Published in final edited form as:

J Drug Target. 2011 September ; 19(8): 606–614. doi:10.3109/1061186X.2010.550921.

Targeting of lysosomes by liposomes modified with octadecyl-rhodamine B

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Abstract

The use of lysosome-targeted liposomes may significantly improve a delivery of therapeutic enzymes into lysosomes for the treatment of lysosome-associated diseases. The aim of this research was to achieve a specific intracellular targeting of lysosomes, by using liposomes modified with the lysosomotropic octadecyl-rhodamine B (RhB) and loaded with a model compound, fluorescein isothiocyanate (FITC)–dextran (FD). Plain and RhB-modified liposomes were prepared by hydration of lipid films and loaded with FD or with 5-dodecanoylamino fluorescein di- β -D-galactopyranoside (C₁₂FDG), a specific substrate for the intralysosomal β -galactosidase. The delivery of these liposomes and their content to lysosomes in HeLa cells was investigated by confocal microscopy, flow cytometry, and subcellular fractionation. Confocal microscopy demonstrated that RhB-liposomes co-localize well with the specific lysosomal markers, unlike plain liposomes. The comparison of the FITC fluorescence of the lysosomes isolated by subcellular fractionation also showed that the efficiency of FD delivery into lysosomes by RhB-modified liposomes was significantly higher compared with plain liposomes. These results were additionally confirmed by the flow cytometry of the intact cells treated with C₁₂FDG-loaded liposomes that also demonstrated increased lysosomal targeting by RhB-modified liposomes. The modification of the liposomal surface with a lysosomotropic ligand, such as octadecyl-RhB, can significantly increase the delivery of liposomal loads to lysosomes.

Keywords

Lysosomal targeting; liposomes; rhodamine B; enzyme replacement therapy

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Declaration of interest

This research was funded by the NIH grant RO1 CA128486 to Vladimir P. Torchilin. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Introduction

Specific delivery of therapeutic agents to individual cell organelles by pharmaceutical carriers is still mainly an unresolved task (Torchilin, 2006), although some attempts have been made to target mitochondria and nuclei using liposomes modified with mitochondriotropic (Boddapati et al., 2008) or nucleotropic (Ko et al., 2009) agents.

Lysosomes, acidic organelles responsible for recycling of cellular constituents, represent another important intracellular target. First, there exists a set of >40 inherited lysosomal storage diseases (LSD) associated with the deficiency of certain lysosomal enzymes, which lead to accumulation of corresponding substrates in lysosomes (Futerman and van Meer, 2004). Taken together, these diseases pose a serious medical problem (Grabowski, 2008; van der Ploeg and Reuser, 2008; Zarate and Hopkin, 2008). The main approach for the treatment of LSD is the enzyme replacement therapy (ERT) based on the administration of exogenous enzymes (Grabowski and Hopkin, 2003). This procedure still remains limited and expensive because of poor delivery and low stability of therapeutic enzymes. The use of liposome-immobilized enzymes opened new opportunities for ERT, which was understood long ago (Gregoriadis, 1978), especially in the treatment of diseases localized in liver cells, which are natural targets for liposomes. Still, liposome-based preparations are not in general clinical use for ERT, and there is a clear need to sharply increase the efficiency of the delivery of the lysosomal enzymes to lysosomes inside cells, that is to achieve lysosomal targeting.

Second, lysosomes are involved in the cellular apoptosis due to the lysosome-dependent cell death pathway (Kirkegaard and Jäättelä, 2009). Previously, it has been proven that a moderate permeabilization of lysosomal membranes can result in the cell apoptosis of cancer cells (Boya et al., 2003). Thus, cancer therapy also may require lysosome-targeted carriers for delivery of lysosome-destabilizing agents causing cancer cell apoptosis.

Here, we attempted to develop a lysosome-targeted drug delivery system based on liposomes modified with a lysosome-specific ligand assuming that the application of such liposomes should significantly increase the efficacy of the liposomal drug delivery to lysosomes.

From the variety of molecules identified to preferentially target and accumulate in lysosomes, we have chosen octadecyl derivative of rhodamine B (RhB), which is used to monitor membrane fusion (Hoekstra et al., 1984) and to study lysosomal metabolism (Kuwana et al., 1995). As has been demonstrated by von Steyern et al., RhB specifically accumulates in the lysosomes of denervated skeletal muscle (Vult von Steyern et al., 1996). Novel acidic fluorescent probes based on RhB have also been described and used for the optical imaging of the intracellular H^+ (Zhang et al., 2009).

We hypothesized that the application of liposomes modified with a specific ligand capable of targeting lysosomes will significantly increase the efficacy of the liposomal delivery to lysosomes. To check this hypothesis, we prepared liposomes loaded with the model compound, fluorescein isothiocyanate (FITC)-dextran (FD), and modified with RhB and

investigated their intracellular delivery and distribution as well as the efficiency of delivery of FD into lysosomes.

Materials and methods

Materials

Egg phosphatidylcholine and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. FD (4000 MW), octadecyl-RhB, β -galactosidase enzyme (product number G0413), and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). BioGel A1.5M was purchased from Bio-Rad (Hercules, CA). Lysosome Enrichment Kit, Coomassie-based protein assay kit, and DyLightTM 350-conjugated goat anti-mouse IgG were obtained from Pierce Biotechnology (Rockford, IL). LysoTracker Red, fluorescein di- β -D-galactopyranoside (FDG), and 5-dodecanoylamino fluorescein di- β -D-galactopyranoside (C₁₂FDG) were purchased from Invitrogen/Molecular Probes, Inc. (Eugene, OR). Mouse monoclonal (H4B4) anti-lysosome-associated membrane protein antibody (anti-Lamp2) was purchased from Abcam (Cambridge, MA). Mounting medium Fluoromount-G[®] was from SouthernBiotech (Birmingham, AL). Human epithelial cervical cancer cells CCL-2 (HeLa) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cell culture media and supplements were from CellGro (Kansas City, MO). All other chemicals and buffer components were of analytical grade preparations. Distilled and deionized water was used in all experiments.

Preparation of liposomes

The lipid films were first obtained from a mixture of ePC and Chol (7:3 molar ratio) in chloroform (Torchilin et al., 2001) and supplemented with RhB in ethanol (1 or 3 mol%). Chloroform was removed on a rotary evaporator followed by freeze-drying on a Freeze Dry System Freezone 4.5 (Labconco, Kansas City, MO). The films were hydrated with vigorous vortexing with 1 mL phosphate-buffered saline (PBS: 137 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM K₂H₂PO₄, pH 7.4) or PBS supplemented with FD (45 mg/mL). To produce liposomes, the hydrated lipid films were extruded 20 times through 200-nm pore size NucleporeTM polycarbonate membranes (Whatman, Clifton, NJ) using an Avanti hand extrusion device (Avanti Polar Lipids, Alabaster, AL). The liposomes and non-incorporated FD were separated on a BioGel 1.5M column (0.7 × 24 cm).

Alternatively, both plain and RhB-modified liposomes were loaded with C₁₂FDG (Plovins et al., 1994), a 12-carbon lipophilic variant of FDG. C₁₂FDG solubilized in DMSO was added to ePC, CHOL, and RhB mixture in chloroform; after evaporation of the solvents, the lipid film was redissolved in chloroform. After a second chloroform evaporation and freeze-drying of the film, plain and RhB-modified C₁₂FDG-loaded liposomes were prepared as described above. Finally, the C₁₂FDG-RhB-liposomes were purified from the non-incorporated RhB and C₁₂FDG by gel filtration on a BioGel 1.5M column. To estimate C₁₂FDG loading, C₁₂FDG liposomes were resuspended in PBS at 150 μ g/mL and incubated with or without recombinant β -galactosidase (0.635 μ g/mL) for 24 h at 37°C. After the liposome dissolution with 0.2% Triton X-100 (to avoid a possible FRET effect), the

fluorescent intensity of the resultant C₁₂FITC was measured on a microplate reader SynergyTM HT (ex/em: 488/520 nm) and normalized for lipid content.

Physicochemical characterization of liposomes

Liposome size and size distribution were determined by Coulter N4 MD Submicron Particle Size Analyzer (Beckman Coulter, Fullerton, CA). The zeta-potential values of the various liposomal preparations were measured at 25°C in water (0.167–0.33 µg lipids per mL) using the zeta-potential analyzer Zeta-Plus (Brookhaven Instruments, Holtsville, NY).

In vitro stability of liposomes in culture medium

To measure the stability of liposomes during the prolonged incubation with the culture medium, plain and RhB-modified liposomes were prepared by hydration of lipid films in PBS supplemented with calcein (50 mM). Liposomes were formed by extrusion as described above and then separated from the free calcein by gel filtration (column 0.7 × 25 cm, BioGel A1.5M, PBS). *In vitro* liposomal stability was examined by the incubation of calcein-loaded plain or RhB-modified liposomes (50 µg/mL) with either PBS or Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 10% v/v) at 37°C. Equal volumes of the liposomal samples were taken from the liposome/DMEM suspension after the incubation for 0, 4, and 24 h and then solubilized by Triton X-100 treatment (50 µL of 10% Triton X-100 to 2 mL of the liposomal suspension). The high concentration of the encapsulated calcein leads to self-quenching of its fluorescence, resulting in a low background fluorescence intensity of the liposomes. The calcein released from leaked liposomes increases the background fluorescence allowing monitoring of the stability of the liposomes. To measure release of calcein, the percent of increase in fluorescent intensity was measured by the fluorescence spectroscopy (ex/em 488/520 nm) and calculated according to formula: $100 \times [FI_{\max} - FI_0] / FI_{\max}$, where FI_0 is the fluorescence intensity of liposomes before treatment with Triton X-100 and $FI_{\max}$ is the fluorescence intensity of liposomes after treatment with Triton X-100.

Cell culture

HeLa cells were grown at 37°C in 5% CO₂ and 95% humidity in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine. The cell cultures were detached by trypsinization with 0.5% trypsin in PBS containing 0.025% EDTA. The cells were used for all experiments up to passage 15.

Interaction of liposomes with cells in vitro

Cells grown to 60–80% confluence were incubated with liposomes (at 50 µg/mL lipid concentration) in complete DMEM (10% FBS and antibiotics) for 4 h, washed three times with DMEM to remove non-bound liposomes, and used for microscopy or fluorescence-activated cell sorter (FACS) experiments. When required, cells treated with liposomes for 4 h and washed with DMEM were additionally kept for 20 h in the liposome-free complete DMEM.

FACS analysis

The fluorescence of liposome-treated cells was measured in a Becton Dickinson FACScanTM (Becton Dickinson, San Jose, CA), and the data analysis was performed using CellQuest software (Becton Dickinson). The green fluorescence was determined at the emission wavelength of 520 nm (channel FL-1), whereas, the red fluorescence was recorded at the emission wavelength of 580 nm (channel FL-2). To eliminate possible overlap of RhB fluorescence with channel FL-1, the compensation between FL-1 and FL-2 channels (1–2%) was applied using the cells treated with RhB-modified FD-free liposomes as an additional control. A total of 10,000 events were acquired for each sample.

Confocal immunofluorescence microscopy

To estimate intracellular localization of RhB-modified liposomes, HeLa cells grown on glass coverslips to 60–70% confluence were incubated with liposomes as described above and then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature (RT), followed by a quenching with NaBH₄ in for 5 min. The cells were then permeabilized by incubation with 0.2% saponin and 1% bovine serum albumin (BSA) in PBS for 10 min at RT, washed three times with a blocking solution (1% BSA in PBS, pH 7.4), and kept for 30 min in the same buffer. Next, the cells were stained with the mouse anti-human Lamp2 mAb diluted with blocking solution (1:50) for 60 min at RT, and washed five times with the blocking solution. Visualization was achieved by cell incubation with DyLightTM 350-conjugated goat antimouse IgG (1:100 dilution) for 60 min at RT followed with five washes with the blocking solution.

As an alternative to staining with the antibodies, HeLa cells treated with liposomes were incubated with 100 nM LysoTracker Red (ex/em: 577/590 nm) for 60 min in completed DMEM. The cells were washed five times with PBS and then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min RT, followed by a PBS wash. The coverslips were mounted on glass slides with Fluoromount-G[®] medium and sealed using a nail polish. The slides were observed with a Zeiss LSM 510 confocal microscope (Thornwood, NY) equipped with a 63×, 1.4-numerical aperture planapochromat oil-immersion objective. To characterize the co-localization of liposomes and lysosomal markers, Pearson's correlation coefficient (PCC) and Mander's overlap coefficient (MOC) were calculated using the ImageJ 1.42 software (NIH).

Preparation of lysosome-enriched fractions

Lysosomes were isolated using a Lysosomal Enrichment Kit from Pierce Biotechnology according to the manufacturer's protocol. In brief, HeLa cells were incubated with liposomes (50 µg/mL as lipid) in complete DMEM for 4 h, washed twice to remove non-bound liposomes, and additionally incubated for 20 h in liposome-free DMEM. The cells (~0.7–1 × 10⁸ cells per experimental treatment) were collected by trypsinization, washed once with ice-cold PBS, and the cell pellet was resuspended in 1 mL of the lysosomal enrichment reagent A complemented with a protease inhibitor cocktail. After 2-min incubation on ice, the cells were lysed by sonication (20 bursts, 3 sec each, at 6 W). The cell lysate was treated with 1 mL of the lysosome enrichment reagent B. The mixture was gently shaken several times and centrifuged at 500 g for 10 min at 4°C to pellet nuclei and any

remaining intact cells. The supernatant fraction was then adjusted to 15% (v/v) OptiPrep™ gradient medium (Pierce Biotechnology), loaded onto the top of discontinuous density gradient with the following steps from top to bottom: 17%, 20%, 23%, 27%, and 30% OptiPrep™, and subjected to ultracentrifugation at 145,000 *g* for 2 h at 4°C (SW41Ti; Beckman Coulter Optima XL, Krefeld, Germany). All individual fractions were collected, washed from the gradient medium with PBS, and pelleted at 30,000 *g* for 30 min at 4°C (TLA-100.3, Beckman Coulter Optima TLX Tabletop). In order to normalize the amount of delivered FD to amount of cells, the protein concentration in each fraction was measured using a Coomassie-based protein assay. To measure FD delivery to lysosomes and RhB distribution through the subcellular fractions, the fluorescent intensity of equal volumes of each fraction was measured in triplicates with a Synergy™ microplate reader (BioTek® Instruments, Winooski, VT) at 488/520 nm (ex/em) for FD or at 530/590 nm (ex/em) for RhB and normalized to the protein content.

In order to identify the lysosomal fraction, β -galactosidase activity was evaluated using FDG, a specific fluorescent substrate for the lysosomal β -galactosidase (Rotman et al., 1963). The lysate obtained from the control (untreated) HeLa cells was subjected to the subcellular fractionation at the same conditions as described above. Fifty microliters of the each of five fractions was dispersed in 150 μ L water supplemented with 15 μ M of FDG and incubated for 18 h at 37°C. The fluorescence intensity of the resulted fluorescent product was measured using a microplate reader, normalized to protein content, and the relative activity of β -galactosidase in each fraction was presented as a percent of total β -galactosidase activity in all fractions. An additional method to identify a lysosome-enriched fraction included the treatment of HeLa cells with LysoTracker Red (1 μ M) for 45 min at 37°C in complete DMEM with the subsequent cell lysis and separation of the individual subcellular fractions at the same experimental conditions as already described. The fluorescence intensity of LysoTracker Red in each individual fraction was measured at 530/590 nm (ex/em), normalized to protein content, and then the fractional distribution of LysoTracker Red was calculated as the percent of the fluorescence in each fraction of the total fluorescence of all fractions.

Statistical analysis

The data were tested for statistical significance using the paired Student's *t*-test. *P*-values, calculated with the SPSS 10.00 software package, were considered significant at *P* < 0.05.

Results

Characterization of liposomes

All liposomal formulations were characterized in terms of size, zeta-potential, and FD content (Table 1). Our calculation showed that at 1 mol% of RhB in the lipid formulation, about 3300 molecules of RhB are incorporated in one liposome. Since RhB was dissolved in the chloroform solution of the lipids and in the process of liposome preparation distributes randomly between inner and outer lipid monolayers, only one-half of RhB molecules will be exposed on the liposomal outer surface. The presence of RhB in formulations did not influence the liposome size and the amount of FD loaded into the liposomes. The presence

of positively charged RhB in the membrane of FD-free liposomes increased their zeta-potential (Table 1). However, RhB-modified and FD-loaded liposomes bear a slight negative charge, which is the result of the positive charge neutralization with the negatively charged FD (Berth et al., 2002).

Liposome stability

In order to avoid an inaccurate interpretation of the experimental data arising from a possible cell interaction with free FD released from “burst” liposomes during their prolonged incubation with the completed culture medium, we measured the ability of the liposomes to keep their content under the experimental conditions used. For this purpose, we used plain and RhB-modified liposomes loaded with 50 mM calcein (see the section “Materials and methods”). Calcein-loaded liposomes were incubated with PBS or DMEM supplemented with FBS (10% v/v) at 37°C for 0, 4, and 24 h. The results revealed that the calcein encapsulation in all liposomal compositions was unchanged even after 24 h incubation with complete DMEM and was similar to the level of calcein encapsulation in the liposomes incubated with PBS only (data not shown).

Cell–liposome interaction

The interaction of RhB-modified liposomes with cells was evaluated by flow cytometry. Figure 1 shows that cells treated with FD-loaded plain liposomes (Lip-FD) or liposomes modified with 1 or 3 mol% of RhB (Lip-FD-RhB1 and Lip-FD-RhB3), whatever the intracellular distribution of liposomes was, demonstrated a similar total level of FITC fluorescence. Additional cell incubation for 20 h in the liposome-free complete DMEM after 4 h of the incubation with liposomes caused a decrease in cell fluorescence in both cases that can be explained by the partial quenching of FITC fluorescence due to the hydrolysis of FD upon prolonged incubation times. Nevertheless, the fluorescence of the cells incubated for 24 h was still significantly increased compared with control (untreated) cells.

Intracellular localization of RhB-modified liposomes

Figure 2 shows representative confocal fluorescence micrographs of HeLa cells treated with Lip-FD or Lip-FD-RhB1 (50 µg/mL) for 4 h or for 4 h followed by wash and additional incubation for 20 h in liposome-free complete DMEM. The co-localization of FD (green channel) and LysoTracker Red (red channel) was followed for the Lip-FD-treated cells, whereas the co-localization of RhB (red channel) and anti-Lamp2 (a specific lysosomal membrane protein) mAb (blue channel) was used in the case of the Lip-FD-RhB1-treated cells. The results of the confocal microscopy (Figure 2D–2F) demonstrated that cell incubation with Lip-FD-RhB1 for 4 h led to the localization of RhB fluorescence mostly in the lysosomes with a high rate of the co-localization with the lysosomal marker (PCC = 0.7; MOC = 0.8). The cells treated with the same concentration of Lip-FD demonstrated much lower localization of FD in the lysosomes (PCC = –0.1; MOP = 0.2). It should be noted that in contrast to RhBmodified liposomes, the significant part of Lip-FD was not internalized after 4 h incubation with cells and remained on the cell surface (Figure 2A).

An additional incubation of Lip-FD-treated cells for 20 h in the liposome-free complete DMEM led to a certain increase of FD co-localization with LysoTracker Red-labeled

lysosomes (PCC = 0.3, MOC = 0.4). However, at the same time point, it was still far less than the colocalization of RhB-modified liposomes with anti-Lamp2 mAb-labeled lysosomes (PCC = 0.5, MOC = 0.6). The results of the confocal microscopy also showed the high level of the co-localization of the liposomal RhB and FD components (PCC = 0.7, MOC = 0.8) in the Lip-FD-RhB1-treated cells (4 + 20 h), indicating that both the lipophilic (RhB) and the hydrophilic (FD) components of the liposomal preparation are preferentially concentrated into lysosomes.

Lysosomal delivery of FD by liposomes

To evaluate the efficiency of FD delivery into lysosomes, we have compared the fluorescence intensity of the lysosome- enriched fractions isolated by ultracentrifugation from cell lysates loaded on a gradient of iodixanol (see the section “Materials and methods”). The lysosomal fraction was identified by β -galactosidase activity (Figure 3A), or by fractional distribution of LysoTracker Red (Figure 3B). Figure 3A and 3B shows that most of the lysosomes were concentrated in the fraction #1 (about 70%) with the minor part (about 15–20%) in the fraction #2. The fractional distribution of the liposomal RhB (Figure 3B) correlated well with the fractional distribution of LysoTracker Red and showed the preferential RhB accumulation (60%) in the lysosome-enriched fraction (Figure 3C). The efficiency of FD delivery into lysosomes mediated by RhB-modified liposomes and evaluated following the FITC fluorescence intensity of the lysosomal fractions was 2-fold higher as compared with plain liposomes (Figure 3D). The observed increase was not determined by a possible spectral overlap of RhB and FITC channels because the comparison of the liposomal fractions from the cells treated with RhB-modified but FD-free liposomes revealed the same level of the autofluorescence as in control untreated cells. Moreover, the elevation of RhB content in the liposomal membrane from 1 to 3 mol% did not affect the FITC fluorescence of the liposomal fraction, thus eliminating a possibility of misinterpretation of the results due to RhB/FITC fluorescence overlap.

Flow cytometric detection of lysosomal targeting using RhB-modified liposomes loaded with C₁₂FDG

To additionally confirm the ability of RhB-modified liposomes to target lysosomes, we developed a new method for evaluation of the lysosomal targeting by flow cytometric analysis of live cells using C₁₂FDG, a lipophilic substrate for the lysosomal β -galactosidase (Rotman et al., 1963). We assumed that upon undergoing the endocytosis (Figure 4), C₁₂FDG-loaded liposomes will eventually deliver their contents into lysosomes, and the intralysosomal β -galactosidase will hydrolyze the nonfluorescent C₁₂FDG into the fluorescent C₁₂FITC, which will be retained inside lysosomes because of its lipophilic moiety. Thus, using a standard flow cytometry procedure, the lysosomal targeting can be quantified following the fluorescence intensity of live intact cells.

With this in mind, we have prepared plain and RhB-modified liposomes loaded with C₁₂FDG and characterized their size, zeta-potential, and the percentage of C₁₂FDG incorporation (Table 1). The loading of C₁₂FDG into RhB-modified liposomes was ~10 times less than into the plain liposomes. Such a decrease in the C₁₂FDG loading can be attributed to a stoichiometric competition between C₁₂FDG and RhB in the liposomal

membrane leading to the partial loss of C₁₂FDG due to its shorter lipophilic moiety. For this reason, two different concentrations of plain liposomes (20 and 200 µg/mL) were used for cell treatment to achieve the same amount of C₁₂FDG as with RhB-modified liposomes (200 µg/mL). Untreated cells were used to determine the level of cell autofluorescence. The cells treated with RhB-modified but C₁₂FDG-free liposomes were used as an additional control to eliminate any effect of RhB fluorescence due to the possible overlap of channels FL-2 and FL-1.

Figure 5 shows that the treatment of cells with different concentrations of C₁₂FDG-loaded plain liposomes (20 and 200 µg/mL) led to a dose-dependent increase in their FITC fluorescence relative to the control (untreated) cells. These data suggest that endocytosed liposomes actually deliver C₁₂FDG into lysosomes in amounts sufficient to be detectable by the flow cytometry. As seen in Figure 5A and 5B, the cells treated with 200 µg/mL of RhB-modified liposomes demonstrated significantly increased C₁₂FITC fluorescence compared with the cells treated with both 20 and 200 µg/mL of the plain liposomes. Therefore, these findings on intact cells clearly show that the modification of the liposomal membrane with RhB increases the lysosomal targeting compared with nonmodified liposomes.

Discussion

Earlier, Huth et al. (2004) noted that pH-sensitive liposomes loaded with FD or labeled with the lipophilic membrane marker rhodamine-B–phosphoethanolamine (RhB-PE) demonstrate a different intracellular distribution pattern after being endocytosed by cells: the highly lipophilic RhB-PE was co-localized with the lysosomotropic dye LysoTracker Red, whereas liposomal FD was not accompanied by LysoTracker Red in all cases.

In this study, we attempted to demonstrate that liposomes modified with octadecyl-RhB, a lysosomotropic targeting ligand, acquire the ability to specifically target lysosomes and allow for an increased delivery of liposome- entrapped substances to these clinically important organelles. FD was chosen as a model compound (to mimic the enzyme load for ERT) because of its relatively high molecular mass (MW 4400) and relative stability against digestion in the hydrolytic intralysosomal compartment (Thorball, 1981). The RhB-modified and plain nonmodified liposomes were loaded with FD and their interaction with HeLa cells and the ability to deliver FD to lysosomes were investigated using flow cytometry, confocal microscopy, and subcellular fractionation.

By comparing the fluorescence of intact lysosome-enriched fractions, we have clearly demonstrated an increased (at least 2-fold) lysosomal delivery of FD in cells treated with RhB-modified liposomes compared with the cells treated with control nonmodified liposomes (Figure 3). At the same time, the results of flow cytometry (Figure 1) revealed that the treatment of cells with the equal amounts of plain or RhB-modified liposomes led to the same level of cell total FITC fluorescence, which clearly indicates that the increased lysosomal accumulation of FD in the case of the cell treated with RhB-modified liposomes cannot be explained by different amounts of cell-introduced FD and results from different intracellular fate and distribution of plain and RhB-modified liposomes.

The lysosomal targeting of RhB-modified liposomes was also confirmed by the results of confocal microscopy showing an elevated co-localization of the liposomal RhB and specific lysosomal marker Lamp2 (revealed by anti-Lamp2 antibodies). It is important to mention here good intracellular co-localization of RhB and FD suggesting a cohesive accumulation of both the lipophilic (RhB) and hydrophilic (FD) liposomal markers (i.e. intact loaded liposomes) within lysosomes. Confocal microscopy also revealed that after 4 h of cell–liposome interaction, the significant part of plain liposomes was still associated with the cell surface indicating a slower rate of their internalization. Although, following additional 20 h incubation, the lysosomal accumulation of the plain liposomes increased, it remained significantly below that for RhB-modified liposomes.

Since methods such as cell staining for microscopy or subcellular fractionation of cell lysates are quite disruptive, it was important to confirm the results in intact live cells. Here, for the first time, we have employed a new method allowing tracking the lysosomal accumulation of the internalized liposomes in intact live cells by flow cytometry (Figure 5). Using C₁₂FDG, a substrate for the intralysosomal β -galactosidase, we clearly showed its increased delivery inside lysosomes by RhB-modified liposomes, that is, the lysosomotropic properties of such liposomes, confirming the data obtained by microscopy and subcellular fractionation methods.

There are a variety of different endocytotic pathways, by which liposomes can be internalized into cells, including clathrin-mediated endocytosis (Mousavi et al., 2004), caveolae-mediated endocytosis (Kiss and Botos, 2009), phagocytosis, and macropinocytosis (Swanson, 2008), and non-clathrin–non-caveolae-dependent endocytosis (Torchilin, 2008). Thus, it has been shown that HUVEC and COS-7 cells internalize liposomes via both, caveolae- and clathrin-mediated endocytosis (Huth et al., 2006). It is also known that the endocytosis by caveolae-dependent mechanism ends in caveosomes, intracellular vesicles with a neutral pH compartment that lacks markers of endosomes or lysosomes, and can be transported directly to smooth endoplasmic reticulum (Pelkmans et al., 2001). Because lysosomes are highly dynamic organelles that can fuse with different cellular compartments (Luzio et al., 2007), the use of lysosomotropic ligands may allow avoidance of the trafficking pathway from endosomes to the Golgi apparatus or endoplasmic reticulum thus increasing the lysosomal accumulation. We assumed that if liposomes are internalized by caveolae- mediated endocytosis, then a lysosomotropic ligand such as RhB could improve the lysosomal targeting due to more effective fusion of the endocytosed liposomes with lysosomes.

In this study, we used non-PEGylated liposomes, since such liposomes are easily taken up by the liver upon the i.v. administration, which is among the primary targets for the therapy of LSD. Still, in our future research we also plan to use PEGylated liposomes with different lysosomotropic targeting ligands coupled to the distant tip of PEG chains for studies related to delivery in organs other than the liver.

Conclusions

The modification of the liposomal surface with the lysosomotropic octadecyl-RhB significantly increases the delivery of liposome-loaded model marker, FD to lysosomes. Such lysosome-targeted liposomes are of clear interest as pharmaceutical carriers for the lysosomal drug delivery including the ERT.

Abbreviations

C₁₂FDG	5-dodecanoylaminofluorescein di-β-D-galactopyranoside
DMEM	Dulbecco's modified Eagle's medium
ERT	enzyme replacement therapy
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FD	FITC-dextran
FDG	fluorescein di-β-D-galactopyranoside
LSD	lysosomal storage diseases
MOP	Mander's overlap coefficient
PBS	phosphate-buffered saline
PCC	Pearson's correlation coefficient
RhB-PE	rhodamine-B-phosphoethanolamine
RhB	octadecyl-rhodamine B

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Figure 1.

Cellular uptake of liposomes by flow cytometry. Fluorescence intensity of HeLa cells treated with equal amounts of different liposome preparations was determined by flow cytometry (channel FL-1). The data are a mean of four different experiments $\pm$ SEM. Significant differences from control (untreated) cells: * $P = 0.048$; † $P = 0.043$; ‡ $P = 0.031$; § $P = 0.048$; ¶ $P = 0.006$; || $P = 0.004$.

Figure 2.

Confocal microscopy of liposome-treated cells. HeLa cells were treated with Lip-FD (A–C) or Lip-FD-RhB1 (D–F) for 4 h, followed by washing or by washing and additional incubation for 20 h in liposome-free DMEM. The treated cells were stained with lysosomal markers and analyzed by confocal microscopy. (A) FD-loaded plain liposomes (green). (B) LysoTracker Red-stained lysosomes (red). (C) Overlay of A and B images with their respective DIC image. (D) RhB (red). (E) anti-Lamp2 mAb-stained lysosomes (blue). (F) Overlay of D and E images with their respective DIC image. Bar = 10 μ m.

Figure 3.

Lysosomal delivery of FITC-dextran by liposomes. HeLa cells were treated with Lip-FD, Lip-FD-Rh1 or Lip-FD-Rh3 for 4 h, followed by washing and additional incubation for 20 h with liposome-free DMEM. Five individual organelle fractions isolated from the nuclear-free cell lysates by subcellular fractionation were characterized for: (A) β -galactosidase activity ($n = 3$); (B) fractional distribution of LysoTracker Red ($n = 3$); (C) fractional distribution of liposomal RhB ($n = 8$); (D) FITC-dextran ($n = 8$). The data are a mean of n experiments $\pm$ SEM. The results of paired Student's t -test: $*P = 0.016$; $^{\#}P = 0.001$.

Figure 4.

Scheme of lysosomal targeting by C₁₂FDG-loaded liposomes. Intralysosomal β -galactosidase (β -Gal) hydrolyzes nonfluorescent C₁₂FDG delivered by RhB-modified liposomes into the fluorescent C₁₂FITC, which accumulates inside lysosomes because of its lipophilic moiety.

Figure 5.

Flow cytometry of lysosomal targeting by liposomes loaded with C₁₂FDG. HeLa cells were incubated with plain liposomes (20 and 200 µg/mL), Lip-RhB1 (A), or Lip-RhB3 (B) (200 µg/mL). The liposomes were loaded with C₁₂FDG (1.5% mol/mol), a fluorescent substrate for the intralysosomal β-galactosidase. After 4 h incubation with liposomes, the cells were washed with and additionally incubated for 20 h with liposome-free DMEM. The fluorescence intensity of FITC (channel FL-1) was determined by the flow cytometry. Each value is the mean ± SD of two different experiments.

Table 1

Composition and properties of RhB-modified liposomes.

Abbreviation	Composition (mol%)				Size $\pm$ SD (nm)	Zeta $\pm$ SD (mV)	FD/lipids (mg/mg)	C ₁₂ FDG loading (% to plain Lip)
	ePC	Chol	Rh	C ₁₂ FDG				
Lip-FD	70	30	—	—	189 $\pm$ 23	-9.3 $\pm$ 3.3	0.48 $\pm$ 0.13	—
Lip-FD-Rh1%	70	30	1	—	187 $\pm$ 16	-8.5 $\pm$ 2.9	0.49 $\pm$ 0.10	—
Lip-FD-Rh3%	70	30	3	—	193 $\pm$ 15	-5.7 $\pm$ 2.3	0.47 $\pm$ 0.11	—
Lip-C ₁₂ FD	70	30	—	1.5	189 $\pm$ 60	-10.1 $\pm$ 1.2	—	100
Lip-C ₁₂ FDG-Rh1%	70	30	1	1.5	190 $\pm$ 65	-10.2 $\pm$ 1.0	—	16
Lip-C ₁₂ FDG-Rh3%	70	30	3	1.5	191 $\pm$ 64	21.6 $\pm$ 5.6	—	11
Lip-Rh1%	70	30	1	—	233 $\pm$ 29	17.0 $\pm$ 4.9	—	—
Lip-Rh3%	70	30	3	—	209 $\pm$ 13	21.9 $\pm$ 5.9	—	—

ePC, egg phosphatidylcholine; Chol, cholesterol; RhB, rhodamine B, C₁₂FDG, 5-dodecanoylaminofluorescein di- β -D-galactopyranoside, Lip, liposomes.