivery of the drug to mitochondria and subsequent improvement in drug action. To test this hypothesis, we first incorporated 0.5 mol % rhodamine labeled phosphatidylethanolamine (Rh-PE) into the STPP-modified nanocarriers. STPP-nanocarriers were prepared as described earlier.[11] Briefly, a dry lipid film consisting of a mixture of DOPC (Avanti Polar Lipids), cholesterol (Avanti Polar Lipids) and STPP (DOPC/Ch/STPP = 83.5/15/1.5 mol %; total lipid 25 mg/mL) was hydrated with 5 mM HEPES (pH 7.4) and probe sonicated. The preparation was then centrifuged for 10 min at 800*g* to remove any titanium particles shed from the probe during sonication. Finally, nanocarriers were purified by gel filtration chromatography on a Sephadex G-15 column. Size distribution and zeta potential was then measured on a Brookhaven Instruments Zeta Plus. The preparation protocol consistently yielded liposomes in the expected nanosize range (54 ± 22 nm). Rh-PE is a fluorescently labeled phospholipid that remains associated with the lipid bilayer in much the same way as a drug molecule would. Rh-PE is thus a convenient model compound that enables us to use established fluorescence detection methods to demonstrate the mitochondria specific delivery mediated by our STPP-modified carrier system. To rule out effects of charge-mediated cell association, we also studied the distribution

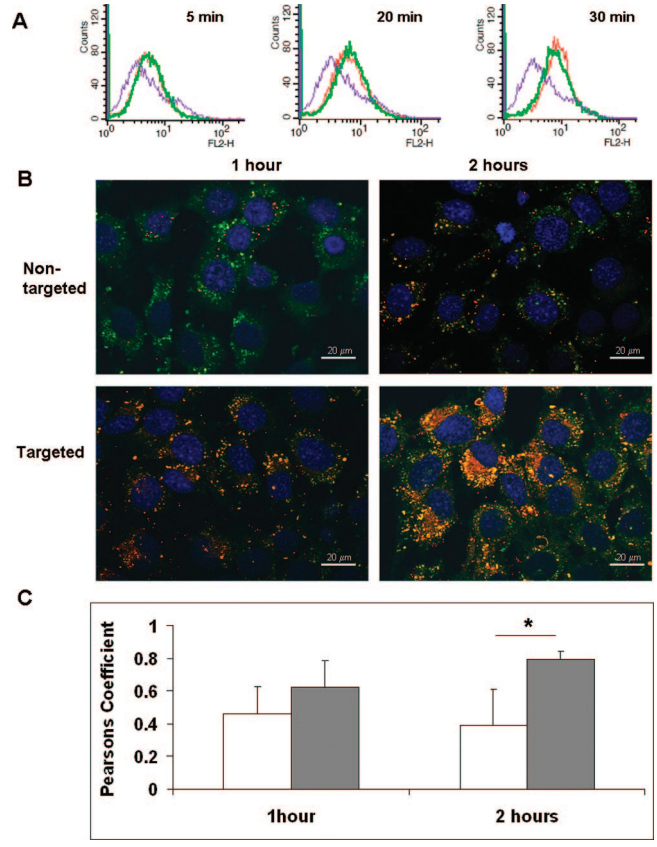

Figure 1. Interaction of liposome formulations with cells. (A) Flow cytometric analysis of nanocarrier binding to 4T1 mouse mammary carcinoma cells: Nanocarriers prepared with rhodamine labeled-PE and either 1.5 mol % DOTAP or 1.5 mol % STPP were incubated with 4T1 Cells for 5, 20 or 30 min and the amount of cell associated fluorescence after washing was measured by flow cytometry. Purple line shows unstained cells, red line shows nontargeted (DOTAP) nanocarriers and 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.)

of Rh-PE delivered by a carrier system (liposomes with 1.5 mol % of the cationic lipid DOTAP instead of STPP) bearing the same surface charge (+30 ± 12 mV) as the STPP-modified formulation being tested.

To study the cell association of the nanocarriers, 4T1 cells grown in flasks were incubated in serum free medium with either Rh-PE containing STPP nanocarriers or DOTAP nanocarriers for 5, 20 and 30 min. After incubation the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. Flow cytometry was performed on a BD FACS Calibur Flow Cytometer and revealed that over the time period analyzed, DOTAP and STPP are identical in their influence on cell association of rhodamine-PE (Figure 1A). To more closely analyze the interaction of the nanocarriers with the cells we used confocal microscopy. 4T1 cells were

grown to 75−85% confluence on 22 mm coverslips in 6 well cell culture plates. The cells were exposed to the 0.5 mol % Rhodamine-PE ($\lambda_{ex} = 550$ nm, $\lambda_{em} = 590$ nm; Avanti Polar Lipids) in nanocarrier preparations for time periods of 1, 2 and 4 h in serum free medium. Following incubation the cells were washed with phosphate buffered saline (PBS) and stained with MitoFluor Green (Molecular Probes/Invitrogen) for 30 min and Hoechst 33342 (Molecular Probes/Invitrogen) as recommended by the manufacturer. The cells were washed with PBS, mounted in Fluoromount G medium and then examined on a Zeiss Meta 510 LSM with Zeiss software. Fluorescence micrographs of the blue, red and green channel within the same field were overlaid and colocalization of red fluorescence of Rh-PE with green fluorescence of stained mitochondria was rendered in yellow using Zeiss confocal imaging software.

Figure 1B shows representative composite confocal fluorescence micrographs that represent the comparative cellular uptake and distribution pattern of Rh-PE mediated by the formulations tested at different time points. Rh-PE fluorescence is seen in red with mitochondria stained green Mitofluor Green and nuclei stained blue with Hoechst 33342. Yellow indicates detected colocalization of Rh-PE fluorescence with the stained mitochondria. As can be seen from Figure 1C, analysis of colocalization (based on ImageJ software analysis of 6 images in each case) clearly shows that at 2 h, the mitochondria-targeted STPP formulation efficiently delivered Rh-PE to the mitochondria (Pearson Coefficient $= 0.792 \pm 0.051$). In contrast, the nontargeted DOTAP formulation was significantly (P two tail <0.005) less specific in delivering Rh-PE to mitochondria (Pearson coefficient $= 0.39 \pm 0.219$). Taken together, the data from flow cytometry and confocal fluorescence microscopy demonstrate that although the surface charge of the nanocarrier may mediate cell association per se, an appropriate targeting ligand on the surface of the nanocarrier can dramatically influence the subsequent subcellular delivery of the drug. Such efficient and specific subcellular delivery mediated by a nanocarrier system is unprecedented and represents a significant achievement in delivery.

Next, to show that such specific delivery to a subcellular compartment can offer a significant improvement in drug action, we used our mitochondria-targeted delivery system with ceramide as a model drug. Ceramide is a sphingolipid signaling molecule that has been shown to mediate a diverse range of biological responses to extracellular stimuli such as proliferation, differentiation, immune response, senescence, and growth arrest.[22–24] It is well-known that mitochondria are the link between the increased levels of ceramide generated by chemotherapeutic drugs and the induction of apoptosis. An increased level of ceramide is necessary for the formation of ceramide channels in the mitochondrial membrane and the subsequent release of cytochrome-C from the mitochondrial intermembrane space.[25,26] More importantly, the formation of these channels and the induction of apoptosis is also prompted by the intracellular delivery of exogenous ceramide.[27] We hypothesized that the specific delivery of ceramide to mitochondria

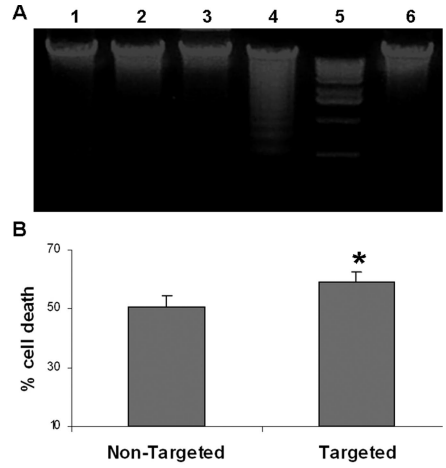

Figure 2. Effect of ceramide formulations on cell death. (A) Agarose gel electrophoresis of total DNA extracts from COLO205 cells treated with various formulations. COLO205 (human cell line) grown to 60% confluence were incubated with the indicated preparations for 18 h, DNA was extracted using DNeasy Tissue Kit obtained from Qiagen. 2 μg of DNA was loaded and run on a 1.4% agarose gel containing 0.5 μg/mL of ethidium bromide. Lane 1: untreated. Lane 2: ceramide in DMSO. Lane 3: ceramide in DOTAP liposomes. Lane 4: ceramide STPP liposomes. Lane 5: 1 kb DNA ladder. Lane 6: empty STPP nanocarrierss. (B) Metabolic toxicity in 4T1 cells ($n = 8$) treated with mitochondria-targeted (targeted), or nontargeted (nontargeted) ceramide concentration of 25 μM. (* indicates a P value of <0.005.) 4T1 cells were grown in 96 well plates to 50−60% confluence and incubated with the preparations for 48 h. Following, incubation the percent cell death $\pm$ standard deviation was determined using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

should lead to an increased apoptotic action in comparison to nontargeted delivery. Figure 2A shows that in our experiments, only cells treated with mitochondria-targeted ceramide undergo apoptosis as indicated by the characteristic DNA ladder that is a definitive indicator of apoptotic cell death.[28] Cells exposed to either free ceramide or ceramide incorporated in a nontargeted formulation did not show any detectable DNA ladder. Thus, specific delivery of ceramide to the mitochondria elicited a robust apoptotic response while nontargeted delivery failed to do so. Further, mitochondria-specific delivery significantly (P two tail <0.005) improved the metabolic toxicity of a dose of ceramide by almost 20% compared to the nontargeted delivery (Figure 2B). Considering that nontargeted ceramide might elicit a cytotoxic effect by acting on other targets besides mitochondria, the change in cytotoxic response is even more striking.

As the final step in our study we investigated the applicability of mitochondria-targeted delivery in improving the antitumor action of ceramide in vivo. Animal studies were performed with nanocarriers that contained 3 mol % PEG5000-PE. After determining a safe injectable dose, we verified that STPP did not significantly change the expected biodistribution of the nanocarriers in comparison to non-STPP nanocarrier (Figure 3). Most importantly, the tumor accumulation was not changed. These data ruled out any biodistribution-mediated difference in the activity of the mitochondria-targeted ceramide on tumor growth. Any change in the in

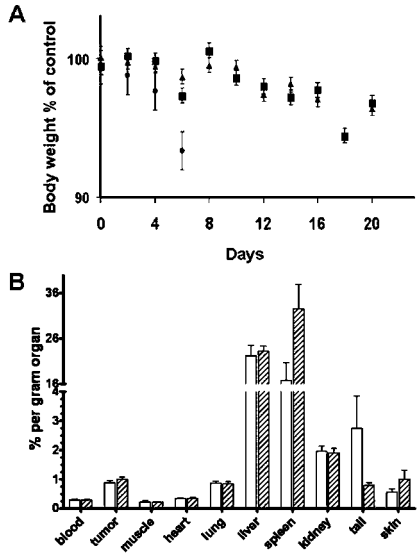

Figure 3. In vivo behavior of STPP nanocarriers. (A) Determination of maximal tolerated dose (MTD) in mice ($n = 6$). 0.45 mg/kg STPP (■), 1.5 mg/kg STPP (▲), 4.5 mg/kg STPP (●). Female Balb/C mice (Charles River Laboratories) were divided into groups of 6 mice. STPP nanocarriers containing STPP equivalent to 0.045, 01.15, 0.4.5 and 1.5 mg/kg were injected in vivo (100 μL/mouse via tail vein) on alternate days for a total of 10 doses over a 21 day period. The animals were monitored for changes in body weight, hydration, ataxia and abnormal behavior. Doses of STPP were considered safe if none of the mice in the group demonstrated any adverse effects during the 21 day test period. (B) Biodistribution of nontargeted carriers (open bars) and STPP nanocarrier (shaded bars) ($n = 6$). STPP liposomes labeled with ^{111}In were prepared by the inclusion of 3 mol % PEG5000PE and 0.5 mol % DTPA-PE during preparation. Following the preparation of the liposomes containing DTPA-PE, liposomes-DTPA-PE were supplemented with 1 M citrate buffer and incubated for 1 h with ^{111}In−citrate complex at room temperature, and then dialyzed overnight against HBS at 4 °C to remove free label. PEGylated liposomes containing no STPP were used as a control. Female C57BL mice bearing LLC (Lewis Lung Carcinoma) tumors were injected in vivo (via tail vein) with 5 μCi of the radiolabeled liposome dispersions (5 animals per group). Animals were sacrificed after 24 h and the indicated organs were removed, weighed and the radioactivity quantified as CPM ± standard deviation using a Beckman 5500B gamma-counter.

vivo activity of ceramide incorporated in STPP-modified nanocarriers is expected to be due to mitochondria-specific delivery in the tumor cells and not due to any changes in the amount of ceramide accumulating in the tumor area. Female BALB/c mice (Charles River Laboratories) were inoculated by subcutaneous injection in the right hind flank region with mouse mammary carcinoma 4T1 cells for the generation of tumors. Upon formation of measurable tumors, the mice were divided into 3 groups of 5 mice each for injection. The injections of a dose of liposomal ceramide equal to 6 mg/kg were repeated at a frequency of one injection per two days as previously reported.[13] Tumor growth was measured over the period of the injections and the tumor volumes of the groups compared.

Figure 4A shows the tumor volumes recorded over the course of the tumor growth inhibition study. In the case of buffer-treated as well as empty STPP-modified nanocarrier-treated groups 50% of the animals developed necrotic morbidity at day 12 and had to be euthanized as required by

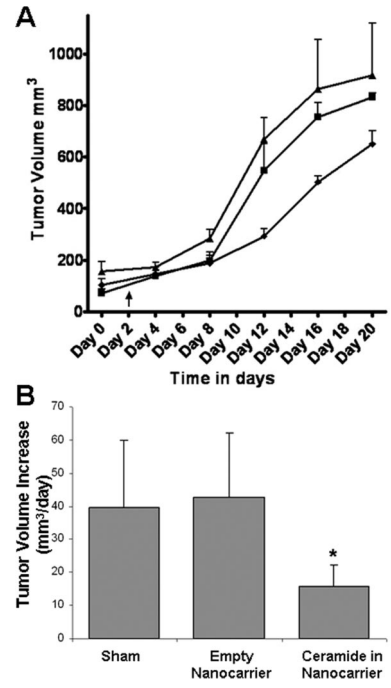

Figure 4. Tumor growth inhibition. (A) Tumor volume (mm^3 ± standard deviation) measured over time period of treatment in Balb/c mice bearing murine 4T1 mammary carcinoma tumors ($n = 6$): after treatment with buffer (■), empty STPP nanocarrier (▲), and ceramide in STPP nanocarrier (♦). The arrow indicates the start of treatment. (B) Tumor growth in mm^3/day ± standard deviation at day 12. ($n = 6$) (* indicates a P value of <0.05.)

institutional humane treatment policy. All mice treated with ceramide in STPP-modified nanocarrier showed no morbidity even at 18 days. Statistical analysis of tumor growth rate at the 12 day time point ($n = 6$) showed that the treatment with ceramide in STPP-modified nanocarrier significantly inhibited tumor growth rate compared to sham treatment (Figure 4B). Further, there was no significant reduction in tumor growth rate upon treatment with empty STPP-modified nanocarriers when compared to sham treatment indicating that STPP-modified nanocarriers alone have no antitumor effect. Finally, the dose used in our study is 6 mg/kg 6 times less than effective doses (36 mg/kg up to 72 mg/kg) reported in previous studies[29] and even at this low dose we saw a significant reduction in tumor growth rate. Taken together the improved animal survival and the retardation of tumor growth rate suggest a notable improvement in the action of ceramide as a result of intracellular delivery by the mitochondria-targeted nanocarrier.

In summary, we have clearly demonstrated that pharmaceutical nanocarriers can be targeted to subcellular compartments. Such nanocarriers offer a significant benefit because they allow the specific delivery of drugs to subcellular targets without the need for chemical modification of the drug molecules. Most importantly, we have shown that such organelle-specific drug-loaded nanocarriers can significantly enhance therapeutic effect. With suitable ligands, the strategy described herein could be applied to other organelle targets, thereby offering improved therapy for a number of diseases associated with organelle dysfunction.

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