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Surface modification of liposomes with rhodamine-123-conjugated polymer results in enhanced mitochondrial targeting

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Abstract

A novel mitochondrial-targeted liposomal drug-delivery system was prepared by modification of the liposomal surface with a newly synthesized polymer, rhodamine-123 (Rh123)-PEG-DOPE inserted into the liposomal lipid bilayer. This novel polymer was synthesized by conjugating the mitochondriotropic dye Rh123, with the amphiphilic polyethylene glycol–phosphatidylethanolamine (PEG-PE) conjugate. The modified liposomes showed better uptake by cells (HeLa, B16F10) estimated by fluorescence microscopy and FACS analysis. The co-localization study with stained mitochondria as well as with the isolation of mitochondria of the cultured cells after their treatment with Rh123 liposomes showed a high degree of accumulation of the modified liposomes in the mitochondria. We also prepared mitochondrial-targeted and nontargeted paclitaxel (PCL)-loaded liposomes. Mitochondrial-targeted PCL-loaded liposomes demonstrated enhanced cytotoxicity toward cancer cells compared with nontargeted drug-loaded liposomes or free PCL. Thus, Rh123-modified liposomes target mitochondria efficiently and can facilitate the delivery of a therapeutic payload to mitochondria.

Keywords

Liposomes; rhodamine-123; amphiphilic polymer; mitochondrial targeting; drug delivery; paclitaxel; cytotoxicity

Introduction

Delivery of biologically active molecules including small molecules into individual subcellular compartments, such as mitochondrial, lysosomal, nuclear, is required to achieve better therapeutic activity and lesser side effects compared with traditional random intracellular distribution of bioactives (Torchilin, 2006; Boddapati et al., 2008). However, the specific subcellular delivery of bioactive molecules is still a challenging issue. One possible approach is the conjugation of a drug molecule with another compound having a specific affinity toward the organelle of interest. Even more productive may be the modification of a drug-loaded pharmaceutical (nano) carrier with such compound, since chemical modification of the drug itself is not required.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Recent progress in genetics and molecular biology has provided some insight into the molecular mechanism of many human disorders. In particular, it has become evident that mitochondrial dysfunction is responsible for a variety of human diseases, including neurodegenerative and neuromuscular ones, obesity, diabetes, ischemic–reperfusion injury, and cancer (Holt et al., 1988, 1990; Wallace, 1999; Schon and DiMauro, 2003; King et al., 2006; Yamada et al., 2007). In addition to supplying cellular energy via the production of ATP, mitochondria are involved in a range of other cellular processes such as signaling, differentiation, cell death, as well as control of the cell cycle and cell growth (Borutaite, 2010; Norberg et al., 2010). Thus, the mitochondrion represents a clinically important intracellular target.

Certain small molecules target mitochondria due to the high mitochondrial negative membrane potential (Horobin et al., 2007). Lipophilic cations, such as triphenylphosphonium (TPP), accumulate in mitochondria. Thus, in earlier studies on mitochondrial-specific delivery strategies, the potential of pharmaceutical nanocarriers modified with cationic compounds for specific delivery of drugs and DNA to mitochondria was explored (D'Souza et al., 2003; Boddapati et al., 2005; Boddapati et al., 2008; Patel et al.). Conjugating mitochondriotropic TPP cation with lipophilic stearyl moiety and incorporating the resulting amphiphilic stearyl TPP (STPP) into the lipid bilayer of liposomes made the liposome mitochondriotropic (Boddapati et al., 2008). Incorporation of drugs, such as paclitaxel (PCL) or ceramide, which act at the mitochondrion, into the mitochondrial-targeted nanocarrier enhanced the drug's action (Boddapati et al., 2008). Here, we tested the mitochondriotropic dye rhodamine-123 (Rh123) as a possible mitochondrial-targeting ligand in a liposomal drug carrier. With this in mind, Rh123 was conjugated with the amphiphilic polymer polyethylene glycol–phosphatidylethanolamine (PEG-PE). The PE moiety should provide an efficient incorporation of the resulting Rh123-PEG-PE conjugate into the liposome membrane, whereas PEG moiety served a spacer arm to allow sufficient freedom for Rh123 interaction with the mitochondria. This construct should result in enhanced accumulation of modified (drug-loaded) liposomes in mitochondria.

Materials and methods

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG₂₀₀₀-PE), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt, Rh123-PE), egg L- α -phosphatidylcholine (Egg PC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-DOPE) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. *p*-Nitrophenyloxycarbonyl (*p*NP-PEG₂₀₀₀-PE) was synthesized according to a previously reported procedure (Torchilin et al., 2001). Cholesterol, Rh123, chloroform-*d*, and Sepharose CL-4B (40–165 μ M) were purchased from Sigma (St. Louis, MO). Sephadex G25 superfine medium was from GE Healthcare Biosciences Corp. (Piscataway, NJ). The mitochondrial isolation kit for cultured cells and the BCA protein assay kit were purchased from Pierce (Rockford, IL). Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). Fluoromount-G was obtained from Southern Biotech (Birmingham, AL).

For cell culture, human cervical carcinoma HeLa cells and murine melanoma cells B16F10 were purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin–amphotericin B solution were purchased from CellGro (Kansas City, MO). Cells were grown in DMEM supplemented with 10% FBS, 100 IU/mL of penicillin/streptomycin, and 250 ng/mL

amphotericin B at 37°C and 5% CO₂. Mitotracker deep red FM and Hoechst 33342 were purchased from Molecular Probes Inc. (Eugene, OR). Trypan blue solution was obtained from Hyclone (Logan, Utah).

Methods

Synthesis, purification, and characterization of the amphiphilic polymer

Rh123-PEG₃₄₀₀-DOPE—pNP-PEG-DOPE was prepared according to a previously reported procedure with some modifications (Torchilin et al., 2001). In brief, 24 mg (32.2 μM) of DOPE in chloro-form were added to a solution of 1 g of NPC-PEG₃₄₀₀-NPC in chloroform followed by 80 μL of triethylamine. The reaction mixture was stirred overnight at room temperature in a nitrogen atmosphere. Chloroform was removed completely with a rotary evaporator and freeze-drier. The crude reaction mixture was hydrated with water containing 0.01 M HCl, 0.15 M NaCl using water bath sonication and passed through a size exclusion chromatography column (CL-4B) to form pNP-PEG-PE micelles. The additional column chromatography was performed 2–3 times for pooled fractions from the previous column. The initial pure fractions of pNP-PEG-PE were pooled and freeze-dried. The powder was suspended in chloroform, sonicated, filtered, and evaporated to yield pure pNP-PEG-PE.

Rh123 (1 mg, 2.63 μM) dissolved in 25 μL of methanol and 10 μL of triethylamine were added to the solution of pNP-PEG₃₄₀₀-DOPE (5 mg, 1.14 μM) in 500 μL of chloroform. The reaction mixture was stirred for 4 h at room temperature under nitrogen. The chloroform was removed, and the reaction mixture was freeze-dried. The crude reaction mixture was dissolved in water and purified by dialysis against deionized water using a cellulose ester membrane for 2 days (MWCO 1 kDa; Spectrum Medical Industries, Houston, TX) with 4–5 times changing water. The contents of the dialysis bag were freeze-dried, weighed, dissolved in chloroform at 5 mg/mL, and kept at –80°C for future use.

Rh123-PEG₃₄₀₀-DOPE (Rh123-PEG-PE) was dissolved in chloroform-*d* and analyzed by ¹H-NMR using a Varian 500 MHz spectrophotometer.

Preparation and characterization of liposomes—Liposomes were prepared by the lipid film hydration technique. In brief, a dry lipid film was prepared from mixture of Egg PC, cholesterol, and Rh123-PEG-DOPE (Egg PC:Ch:Rh123-PEG-DOPE = 69:30:1) or Egg PC, cholesterol, PEG₂₀₀₀-PE, and NBD-PE (Egg PC:Ch: PEG₂₀₀₀-PE:NBD-PE = 68.24:30:1:0.78) by rotary evaporation of the chloroform solution of the combined ingredients followed by freeze-drying (Labconco, Freeze dry system, Freezone) for at least 4 h to remove traces of solvents. The dry lipid film was hydrated in phosphate-buffered saline (PBS) at pH 7.4 and at a 4 mg/mL lipid concentration. The hydrated mixture was vigorously vortexed for 5 min to make multilamellar vesicles from the dry film and subsequently extruded 20 times through a 200-nm pore size polycarbonate membrane filter (Avanti Polar Lipids Inc.) to yield liposomes of uniform particle size. The liposomes were dialyzed overnight against PBS, pH 7.4, using cellulose ester membrane (MWCO 14 kDa) to remove unincorporated materials. For all experiments measuring fluorescence intensity, the plain liposomes (PL) were modified with the fluorescent lipid NBD-PE. The fluorescence of both formulations was adjusted to a comparable same level each time before performing the fluorescence experiments and read using the filter (ex. 485/20, em. 528/20) in fluorescent spectrophotometer. A standard curve was constructed of PLs prepared with different mole fractions of NBD-PE. The fluorescence of liposomes modified with 1 mol% Rh123-PEG-DOPE matched the fluorescence of PL modified with 0.78 mol% NBD-PE. PCL-loaded liposomes were also prepared by hydrating the lipid film consisting of Egg PC, Chol, Rh123-PEG-PE (at 69:30:1) and PCL for liposomes and Egg PC:Chol:PEG₂₀₀₀-PE (at

69:30:1) and PCL for nontargeted liposomes. In both the formulations, PCL in a methanol solution was added into the chloroform solution as 1% w/w of the amount of Egg PC in the formulation.

Preparation of PCL-loaded Rh123-L and PL—PCL stock solution in methanol (0.5 mg/mL) at concentration of 1% w/w of the total lipid was added to a chloroform solution of the other liposomal ingredients. The solvent was removed by rotary evaporation and freeze-drying. The rest of the procedure was same as described before. The unincorporated PCL was removed by filtration of the liposome suspension through a 0.2- μ m nylon membrane (Millipore Co., Bedford, MA).

Liposome size and zeta potential (ζ)—The liposome size and size distribution were measured by dynamic light scattering (DLS) using a Zeta Plus Instrument (Brookhaven Instrument Corporation, Holtsville, NY). The zeta potential of formulations was measured by a zeta phase Analysis Light Scattering (PALS) system with an ultrasensitive zeta potential analyzer (Brookhaven Instruments, Holtsville, NY). The liposome suspension was diluted as needed with a 1 M KCl solution. All zeta potential measurements were performed in triplicate (Table 1).

Measurement of PCL load in Rh123-L and PL—The content of PCL in Rh123-L and PL was measured by reverse phase HPLC (D-7000 HPLC system, Hitachi, Japan) using an Xbridge column (C18, 4.6 mm $\times$ 250 mm; Waters, Milliford, MA). An aliquot of liposomes was dissolved in a solvent mixture of acetonitrile:water (7:3) and then injected into the HPLC system using acetonitrile:water (7:3) as the mobile phase at flow rate 1 mL/min with detection at 227 nm. Each run was done in triplicate. The PCL loading was determined using a calibration curve (AUC versus concentration of PCL solution injected) obtained in the same conditions using known concentrations of PCL in the same solvent. The correlation factor for the standard curve (R^2) was 0.99 (data not shown). The loading was determined as follows:

$$\% \text{ of PCL loading} = \frac{\text{Amount of PCL in liposomes obtained by HPLC analysis}}{\text{Amount of PCL added at the time of preparation}} \times 100$$

Concentration of the liposomal solution for cell studies was determined depending on the loading in PL and Rh123-L.

Stability of liposomes—Stability of Rh123-L was monitored over a time period of 3 months at 4°C. Changes in liposome size and size distribution were followed using DLS instrument.

Cellular uptake of liposomes by FACS analysis—In order to assess the cell-targeting capacity of nanocarriers, HeLa and B16F10 cells incubated with liposomal formulations (Rh123-L and PL) were subjected to FACS analysis. Approximately 200–300,000 HeLa and B16F10 cells/data point in triplicate were incubated in the presence of a test formulation at a lipid concentration 0.2 mg/mL in serum-free media for 15 min, 30 min, and 1 h at 37°C. The cells were centrifuged, and washed with ice-cold PBS at least three times before analysis using a BD FACS Caliber flow cytometer. The cells were gated using forward versus side scatter to exclude debris and dead cells before analyzing in FACS with 10,000 cell counts. The data were analyzed with BD Cell Quest Pro software.

Liposomal internalization by cell lysis method—HeLa (90,000 cells/well) and B16F10 (75,000 cells/well) cells were grown in 12-well plates. Twenty-four hours after the cell seeding, cells were incubated with Rh123-L or PL at a total lipid concentration of 0.2 mg/mL for 1, 2, or 4 h in serum-free media. The cells were washed with PBS, incubated with 350 μ L of 5 N NaCl solution for 15 min, and pipetted vigorously to facilitate cell detachment and lysis. The fluorescence intensity was measured using a multidetection microplate reader (Bio-Tek, Winooski, VT) using 485/528 nm excitation/emission wavelengths. Protein content of each well was determined by BCA protein assay following the manufacturer's protocol (Pierce).

Fluorescence microscopy—To analyze the interaction of nanocarriers with the cells and assess the mitochondrial-targeting property of the nanocarriers, fluorescence microscopy was performed using a Zeiss Meta 510 LSM confocal microscope. HeLa cells were grown to 75–80% confluence on 22-mm coverslips in six-well cell culture plates. The cells were exposed to Rh123-L and PL in serum-free media for 2 h and incubated with Mitotracker deep red FM for 0.5 h at a concentration of 100 nM and Hoechst 33342 for 5 min at 5 μ g/mL. The cells were washed several times with PBS and fixed with a 4% paraformaldehyde solution. The cells were mounted on Fluoromount-G medium and examined on a Zeiss Meta 510 LSM confocal microscope. The data were analyzed by ImageJ software. Fluorescent micrographs of the blue, green, and deep red channels within the same field were overlaid so that co-localization of the green fluorescence of Rh123-L and PL with deep red fluorescence of stained mitochondria was rendered yellow using ImageJ software.

Mitochondrial isolation and analysis of fluorescence—Mitochondria were isolated from HeLa cells following the manufacturer's protocol. HeLa cells were grown in T-150 flasks to 85–90% confluence. Twenty-five to 30 million cells were used for each data point. Control, PL, Rh123-L samples were tested in duplicate and the full experiment was performed three times. The isolated mitochondrial pellet was dissolved in 120 μ L of CHAPS buffer (2% CHAPS in TBS, pH 7.2). The protein content of the mitochondrial fractions was determined using the BCA protein assay kit (Pierce).

Cytotoxicity assay—To indirectly evaluate the ability of Rh123-L to deliver PCL into the target site, we determined cell viability using the trypan blue exclusion assay. PCL was incorporated as 1% w/w of the total lipid in the liposomes. The loading of PCL in Rh123-L and PL were determined by HPLC using reverse phase C18 column as described in the "Preparation and Characterization of Liposomes" section. HeLa cells were seeded in 12-well cell culture plates (80,000 cells/well). After 24 h, cells were incubated with PCL-Rh123-L, PCL-PL, or free PCL at concentrations of 1.5 and 0.5 μ g/mL of PCL for 4 h in serum-free media. The media containing the formulation was removed; the cells were washed and incubated in complete media overnight before performing the cytotoxicity assay. Cells were trypsinized (300 μ L), supplemented with serum (100 μ L) to neutralize trypsin action, and treated with trypan blue (50 μ L). Live cells were counted three times using hemo-cytometer and averaged. The experiment was performed three times in triplicate.

Results and discussion

Synthesis of the amphiphilic conjugate

Rh123 was linked with the PEG chain of amphiphilic polymer PEG-PE via amide bond formation (Figure 1). The polymer was characterized by ^1H -NMR in *d*-chloroform using a Varian 500 MHz spectrometer.

$^1\text{H-NMR}$: δ 0.86–0.92 (t, 6H, $[\text{CH}_2]_n\text{-CH}_3$)₂ of lipophilic chain in phospholipid), 1.28–1.31 (m, $[\text{C H}_2]_n\text{-CH}_3$)₂ of DOPE), 2.01–2.02 (m, 8H, $[\text{C H}_2\text{-CH=CH-CH}_2]$ ₂ of DOPE), 2.29–2.32 (m, from DOPE), 2.38–3.85 (m, PEG, DOPE), 4.06–4.48 (2 bs, 1 m, DOPE), 5.25 (bs, 1H, $\text{-O-CH}_2\text{-CH(CH}_2\text{-)-O-}$ of DOPE), 5.35–5.37 (m, 4H, $[\text{CH}_2\text{-CH=CH-CH}_2]$ ₂, 6.91–7.07 (m, 4H, Ar-*H* of Rh123), 7.41–7.43 (m, 2H, Ar-*H* of Rh123), 7.66–7.80 (m, 1H, Ar-*H* of Rh123), 8.07–8.08 (d, $J = 8.5$ Hz, Ar-*H* of Rh123), 8.30–8.32 (d, 2H, $J = 9.0$ Hz, Ar-*H* of Rh123). The presence of new peaks in the aromatic region from the aromatic protons of Rh123 indicated successful conjugation.

Characterization of liposomes

The synthesized amphiphilic copolymer Rh123-PEG-PE was incorporated into the liposomal lipid bilayer. For cell-based experiments, the surface modification of liposomes with 1 mol% of Rh123 polymer was enough to impart a targeting effect. Size distribution, mean diameter, and zeta potential values for targeted and nontargeted liposomes as well as for PCL-loaded targeted and nontargeted liposomes were all rather similar (Table 1 and Figure 2).

FACS analysis of cellular uptake of liposomes

Uptake of liposomes by HeLa and B16F10 cells was performed under the same conditions. In all fluorescence experiments, PL were modified with the green fluorescence marker NBD-PE, and the fluorescence of PL was normalized prior to cell treatment by adjustment of the amount of NBD-PE in the liposomal formulation to produce an equal intensity of fluorescence with the Rh123-L. The fluorescence intensity of PL modified with 0.78 mol% of NBD-PE corresponded to the fluorescence intensity of Rh123-L modified with 1 mol% of Rh123-PEG-PE.

Flow cytometry data revealed a significantly higher uptake of Rh123-L as little as 15 min of treatment compared with PL (Figure 3). The targeted liposomes were internalized in significantly higher amounts at all time points compared with PL ($P < 0.001$). A time-dependent increase in cell-associated fluorescence of Rh123-L occurred in both cell lines as seen from the comparison of the geometric mean of fluorescence at the three time points of 15, 30 min, and 1 h (Figure 4A and 4B). However, no time-dependent increase occurred in the uptake of PL after 1 h.

Estimation by cell lysis

Cellular internalization of formulations was also studied with a cell lysis assay after cell treatment with liposome preparations for 1, 2, and 4 h, and subsequent analysis of samples by fluorescence spectroscopy of HeLa and B16F10 cell lines (Figure 4). The data are presented as fluorescence intensity/microgram of protein versus the incubation time. The uptake pattern was similar to the result obtained using FACS analysis. Rh123-L were taken up by cells in a significantly higher amount than by PL at all time points. In fact, the uptake of PL did not change significantly over 4 h time period in both the cell lines. However, it was not certain whether the formulation was associated with the cell membrane or internalized and directed into the subcellular compartments.

Confocal microscopy

To assess more closely the intracellular trafficking of the liposomal formulations, confocal microscopy was performed with stained mitochondria and co-localization of the labeled formulations was followed with mitotracker. Overlaid multichannel confocal fluorescence micrograph showed significantly higher co-localization of Rh123-L in the mitochondria compared with PL as indicated by yellow color in the merged picture of deep red-stained

mitochondria and green fluorescence-labeled formulations in the same field (Figure 5). Analysis of co-localization (ImageJ software) showed significant accumulation of targeted liposomes in the mitochondria (Pearson's coefficient 0.55, Mander's coefficient 0.75 for Rh123-L compared with Pearson's coefficient -0.083, Mander's coefficient 0.007 for PL). Thus, the confocal microscopy co-localization data strongly supports our hypothesis that the Rh123-conjugated pharmaceutical nanocarrier targets mitochondria.

Mitochondrial isolation

Mitochondria were isolated from the cultured HeLa cells after Rh123-L and PL treatment for 4 h, and mitochondrial-associated fluorescence was measured (Figure 6). Significantly higher fluorescence occurred in the mitochondrial fraction isolated after Rh123-L treatment compared with control mitochondria and PL-treated mitochondria, which again clearly demonstrated the mitochondrial-targeting property of the Rh123-L formulation. The fluorescence of the cytosol fraction was also significantly higher in Rh123-L-treated cells compared with PL-treated ones.

Cytotoxicity

To demonstrate that specific delivery of the drug to the desired subcellular compartment can significantly enhance drug action, the mitotic inhibitor PCL was used since much evidence of PCL acting on and altering the function of mitochondria to produce apoptotic action have been observed (André et al., 2002; Kidd et al., 2002; Ferlini et al., 2009). PCL-Rh123-L treatment at PCL concentration 1.5 $\mu\text{g/mL}$ produced significantly higher cytotoxicity than free PCL and PCL-PL (Figure 7). The 35–40% reduction of cell survival was observed with PCL-Rh123-L compared with nontargeted PCL formulations.

PCL is also well-known as microtubule-destabilizing agent (Schiff et al., 1979). However, apart from its interaction with beta tubulin, PCL also activates the intrinsic mitochondrial apoptotic pathway leading to opening of the permeability transition pore channel (PTPC) and subsequent release of proapoptotic factors, which ultimately leads to apoptosis (Bhalla, 2003; Ferlini et al., 2009). PCL directly inhibits the anti-apoptotic protein BCL-2 in the loop domain that participates in the regulation of PTPC (Shimizu et al., 1998) and thereby facilitates the initiation of apoptosis. Therefore, much evidence indicates a possible apoptotic action of PCL mediated by alteration of mitochondrial function. This study demonstrated the efficient delivery of the therapeutic cargo PCL into the intracellular site of action as evidenced by remarkable increase in PCL-associated cytotoxicity with mitochondrial-targeted PCL-Rh123-L.

Conclusions

We successfully conjugated a mitochondriotropic dye Rh123 with amphiphilic polymer PEG-PE and incorporated the modified polymer into a liposomal drug-delivery system and achieved its targeting to mitochondria. Our results showed that the uptake of targeted liposomes was significantly higher than that of nontargeted PL at all time points as determined by FACS analysis or by cell lysis method.

We assessed the ability of the targeted liposomes to efficiently deliver a therapeutic cargo into the mitochondrial subcellular compartment and determined the cytotoxicity of PCL delivered to the cells by targeted and nontargeted formulations. The result strongly supports the mitochondrial-targeting property of Rh123-L as evident from the enhanced cytotoxicity.

Overall, our results demonstrate that surface modification of liposomes with the novel amphiphilic Rh123-PEG-PE copolymer not only facilitates cellular association and

internalization, but also directs the trafficking of the nanoparticles to a specific subcellular compartment, mitochondria. The substantial increase in the cell killing ability of PCL-Rh123-L compared with PCL-PL confirms the efficient delivery of PCL into the targeted site of PCL's action and suggests that this novel liposomal formulation could serve more generally as mitochondrial-targeted drug-delivery system.

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Figure 1.
Synthesis of Rh123-PEG₃₄₀₀-DOPE.

Figure 2. Size distribution of PL, Rh123-L, PCL-PL, and PCL-Rh123-L. No significant change in size distribution was observed after PCL incorporation.

Figure 3.

Cell uptake of Rh123-L and PL at different time points using two different cell lines (HeLa and B16F10) by flow cytometry. Cells were incubated with the Rh123-L or PL at lipid concentrations of 0.2 mg/mL for 15 min, 30 min, and 1 h. After incubation, cells were washed to remove unbound formulations. Cell-associated fluorescence was measured by FACS. A significant increase in the uptake of Rh123-L occurred compared with PL.

Figure 4.

Cellular internalization of Rh123-L and PL in HeLa and B16F10 cells. Geometric mean fluorescence obtained from flow cytometry data of (A) HeLa cells and (B) B16F10 cells. Cellular internalization estimated from the fluorescence after incubation for 1, 2, and 4 h with Rh123-L or PL followed by cell lysis in (C) HeLa cells and (D) B16F10 cells by the cell lysis method. High internalization of Rh123-L occurred compared with PL. Two-way ANOVA analysis followed by Bonferroni post-comparison test showed that the difference was statistically significant. *** $P < 0.001$, $n = 3$.

Figure 5.

Intracellular co-localization by fluorescence confocal microscopy. (A) HeLa cells treated with PL and (B) cells treated with Rh123-L. Left panel: Cell treatment with PL or Rh123-L in the green channel (ex. 505 nm, em. 530 nm); Middle panel: Cell staining treatment for visualization of mitochondria with Mitotracker deep red in the deep red channel (ex. 644 nm, em. 665 nm); Right panel: Merged left and middle panels and Hoechst-stained nuclei (blue channel, ex. 385 nm, em. 470 nm). Yellow color indicates co-localization of mitochondria and Rh123-L. Analysis of co-localization (ImageJ software) confirmed significant accumulation of targeted liposomes in the mitochondria (Pearson's coefficient 0.55, Mander's coefficient 0.75 for Rh123-L compared with Pearson's coefficient -0.083, Mander's coefficient 0.007 for PL).

Figure 6.

Fluorescence associated with isolated mitochondrial fractions of HeLa cells treated with Rh123-L or PL for 4 h. One-way ANOVA analysis was performed followed by Bonferroni's multiple comparison test that indicated that differences of control versus Rh123-L and PL versus Rh123-L were statistically significant (***) ($P < 0.05$) ($n = 3$).

Figure 7.

Cytotoxicity of PCL in targeted and nontargeted formulations on HeLa cells by trypan blue assay. Cells were treated with free PCL, PCL-PL, or PCL-Rh123-L for 4 h followed by 24 h incubation before assessment of cell viability. Two-way ANOVA analysis was performed followed by Bonferroni's post-hoc test that showed a significant difference between free PCL and PCL-Rh123-L at 0.5 and 1.5 μg/mL doses (** $P < 0.01$, * $P < 0.05$, respectively). Comparison of PCL-PL versus PCL-Rh123-L at a 1.5 μg/mL PCL dose was statistically significant (** $P < 0.01$) ($n = 3$).

Table 1

Properties of liposomes.

Liposomes	Mean diameter $\pm$ SD (nm), (<i>n</i> = 3)	Polydispersity index $\pm$ SD (<i>n</i> = 3)	Zeta potential $\pm$ SD (mV), (<i>n</i> = 3)	Loading efficiency of PCL (%)
PL	174.9 $\pm$ 34.9	0.039 $\pm$ 0.05	−18.27 $\pm$ 3.72	na
Rh123-L	190.9 $\pm$ 43.7	0.066 $\pm$ 0.01	−17.93 $\pm$ 2.41	na
PCL-PL	163.9 $\pm$ 37.6	0.072 $\pm$ 0.04	−18.78 $\pm$ 1.32	83 $\pm$ 1.05
PCI-Rh123 -L	188.2 $\pm$ 43.2	0.071 $\pm$ 0.03	−16.23 $\pm$ 0.39	92 $\pm$ 1.20