DEVELOPMENT OF CELL-SPECIFIC AND ORGANELLE-SPECIFIC DELIVERY SYSTEMS BY SURFACE MODIFICATION OF LIPID-BASED PHARMACEUTICAL NANOCARRIERS

Doctoral Thesis Presented

By

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Abstract

Objective
The use of lipid-based pharmaceutical nanocarriers, such as lipid-core polymeric micelles and liposomes have improved the pharmacokinetic and pharmacodynamic properties of many pharmaceuticals, especially in chemotherapy. These delivery systems have not only helped in increasing the solubility of poorly soluble chemotherapeutic drugs but have also acted as reservoir of large molecular weight active pharmaceutical ingredients (API) like proteins. The objective of the thesis was to explore surface modification options to further enhance the delivery of pro-apoptotic small molecules for cell-specific delivery and proteins for intracellular delivery. The thesis has been divided into two sections.

Section a
The purpose of this study was to enhance the delivery of new pro-apoptotic small molecule, N-[(2-hydroxy-5-nitrophenyl)amino]carbonothioyl]-3,5-dimethylbenzamide (DM-PIT-1), which is the non-lipid antagonist of phosphatidylinositol-3.4.5-triphosphate and inhibitor of the PI3-kinase pathway. Micelle-forming PEG$_{2000}$-PE was used to solubilize DM-PIT-1, which has an aqueous solubility of 30 μM. To further improve the delivery of the micellar DM-PIT-1, cancer-targeting anti-nucleosomal mAb2C5 antibodies and/or Tumor necrosis factor-Related Apoptosis Inducing Ligand (TRAIL) were attached to the surface of polymeric micelles.

Methods. Plain micelles and surface-modified micelles (mAb2C5 and TRAIL) loaded with DM-PIT-1 were prepared and characterized by dynamic light scattering. Drug
loading efficiency was determined by HPLC. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) method.

**Results** DM-PIT-1 was effectively incorporated (> 70%) into 14–16 nm micelles, which had a negative surface zeta potential of 4–5 mV. Micellar DM-PIT-1 demonstrated high *in vitro* cytotoxicity against various cancer cells. An improved potency of the dual-activity DM-PIT-1/TRAIL combination nanoparticles in inducing death of TRAIL-resistant cancer cells was noticed. Similar effect was found mAb 2C5 conjugated DM-PIT-1 loaded micelles. The efficacy of the TRAIL therapy was enhanced by combining it with the mAb 2C5 DM-PIT-1 loaded micelles.

**Section b Part 1**

The purpose of this study was to develop octadecyl-rhodamine B (Rh)-modified liposomes loaded with fluorophore, FITC-dextran (FD), to achieve efficient lysosomal targeting and delivery.

**Methods.** Plain or Rh-modified liposomes (Rh mol 1% and 3 mol %) were prepared from a mixture of phosphatidylcholine/cholesterol (7:3 molar ratio) by hydration of lipid films in PBS supplemented with FD. Alternatively, plain and Lip-Rh were loaded with 5-dodecanoylamino fluorescein di-b-D-galactopyranoside (C_{12}FDG), a specific lipophilic substrate for the lysosomal enzyme β-galactosidase. HeLa cells were incubated with plain or Rh-modified liposomes. Delivery to lysosomes was investigated by confocal microscopy, flow cytometry and subcellular fractionation methods.

**Results.** Incubation of HeLa cells with Lip-FD-Rh led to the accumulation of FD primarily in the lysosomes, which was evident from high rates of co-localization with
specific lysosomal markers (Lysotracker Red and Lamp-2 antibody). The cells treated with the same concentration of plain Lip-FD showed low localization of FD in the lysosomes. Comparison of the fluorescence intensity of lysosome-enriched fractions showed that the efficiency of lysosomal delivery of FD by Rh-modified liposomes was 2-fold higher compared to plain liposomes. These results were confirmed by the flow cytometric analysis of the live intact cells treated with C₁₂FDG-loaded liposomes, which showed increased lysosomal targeting by Rh-modified liposomes.

Section b Part 2

The purpose of this study was to develop octadecyl-rhodamine B (Rh) modified liposomes loaded with VPRIV® (velaglucerase alfa for injection), a lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT), and determine the lysosomal targeting efficiency of the delivery system.

Methods. Plain or Rh-modified liposomes (Rh mol 1%) were prepared from a mixture of phosphatidylcholine/cholesterol (7:3 molar ratio) by hydration of lipid films in PBS supplemented with VPRIV®. Alternatively, plain and Rh1%-lip were loaded with FD40K. Gaucher’s fibroblasts and monocyte derived macrophages (MDMs) were incubated with plain or Rh-modified liposomes. Delivery to lysosomes was investigated by confocal microscopy and flow cytometry methods.

Results. Incubation of Gaucher's fibroblasts and MDMs with Rh1%-liposomes led to accumulation of VPRIV primarily in the lysosomes which was evident from the resultant fluorescence intensity when the treated cells were incubated with a lysosome-specific substrate. The cells treated with the same concentration of plain liposomes showed
lower targeting. Comparison of the fluorescence intensity showed that the efficiency of lysosomal delivery of enzymes by Rh-modified liposomes was 1.5-fold higher compared to plain liposomes.

**Conclusions**

The lipid-core polymeric micelles form increase the solubility of poorly soluble DM-PIT-1. Their surface modification with mAb2C5 or TRAIL enhances the cell killing effect of this novel chemotherapeutic agent. This system can be used for targeted combination therapy against TRAIL-resistant cancers.

The modification of the liposomal surface with octadecyl-rhodamine B significantly increases the delivery of liposome-loaded FITC-dextran to lysosomes. Therefore, the delivery system was applied to Gaucher’s fibroblasts and MDMs, which is a Gaucher’s cell model. The octadecyl-rhodamine-modified liposomes significantly increased the delivery of liposome-loaded VPRIV into the lysosome.

These *in vitro* studies open up avenues for better targeting of therapeutic proteins like VPRIV (known for developing antibodies when administered in free form), encapsulated in reservoir-type nanocarrier systems.
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<td>API</td>
<td>active pharmaceutical ingredients</td>
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<tr>
<td>C12FDG</td>
<td>5-dodecanoylamino fluorescein di-β-D-galactopyranoside</td>
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<tr>
<td>CBE</td>
<td>conduritol-β-epoxide (CBE)</td>
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<td>CHOL</td>
<td>cholesterol</td>
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<td>DcR</td>
<td>Decoy receptors</td>
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<td>DR</td>
<td>death receptors</td>
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<td>DM-PIT-1</td>
<td>N-[(2-hydroxy-5-nitrophenyl)amino] carbonothioyl]-3, 5-dimethylbenzamide</td>
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<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
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<td>Dox</td>
<td>Doxorubicin</td>
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<tr>
<td>ePC</td>
<td>Egg phosphatidylcholine</td>
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<td>ERT</td>
<td>enzyme replacement therapy</td>
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<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
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<td>FITCD or FD</td>
<td>fluoresceinisothiocyanato-dextran</td>
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<td>FITCD 40k</td>
<td>fluorescein isothiocyanate-dextran (FD, 40,000 MW)</td>
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<td>FDG</td>
<td>fluorescein di-β-D-galactopyranoside</td>
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<td>GlcCerase</td>
<td>Glucocerebrosidase</td>
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<td>GlcCer</td>
<td>Glucocerebroside</td>
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<td>Lip-FD</td>
<td>FITC-dextran-loaded plain</td>
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<td>Abbreviation</td>
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<td>mAb2C5</td>
<td><em>Cancer targeting anti-nucleosomal Monoclonal antibody</em></td>
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<td>MDM</td>
<td><em>Monocyte derived macrophages</em></td>
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<td>PEG</td>
<td><em>Polyethylene-glycol</em></td>
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<td>PEG-DSPE</td>
<td><em>1,2-Disteratoyl-sn-glycero-3-phosphoethanolamine- N-[methoxy (polyethylene-glycol)-2000]</em></td>
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<td>PFB-FDGglu</td>
<td><em>5-(pentafluorobenzoylamino) fluorescein diglucoside</em></td>
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<td>PH</td>
<td><em>Pleckstrin Homology</em></td>
</tr>
<tr>
<td>PIP3</td>
<td><em>Phosphatidyl-inositol 3,4,5 triphosphate</em></td>
</tr>
<tr>
<td>PTEN</td>
<td><em>Phosphatase and tensin homolog</em></td>
</tr>
<tr>
<td>PMA</td>
<td><em>Phorbol-12-myristate 13-acetate</em></td>
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<td>Rh</td>
<td><em>Octadecyl-rhodamine B</em></td>
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<td>TNF</td>
<td><em>Tumor necrosis factor</em></td>
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<td>TRAIL</td>
<td><em>TNF-related apoptosis inducing ligand</em></td>
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<td>Flow cytometry of lysosomal targeting by liposomes loaded with C12FDG. HeLa cells were incubated with Lip-FD (20 and 200 μg/ml), Lip-Rh1% (A) or with Lip-Rh3% (B) (200 μg/ml). The liposomes were loaded with C12FDG (1.5 % mol/mol), a fluorescent substrate for the lysosomal 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 FL1) was determined by the flow cytometry. Each value is the mean ± SD of 2 different experiments.</td>
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Flow cytometry of lysosomal targeting by liposomes loaded with VPRIV. Two different cell lines, MDMs [A] and Gaucher’s [B], were incubated with Plain Lip (L) [100 μg/ml lipid and containing < 3.8 mUnits/ml VPRIV], Lip-Rh1% [100 μg/ml lipid and containing 3.8 mUnits/ml VPRIV], Plain Lip (E) [3.8 mUnits/ml VPRIV] or with free VPRIV [3.8 mUnits/ml]. The liposomes were loaded with VPRIV. After 4 h incubation with liposomes, the cells were washed with and additionally incubated for 20 h with liposome-free MEM or RPMI respectively. The fluorescence intensity (channel FL1) was determined by the flow cytometry after treating the cells with PFB-FDGGlu. Each value is the mean ± SD of 3 different experiments. (* p<0.01; ## p<0.01; ** p<0.05)
Chapter 1. Statement of problem and literature review

Section 1.

Enhanced delivery of apoptotic small molecules using surface-modified micellar drug delivery systems.

[1] Site-specific drug delivery

The most important requirement for any type of therapy or treatment involving drugs is the accumulation of these agents in the desired region of the body. Most of a drug administered by injection circulates throughout the body and is eliminated by hepatic metabolism (first pass metabolism) or renal excretion. Thus, only a limited portion of the dose reaches the target sites. Additionally, the dose that goes off target may cause undesirable side-effects. For an optimal medicinal effect, the concentration of the drug must be maintained at an optimum effective level at the target sites, with minimal dose accumulation at off-target sites. Site-specific drug delivery systems that allow control of drug distribution are therefore desirable in advanced medicines. These systems will not only be instrumental in increasing the bioavailability, resulting in increased accumulation of drug in the pathological area of interest but will also lead to minimized premature drug degradation upon administration and preventing undesirable side-effects on normal cells, organs and tissues by the cytotoxic drugs [1].

[2] Cancer

Cancer is the second leading cause of death behind heart disease for the overall population. Currently, one in four deaths in United states is due to cancer. In 2011
alone, a total of 1,596,670 new cancer cases and 571,950 deaths from cancer are projected to occur in the United States [2].

There are three major treatment modalities in cancer therapy

- Surgery
- Radiation therapy
- Chemotherapy

Surgery and radiation therapy serve as local modes of treatment. Chemotherapy is a systemic treatment and is effective even against metastatic tumors. Chemotherapy acts by killing cells that divide rapidly, which is one of the hallmarks of cancer cells. This means that it also harms cells that divide rapidly under normal circumstances.

[3] Problems associated with chemotherapy

Even though localized primary solid tumors can be removed successfully by surgical process, it is known that growing or metastasizing tumors require extensive chemotherapy. Chemotherapy, though effective, is considered to be tricky because under-dosing affects the efficacy of therapy while an overdose of these drugs can cause excessive toxicity. As the cell killing actions of these drugs are not limited to cancerous cells, non-selective distribution of these agents can cause considerable side-effects, in particular nephrotoxicity, neurotoxicity, ototoxicity. e.g. doxorubicin, one of the classic anti-cancer drugs used in chemotherapy, is notorious for its side effects (cardiotoxicity). It has been observed that the risk of developing amenorrhea (absence of a menstrual period in a woman of reproductive age) following a treatment protocol containing doxorubicin ranges from 20% to 80%, depending on the female patient’s
age (higher incidence was found at the age of 40 and older and moderate incidence was found at the age of 30-39) [3]. In a related study, Ben-Aharon et al. observed that a major reduction of both ovarian size and weight occurred when female mice were injected intraperitoneally with 7.5 or 10 mg/kg doxorubicin [4]. Retrospective study of phase III trials \((n = 630 \text{ patients})\) of conventional doxorubicin suggested that doxorubicin-induced congestive heart failure (CHF) may occur at low cumulative doses \((\leq 300 \text{ mg/m}^2)\) [5] and these side effects were usually irreversible, moreover these clinical signs and symptoms persist for months or longer after treatment. All these side effects occur due to non-selective distribution of this agent.

[4] **Need for targeted drug delivery systems**

A targeted drug delivery system that can deliver cytotoxic drugs specifically to the target organs, tissues, or cells may significantly reduce the adverse effects that are caused due to non-selective distribution of these anti-cancer agents. Such systems may help to achieve the recognition of the target on various levels: on the level of a whole organ, certain cells specific for this organ, individual components of these cells (cell surface antigens) or even at organelle level [6] thereby, additionally, enhancing the delivery of encapsulated guest molecules (drugs, enzymes, gene constructs etc). Micelles and liposomes are delivery systems which have been in use for more than two decades and can be surface-modified to develop effective targeted delivery systems.
Lipid-core polymeric micelles

Micelles represent colloidal dispersions (5 – 100 nm) that consist of particulate matter or a dispersed phase, distributed within a continuous phase or dispersion medium. These are spontaneously formed above a certain concentration (the critical micelle concentration) and at certain temperature (critical micelle temperature) by amphiphilic or surface-active agents (surfactants) [7].

Figure 1. Schematic of the core-shell architecture of a polymer micelle and its dimensions.

These amphiphilic unimers (Figure 1), in most cases, include a PEG block with a molecular weight of 1 to 15kDa that act as a corona-forming block and a hydrophobic block with a length closer to or somewhat lower than that of hydrophilic block [8]. At low concentrations, these amphiphiles exist as unimers. However, when the concentration is increased aggregation takes place within a narrow concentration interval. Following the chromatography of the serially diluted samples of PEG (5 kDa)-PE, it has been found that micelles do not dissociate into individual polymeric chains up to polymer concentrations of ca. 1 ug/ml. This corresponds to a micromolar critical
micelle concentration value that is at least 100-fold lower than those of conventional detergents. These polymeric micelles have been used in vivo for several previously existing drugs[9]. Moreover encapsulation of the guest molecules within the polymer core maintains drug stability as it hinders enzymatic degradation and inactivation of the encapsulated guest molecules. Additionally, particle recognition by opsonizing proteins is reduced significantly by the hydrophilic corona of the micelle [10]. In the absence of this hydrophilic corona, the micelles would undergo rapid phagocytic clearance by the reticuloendothelial system (RES) [11]. Polymeric micelles are considered to be close to the ideal drug delivery system [12]. Polymeric micelles made of PEG-lipid conjugates are also referred to as lipid-core polymeric micelles due to the presence of two long fatty acyl groups in the hydrophobic block of this system. The hydrophobic interactions between the polymeric chains in the micelle’s core increases due to the presence of these lipid moieties which in turn provide additional stability to the encapsulated guest molecules. The presence of a lipid core also offers a sound way to solubilize sparingly soluble drugs and minimize many side-effects. Enhanced permeability across the physiological barriers has also been observed [13].

Under certain circumstances, the endothelial lining of blood vessels becomes more permeable than in the normal state. This has been demonstrated for many tumors [14] and in infarcted tissues [15]. Consequently micelles, due to their small size advantage, can leave the vascular lumen and accumulate within the interstitial space.

Figure 2 Schematic representation of different mechanisms by which nanocarriers can deliver drugs to tumours. Passive tissue targeting is achieved by extravasation of nanoparticles through increased permeability of the tumour vasculature and ineffective lymphatic drainage (EPR effect). Active cellular targeting (inset) can be achieved by functionalizing the surface of nanoparticles with ligands that promote cell-specific recognition and binding. [Adapted from [16]]

If these micelles are loaded with active pharmaceutical ingredients (APIs), the APIs can be delivered into the interstitial space. The transport efficacy and accumulation of
micelles, and the API, in the tumor interstitium is thus determined by their ability to penetrate the leaky tumor vasculature endothelium [17]. It is advantageous that the size of these particles can be exploited to control the efficacy of delivery as diffusion and accumulation parameters are strongly dependent on the cut-off size of the tumor blood vessel wall, and that the cut-off varies for different tumors. As diagramed in (Fig. 2), plain micelles encapsulated with drugs can increase the accumulation of a drug in the tumor due to the Enhanced Permeability and Retention (EPR) effect [17]. This increased accumulation of APIs in the areas with leaky vasculature eventually will lead to enhanced accumulation of APIs at desired regions.

[7] Active targeting of a drug carrier

It is a well established phenomenon that in some tumors the endothelial lining of the blood vessel wall becomes more fenestral than the normal tissues [18]. Micelles can diffuse through these fenestrations and accumulate in the interstitial spaces. However, in many pathological conditions, the integrity of the vasculature may not be compromised and therefore this approach to drug targeting cannot be used universally and drug targeting to these sites may be technically more difficult. Binding a targeting moiety to a carrier which aids in specific recognition of the target site to achieve better targeting should be more useful in such cases. This type of targeting is termed as ‘active targeting’. Active targeting approaches can be augmented, by loading more than one pharmaceutical agent onto a single targeting moiety than a simple 1:1 ratio. This is where use of the reservoir-type system such as micelles and other nanoparticle seems more practical. Reservoir-type systems including
microparticles, liposomes, micelles etc provide a number of advantages: (a) increased load of the drug; (b) multiple APIs loaded into the system; (c) possibility of size-control which plays a huge role in its permeability, (d) capacity for surface modification with more than one targeting ligand/moiety to render it multifunctional (Figure 3).

Figure 3. Pharmaceutical micelles. Spontaneous micelle formation from amphiphilic molecules in aqueous media and loading with hydrophobic drug (A), a multifunctional pharmaceutical micelle containing; (a) specific targeting ligand attached to the micelle surface; (b) heavy metal atom loaded onto the micelle via the micelle-incorporated chelating moiety for gamma- or magnetic resonance (MR) imaging application; (c) or a cell-penetrating peptide, CPP, such as TATp attached to the micelle surface to promote enhanced uptake by the cells (B) {Adapted from [19]}

Active targeting strategies, which involve functionalizing the micellar surface with a ligand that recognizes tumor-specific receptors have been much sought after because
they promise greater efficiency and more specific targeting than passively targeting formulations. Targeted micelles will not only promote the local release of a free drug from micelles but also will ensure the stability of drugs thereby reducing the drug toxicity that usually results from the interaction of the drug with non-target organs.

Drug carriers have been conjugated with small organic molecules such as folate and transferrin, which are over-expressed in a variety of tumors [20-24]. Galactose- and lactose-modified micelles made of PEG-polylactide co-polymer specifically interact with lectins, thus modeling targeted delivery of the micelles to hepatic sites [21]. Peptides such as cyclic(Arg-Gly-Asp-D-Phe-Lys) (cRGDfK) peptide, which targets the αvβ3 integrin over-expressed on the surface of angiogenic tumor vessels have been used to modify the surface of micelles [25]. Micelles have also been modified with carbohydrate moieties, other than lactose and galactose, which have high affinity for asialoglycoprotein receptors (ASGPR) that are over-expressed in hepatocellular carcinoma [21, 26, 27].

**[8] Antibodies as ligands for surface modification**

Of all the ligands that have been mentioned, antibodies seem to provide the broadest opportunities for targeting. The capacity to recognize single antigens that are over expressed on cancer cells, with great specificity and affinity, is what makes monoclonal antibodies such powerful targeting ligands,

Early studies have demonstrated the existence of natural antitumor antibodies in healthy individuals. The antinuclear autoantibodies (ANAs) are natural autoantibodies and participate in antitumor immunosurveillance. ANAs selectively recognize the
tumor cell surface, with nucleosome-restricted specificity [28]. These antibodies target the nucleosomes, released from the apoptotically dying tumor cells and bound to the neighboring live tumor cells [29] by certain nucleosome binding sites/receptors present on the tumor cell surface. As a result, ANAs are most reactive against the surfaces of transformed cells but not normal cells. Nucleosome-specific monoclonal ANA 2C5 (mAb 2C5) is a good representative of this group [30]. Low, subtherapeutic concentrations of this antibody have been used as targeting moieties for the tumor-specific delivery of pharmaceutical carriers, such as long-circulating PEG-liposomes and PEG-PE-based micelles, loaded with various therapeutic and diagnostic agents [30, 31]. Several attempts to covalently attach an antibody to a surfactant or polymeric micelle (i.e., to prepare immunomicelles) have been described [20][13, 30, 32]. Studies with fluorescent labels or by SDS-PAGE [30, 33] have shown that 20 to 30 antibody molecules can be attached to a single micelle. Antibodies attached to the micelle corona preserve their specific binding ability, and immunomicelles specifically recognize their target substrates as has been confirmed by ELISA [34].

[9] Chemistry of antibody-micelle conjugation

Immunomicelles could be prepared by using PEG–PE (polyethylene glycol-phosphatidyl ethanolamine) with the free PEG terminus activated with p-nitrophenyl carbonyl (pNP) group. For this purpose, micelles are prepared from PEG–PE with the addition of a small fraction of pNP–PEG–PE. The phosphatidylethanolamine (PE) residues form the micelle core, whereas the water-exposed pNP group, stable at pH values below 6, efficiently interacts with amino groups of various ligands (such as
antibodies and their fragments) at pH values above 7.5, yielding a stable urethan (carbamate) bond. [Figure.4]. To prepare immunotargeted micelles, mAb 2C5 is simply incubated with drug-loaded micelles at pH around 8.0.

Figure 4. A) Schematic structure of PEG–PE micelles containing a small addition of the pNP–PEG–PE component. (B) Coupling of amino group-containing ligands (antibodies) with pNP groups.[30]

[10] TNF-related apoptosis inducing ligand (TRAIL) for micelle modification

TRAIL or TNF-related apoptosis inducing ligand, also known as Apo2L is a member of the tumor necrosis factor (TNF) superfamily and was identified based on its homology
to FasL (type-II transmembrane protein that is a part of tumor necrosis factor). TRAIL induces the activation of cell surface death receptors and may be an attractive therapeutic strategy to promote apoptosis of tumor cells through the activation of the extrinsic pathway of apoptosis. Apo2L/TRAIL binds to DR4 and DR5 inducing oligomerization of the receptors and initiation of a pathway mediated by proteases called caspases [35, 36]. This pathway is also called the extrinsic pathway of apoptosis to denote its responsiveness to extracellular signals. Figure 5 shows how the extrinsic pathway of apoptosis is triggered by interaction of TNF family death ligand (TRAIL) with their corresponding death receptors, such as TRAIL-Rs (DR4, DR5). These proteins recruit adapter proteins to their cytosolic domains, Fas-Associated protein with Death Domain (FADD), which then bind pro-caspase-8. Caspase-8 then activates the downstream effectors (caspase-3, caspase-6, and caspase-7) which cleave a variety of cellular substrates to execute the apoptotic program.

Apo2L/TRAIL engages four types of receptors. DR4 (TRAIL receptor-1) and DR5 (TRAIL receptor-2, KILLER, TRICK2) — are death receptors (DRs) which contain a cytoplasmic death domain and so transduce an apoptotic signal, as described above; decoy receptors (DcR)1, which lacks an intracellular death domain, and DcR2, which contains a truncated non-functional death domain. Decoy receptors (DcRs) do not initiate apoptotic cell death [37]. Though it is obvious that TRAIL induced apoptosis will not be seen if TRAIL interacts with decoy receptors, it is not clear as to why that interaction takes place. It is also known that TRAIL induces toxicity in variety of cancer cell lines but shows little toxicity towards normal cells [38].
Figure 5. TRAIL apoptosis pathway Apo2L/TRAIL interacts with four closely related members of the TNF receptor superfamily. DR4 and DR5 comprises of a cytoplasmic death domain that signal apoptosis; DcR1 is linked to the plasma membrane by a glycoprophosphatidylinositol moiety and lacks signaling activity; DcR2 has a truncated non-functional death domain. OPG is capable of binding weakly to Apo2L/TRAIL, although the physiological significance of its interaction with this ligand is unclear. TNF family death ligand (TRAIL) activates extrinsic pathway by interaction with their corresponding death receptors, such as TRAIL-Rs (DR4, DR5). These proteins recruit adapter proteins to their cytosolic domains, Fas-Associated protein with Death Domain (FADD), which then bind pro-caspase-8. Caspase-8 then activates the downstream effectors (caspase-3, caspase-6, and caspase-7) which cleave a variety of cellular substrates to execute the apoptotic program. {Adapted from [39]}
The observation that Apo2L/TRAIL induces apoptosis in a wide variety of cancer cell lines but show little toxicity toward many types of normal cells [39], encouraged cancer researchers to use it for cancer therapy. It was observed that TRAIL administered as a single agent or in combination with chemotherapy or radiotherapy showed substantial anti-tumor activity [39]. As described above, TRAIL triggers cell death by activating death receptors (DR) and therefore can be termed as DR ligand. DR ligands have potential usefulness for cancer therapy because, unlike many conventional cancer therapeutic agents, they can trigger tumor cell apoptosis independently of the p53 tumor suppressor gene [35]. Hence, DR ligands could be effective against tumors that have acquired resistance to chemotherapy or radiotherapy. Thus they might be useful in combination with conventional therapies against tumors that retain at least partial sensitivity to chemotherapy or radiation and implies that TRAIL may be used synergistically with various chemotherapeutic agents. The fact that Apo2L/TRAIL targets a wide range of cancer cell types and has safety advantages over other DR ligands, encouraged us to employ TRAIL in our system by developing delivery systems surface modified with TRAIL. The chemistry used in the process was same as that used in the mAb2C5 conjugation to micellar surface.


Hydrophobicity and low solubility, that are well known properties of many anticancer drugs [40], aid penetration into the cell and beyond into intracellular targets [41, 42]. In fact, membrane permeability and efficacy of such drugs increases with increasing hydrophobicity but around 40% of potentially valuable drug candidates identified by
high throughput screening are rejected and never enter the formulation development stage due to the same property (poor water solubility) that makes it so permeable [43] [44, 45]. This is because most poorly water soluble agents have poor absorption and low bioavailability, especially upon oral administration [46]. Severe side-effects, such as respiratory failure stemming from embolism, have been encountered due to aggregation of these agents upon intravenous administration [47]. These side-effects can be avoided by solubilizing such poorly soluble drugs. Clinically acceptable co-solvents [48-51] and the formation of salts (e.g. alkali salts of acidic drugs or acidic salts of basic drugs which are more soluble than their parent drugs) can be used to solubilize poorly soluble drugs [52]. Altering the pH of the drug microenvironment is another method [52]. But mere solubilization of these agents does not resolve the issues concerning side-effects and accumulation at undesired sites. Each of the above methods has its set of disadvantages. To cite a few, use of co-solvents themselves cause toxicity or undesirable effects including acute hypersensitivity, and peripheral neuropathy reactions have been reported with administration of Tween 80 [53]. Using surfactants is also not desirable as they cannot retain solubilized material at concentrations lower than their critical micelle concentration as there is a danger of spilling of excess drug in the undesirable areas due to dilution of the solubilized preparation in aqueous environment (i.e. physiological fluids). These factors have encouraged the interest in the use of lipid-core polymeric micellar systems in drug formulation and product development as this will effectively overcome the physical and biological barriers related to poor aqueous solubility of anti-cancer drugs and thus its consequent bioavailability. In a lipid core polymeric micellar system, polar
molecules get adsorbed on the micelle surface, nonpolar molecules are solubilized within the micelle core, and substances with intermediate polarity get distributed along surfactant molecules in intermediate positions.

Over the last decade, poorly soluble drugs such as diazepam and indomethacin [54, 55], anthracycline antibiotics [56, 57] such as doxorubicin [58, 59], and polynucleotides [60, 61] have been effectively solubilized by various polymeric micelles, including Pluronic (block co-polymers of PEG and polypropylene glycol) [62]. Doxorubicin incorporated into Pluronic micelles have demonstrated improved bioavailability when compared with free drug in the experimental treatment of murine tumors (leukemia P388, myeloma, Lewis lung carcinoma) and human tumors (breast carcinoma MCF-7) in mice [63]. PEG-PE micelles have been effectively used to incorporate a variety of sparingly soluble and amphiphilic substances including paclitaxel, tamoxifen, camptothecin, porphyrine, vitamin K3, and others [64-66]. Thus far, micellar delivery systems have proved to be a good delivery system for poorly soluble drugs and were therefore chosen for encapsulating and increasing the solubility of poorly soluble drug in this project.


N-{{{[(2-hydroxy-5-nitrophenyl)amino] carbonothioyl}-3, 5-dimethylbenzamide (DM-PIT-1), (Figure 6) the first-in-class small molecule non-lipid inhibitor of phosphatidylinositol 3,4,5 triphosphate (PIP3) binding to pleckstrin homology (PH) domain, has a
high apoptotic activity, especially against phosphatase and tensin homolog (PTEN) -
deficient cancer cells [67].

Dysregulation of the phosphoinositide-3-kinase (PI3K) pathway has been implicated
in many human diseases. It is known that hyperactivation of this pathway plays an
important role in tumorigenesis. PI3Ks (α, β, and γ) are recruited to the plasma
membrane in response to growth factor and hormone stimulation orchestrates
multiple downstream intracellular signaling events [68].

Figure 6. Chemical structure of N-{{(2-hydroxy- 5-nitrophenyl)aminocarb-
onothioyl} -3, 5-dimethylbenzamide (DM-PIT-1).

PI3Ks does this by mediating the phosphorylation of lipid phosphatidylinositol-4,5-
bisphosphate (PIP2) which leads to the generation of phosphatidylinositol-3,4,5-
trisphosphate (PIP3). PIP3 regulates a number of complex cellular signaling network
such as cell growth, proliferation, and survival. PIP3-target proteins are found in the
cytosol of unstimulated cells and these are recruited to the membrane through
pleckstrin-homology (PH) domain-mediated binding. Membrane translocation and
activation of the PIP3-target proteins initiate a variety of local responses, including assembly of signaling complexes and priming of protein kinase cascades [68] [69].

PIP3 regulates an array of PH domain-containing proteins, such as serine-threonine kinases, Akt and PDK1. This PIP3 signaling is terminated by the phosphatase PTEN, which dephosphorylates PIP3 and thereby regulating PIP3-signalling. Genetic alterations targeting PTEN are among the most frequent mutations in human cancers, indicating a critical role of uncontrolled signaling through PIP3 in tumorigenesis and metastasis [69].

Though the IC₅₀ of DMPIT-1 is 31.03 µM [70], the low aqueous solubility of free DM-PIT-1 (under 30 µM) may cause significant bioavailability problems. This encouraged the interest in the use of lipid-core polymeric micellar system which is well known for the increasing the solubility of poorly soluble drugs. DMPIT-1 substantially sensitizes U87MG cells to killing by TRAIL [70] therefore can be used synergistically with TRAIL, which may enhance the cell killing effects. This encouraged us to consider conjugating TRAIL onto the micellar surface.
Section 1B

Intracellular delivery using liposomes modified with octadecyl-rhodamine B

[1] Introduction

Specific delivery of therapeutic agents to individual cell organelles is still mainly an unresolved task in drug delivery. Organelle specific delivery is important because many drugs act on the molecular targets associated with certain organelles inside mammalian cells. So far, multiple and only partially successful attempts have been made to bring various low-molecular weight and macromolecular drugs and drug-loaded pharmaceutical carriers directly into the cell cytoplasm, bypassing the endocytic pathway. Even after safe delivery into the cell cytoplasm, a drug may still have to find its way to specific organelles (nuclei, lysosomes, mitochondria etc) to be effective. Many drug delivery approaches assume that merely mediating cell association would 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 but that is not the case, for example, in gene delivery. The traditional routes of internalization of DNA carriers, involving pinocytosis or endocytosis, leads to degradation of DNA inside lysosomes which in turn, strongly limits the efficacy of transfection. In this case, a delivery system that protects the genetic construct from the low-pH of the lysosomes and aids in its delivery into the cytoplasm may be highly desirable and may increase the efficacy of transfection. Another example is that of intracytoplasmic delivery in cancer therapy which may help in overcoming an important treatment obstacle such as multi-drug
resistance. The use of drug carrier systems, encapsulated with therapeutic agents, for intracellular delivery should substantially enhance a therapeutic response, since the uptake of a single drug-loaded nanocarrier by the cell brings multiple copies of therapeutic agents.

[2] Lysosomes and Gaucher’s disease

Lysosomes, acidic organelles responsible for recycling of cellular constituents, represent an important intracellular target. Lysosomes contain at least 50–60 soluble hydrolases [71] and at least 7 integral membrane proteins [72]. Mutations in the genes that encode any of these proteins may cause a lysosomal storage diseases (LSD). LSD involves a deficiency or inefficiency in the functioning of any of these lysosomal enzymes which, in turn, leads to the accumulation of their substrates in the lysosomes. Over 40 LSDs, that involve soluble hydrolases, are known [73]. Despite the relative rarity of each of these diseases, taken together, they pose a serious medical problem [74-76].

Gaucher’s disease is a type of lysosomal storage disease. It is named after the dermatologist Phillipe C.E. Gaucher who, in the year 1882, observed a patient with an enlarged spleen and noted the presence of unusually large cells [77] in the patient’s spleen. Reports of additional patients with similar presentations appeared shortly thereafter, and the eponym “Gaucher’s disease” was applied [78]. In addition, the term “Gaucher cell” became commonly used to specify the characteristic engorged cells in the organs of such patients [78]. Very little was known about the Gaucher’s disease.
even until the late 19th century, the involvement of lymph nodes, liver and the bone marrow was reported in beginning of 20th century [79] and it was not until the 1920s that the accumulated substrate was discovered to be a cerebroside. It was then observed that the age at which the signs and symptoms associated with Gaucher disease become manifest varied considerably. Collier reported a patient in 1895 who was 6 years old [79]. Neurologic impairment in infants was first reported by Oberling and Woringer in 1927 [77]. This phenotype eventually became known as the infantile or acute neuronopathic form. These condition is currently classified as Type 2 Gaucher disease [80]. Later, neurologic signs were observed in patients who were in their preteen and early teen years. This phenotype has been called the juvenile or chronic neuronopathic form [80]. These patients are now classified as Type 3 Gaucher disease. The most prevalent phenotype, however, is Type 1 Gaucher disease, an “adult” form of Gaucher disease without overt evidence of central nervous system being involved [81]. In some of these individuals, systemic signs may appear as early as the first year of life. At the other extreme, the occurrence of Gaucher disease has been documented as late as the eighth and ninth decades in individuals who were only mildly affected, or even completely asymptomatic [77]

[3] Enzyme replacement therapy and its limitations

Gaucher’s disease is due to the deficiency of a lysosomal enzyme glucocerebrosidase (GlcCerase) which, in turn, leads to the deposition of the substrate glucocerebroside inside the lysosomes [82]. Residual levels of GlcCerase activity in Gaucher disease patients have been estimated at between 5–25% of normal activity [77]. As a
consequence, glucocerebroside (GlcCer) accumulates in essentially every tissue where its levels have been measured. For instance, GlcCer accumulates to levels of ~30–40 mmol/kg tissue in spleen obtained from all three types of Gaucher disease patients, and glucosylsphingosine (GlcSph), the deacylated form of GlcCer, which is not usually detectable in normal tissues, accumulates to lower but significant levels of ~0.1–0.2 mmol/kg [83]. Though there is no cure for Gaucher disease; there are treatment modalities that lead to temporary correction. Enzyme replacement therapy is the most successful of them. This involves delivering the glucocerebrosidase into the lysosomes where it cleaves the deposited substrate and thus completes the metabolic pathway [82]. This approach is based on the administration of active exogenous enzymes. The seed for this therapy lies in an interesting discovery known as enzymatic “cross-correction” [84, 85]. In the early 1970s a specific trafficking system, governing the transport of most lysosomal proteins to the lysosomes, was discovered. It was seen that specific modification on their N-linked oligosaccharide chains by phosphorylated mannose residues led to its uptake by mannose-6-phosphate (M6P) receptors embedded in the lysosomes [86]. Around the same time, it became known that most cell types release small amounts of lysosomal enzymes and that these “secreted” forms could be internalized via M6P receptors present on the cell surface [86]. These two discoveries led to the development of the concept of enzymatic “cross-correction,” i.e., the idea that small amounts of enzyme released from normal cells could be internalized by diseased cells and correct the metabolic defect in patients with lysosomal storage disorders [85, 87].
Shortly after the demonstration of enzymatic “cross-correction” by Neufeld and colleagues, the concept of treating lysosomal diseases by direct enzyme replacement evolved. The success of these procedures however ‘relied’ on the degree of the accessibility of the defective target cells to the circulating enzymes. Attempts at enzyme replacement therapy were first undertaken in the early 1970s [73, 88]. These early attempts were severely hampered by the limited availability of the purified, normal enzymes but they provided the “proof of principle” for the approach, and showed that intravenously administered enzymes could be taken up by target cells of pathology and that it led to the reduction of pathological substrate accumulation. In addition, these early studies also revealed the critical importance of the need to improve enzyme targeting to specific cell types in individual diseases, e.g. macrophages in Gaucher disease.

Initially, when placental glucocerebrosidase was injected into two patients with this disorder, glucocerebroside that had accumulated in the liver reduced significantly in both the patients [73]. The elevated glucocerebroside in the blood of the recipients returned to normal levels within 3 days following injection of the enzyme. It was also observed that the reduction of glucocerebroside in the circulation lasted many months [89]. To enhance this delivery, it was important to modify the four oligosaccharide chains found on this enzyme so that the enzymes could be targeted to macrophages where glucocerebroside accumulated. To this effect, mannose-terminal glucocerebrosidase was produced [90] as glycoproteins with terminal molecules of mannose molecules are detected by lectins on the surface of macrophages and hence taken up by macrophages [91].
Velaglucerase alfa (VPRIV®, Shire Human Genetic Therapies, Inc, MA, USA), is one of the biosimilars available for the enzyme replacement therapy. This agent is generated by gene activation of the endogenous human glucocerebrosidase gene in an immortalized human fibrosarcoma cell line [92]. The engineered cells are cultured in a medium containing the powerful inhibitor kifunesine, which blocks the action of α-mannosidase I for glycoprotein biosynthesis, and as a result, a human glucocerebrosidase protein displaying terminal mannose sugars is produced [93]. Velaglucerase alfa is a 63 kDa monomeric glycoprotein containing 497 amino acids that are identical to that of the natural placental human protein [92]. Clinical trials with VPRIV® have shown statistically significant improvements from baseline in hemoglobin concentration and platelet counts. Statistically significant improvements were also noted in the mean percentage change from baseline to 9 months for hemoglobin concentration (+19.2%), platelet counts (+67.6%), liver volume (−18.2%) and spleen volume (−49.5%) [94]. Improvements in clinical parameters continued to be observed for long time after treatment [94], and normalization of hemoglobin was observed in all patients.

In general, enzyme replacement therapy (ERT) is considered very safe and effective for patients, many of whom suffered from this life threatening disease but ERT does have its share of limitations. There is room for improvement by modifying the delivery (intravenous infusion) of enzyme to enhance its stability, activity and/or cell-type specific uptake. Improper or sub-optimal glycosylation of these enzymes sometimes result in poor receptor-mediated delivery thereby resulting in inefficient cell targeting and
substrate clearance [95]. The use of surface-modified nanocarrier lipid-based systems such as liposomes, encapsulated with therapeutic agents, for enzyme delivery should substantially enhance the therapeutic response, since the uptake of a single drug loaded nanocarrier by the cell brings multiple copies of therapeutic agents into the cell. Free enzyme uptake and lysosomal delivery is usually by clathrin mediated endocytosis which may be impaired in some cases [79]. It is known that liposomal uptake is by clathrin and caveolae mediated endocytosis [96] and the use of lysosomotropic ligands may allow avoidance of the trafficking pathway from endosomes to the Golgi apparatus or endoplasmic reticulum and thus increase the lysosomal accumulation. Liposomes modified with octadecyl rhodamine B (discussed on page. 32) should improve the lysosomal targeting due to more effective fusion of the endocytosed liposomes with lysosomes. As with all therapeutic proteins, this treatment modality also faces the potential of developing immune response. The development of antiglucocerebrosidase antibodies have been reported among 5 to 15% of patients [97] and handful of cases have been associated with IgE antibodies and anaphylactic-type adverse events [98]. Moreover, severe allergic responses have occurred in patients who received higher dosage administered at a rapid rate [99]. Few patients have also developed infusion-related reactions such as pruritus, urticaria, and chest discomfort [98]. For a rare genetic disease even a handful of anaphylactic events should be considered significant. Use of liposomes practically cause no antigenic reactions or toxic reactions and thus can be considered a good choice for delivery system. Though higher dosing at rapid rate of these enzymes have caused allergic response, high dosing at long dosing intervals have shown statistically significant advantage for two surrogate markers
(chitotriosidase and bone marrow magnetic resonance imaging (MRI) score) of Gaucher’s disease [100]. Higher doses may be achieved by high enzyme encapsulation in liposomes. Since liposomal delivery system is known to ensure the stability of API and given the advantage of higher loaded encapsulation, liposomes may aid in achieving higher payload at the target site.

[4] Liposomes and active targeting strategies

Liposome is a promising nanocarrier, which has been in use for more than three decades as a tool for enhancing the delivery of APIs to their target sites [101]. Liposomes are artificial phospholipid vesicles, varying in size from 50 to 1000 nm, that can be loaded with a variety of guest molecules [102]. They are biologically inert and completely biocompatible. They cause practically no toxic or antigenic reactions and under certain circumstances the liposomes will deliver their contents inside the cell. Moreover, loaded guest molecules are protected from the destructive action of the extracellular environment. Liposomes with specific affinity for an affected organ or tissue increase the efficacy of liposomal pharmaceutical agents; another advantage of using liposome is the ease with which liposomal surface can be modified for targeting purposes. Surface modification of these carriers is often used to control their properties in a desirable fashion and make them simultaneously perform several different functions, for example coupling of transferrin (Tf) to PEG on the PEGylated liposomes render enhanced targetability of drug delivery into solid tumors, which over-express transferrin receptors (TfR) [103]. Targeting tumors with folate-modified liposomes
represents another popular approach, because folate receptors (FR) are over-expressed in a range of tumor cells [104].

[5] Proteins and peptide drugs in liposomes

Liposomes have been used frequently for as pharmaceutical carriers for various proteins and peptide drugs. The increase in the circulation half-life of the liposomal L-asparaginase and the decrease in the antigenicity and susceptibility towards the proteolytic degradation together with the increase in the efficiency have been shown in mice models. Palmitoyl-L-asparaginase in liposomes demonstrated that blood half life can be increased by almost 10-fold [105]. It also demonstrated decrease in acute toxicity and improved anti-tumor activity in vivo. Encapsulation of the L-asparaginase in liposomes also prevents the production of anti-asparaginase antibodies. Among, peptides, cytokines have been frequent guest molecules for liposomal encapsulation. Liposomal encapsulation did increase their lifetime in the body. The incorporation of recombinant interleukin-2 into liposomes increased its blood circulation time by 8-fold [106].

[6] Liposomes in therapy of lysosomal storage diseases

The use of liposome-immobilized enzymes opened new opportunities for ERT [107] especially in the treatment of diseases localized to liver cells, which are natural targets for liposomes. To this effect, the biodistribution studies of β-fructofuranosidase-loaded liposomes were carried out and it demonstrated a good enzyme accumulation in the liver with up to 50% of intracellular enzyme activity localized in the lysosomal fraction.
[107]. Similar results were observed for intravenously administered liposome-encapsulated α-mannosidase [108], neuraminidase [109] and β-glucuronidase [110]. Around the same time, degradation of accumulated glucocerberoside in lysosomes of feline fibroblasts was also demonstrated with liposome-encapsulated β-glucocerebrosidase [111, 112]. The liposomes used in all the experiments lacked surface modification and therefore were not a targeted liposomal system. Considering the success with these non-modified liposomal formulations, liposomal delivery system surface modified with lysosomotropic ligands may enhance the delivery of the guest molecules, enzymes in this case, to lysosomes.

[7] Octadecyl Rhodamine B

Rhodamine B is routinely used for the visualization of lysosomes and other acidic organelles in the live cells [113]. Octadecyl Rhodamine B is a hydrophobized derivative of Rhodamine B and has been widely used for monitoring membrane fusion [114] and for studying lysosomal metabolism [115]. Huth et. al. [116] have already shown the different distribution pattern for hydrophilic dye fluorescein isothiocyanate-dextran (FITC-dextran)-labelled liposomes when compared to the rhodamine-B-phosphoethanolamine (Rh-PE)-labelled liposomes. It was observed that the highly lipophilic Rh-PE-labelled liposomes co-localized with the lysosomotropic dye LysoTracker Red while FITC-dextran-labelled liposomes did not co-localize with the lysosomotropic dye. The authors concluded that Rh-PE might influence the uptake of particles and their intracellular distribution due to its surface-modifying properties. This
experiment showed that modification of liposomal surface with Rh-PE still retained the organelle binding ability of liposomes.
Chapter 2. Rationale for the development of delivery systems

Lipid-based pharmaceutical nanocarriers such as liposomes and micelles have been in use for more than three decades. It is known that these delivery systems not only protect the encapsulated guest molecules from early degradation but also enhance its delivery.

The thesis has been divided into two major sections:

(a) It is a well established phenomenon that in some tumors the endothelial lining of the blood vessel wall becomes more fenestral than the normal tissues. Micelles can diffuse through these fenestrations and accumulate in the interstitial spaces due to size advantage. If these micelles are loaded with active pharmaceutical ingredients (APIs), these APIs can be delivered inside the interstitial space. Further, attaching a targeting moiety to this carrier, which aids in specific recognition of the target site to achieve better targeting, can help enhance the delivery of APIs encapsulated in the micelles. Moreover, use of lipid-core polymeric micelles is also known to effectively overcome the physical and biological barriers related to poor aqueous solubility of this drug and thus its consequent bioavailability. N-[(2-hydroxy-5-nitrophenyl)amino]carbonothioyl]-3,5-dimethylbenzamide (DM-PIT-1), a small molecule non-lipid inhibitor of phosphatidyl-inositol 3,4,5 triphosphate (PIP3) with high apoptotic activity, has a low aqueous solubility (under 30 µM). The use of lipid-core polymeric micelles can help us increase the solubility of this drug, and conjugating the surface of
these micellar formulations with targeting ligands may further enhance the delivery of this drug and its performance.

With this in mind, we decided to develop active targeting micellar drug delivery systems. In particular, we have used TRAIL for this purpose as we observed that DMPIT-1 sensitizes U87MG to killing by TRAIL. Moreover, TRAIL is known to induce apoptosis in a wide variety of cancer cell lines but show little toxicity toward many types of normal cells. Further, TRAIL triggers cell death by activating death receptors (DR) unlike many conventional cancer therapeutic agents that trigger tumor cell apoptosis exploiting the p53 tumor suppressor gene which is inactivated in more than half of human cancers. Thus, we may assume that TRAIL may act as a synergistic tool when used with various chemotherapeutic agents providing enhanced cell killing.

Alternatively, we also prepared micelles conjugated with mAb2C5. Low, sub-therapeutic concentrations of this antibody have been in use as targeting moieties for the tumor-specific delivery of micelles. We used mAb2C5 to enhance the delivery of encapsulated drug (DMPIT-1). Thus, the first approach exploits the functionality and flexibility of micellar delivery system for enhancing the delivery of a pro-apoptotic agent to cancer cells i.e a cell-specific micellar delivery system.

(b) Organelle-specific delivery of therapeutic agents to individual cell organelles is still mainly an unresolved task in drug delivery. The use of drug carrier systems, loaded with therapeutic agents for intracellular delivery should substantially enhance a therapeutic response, since the uptake of a single drug loaded
nanocarrier by the cell brings multiple copies of therapeutic agents into the target cell. In case of Gaucher’s disease, the delivery of enzymes into the lysosomes is highly desirable. Gaucher’s disease is a lysosomal storage disease, which involves deficiency of lysosomal enzyme (glucocerebrosidase) due to genetic mutations. Enzyme replacement therapy is one of the most successful treatment modalities available for Gaucher’s disease. Velaglucerase alfa (VPRIV®, Shire Human Genetic Therapies, Inc, MA, USA), is one of the biosimilars used for the enzyme replacement therapy for Gaucher’s disease. This agent is generated by gene activation of the endogenous human glucocerebrosidase gene in an immortalized human fibrosarcoma cell line. As discussed in the previous chapter, there are limitations to using free enzymes for infusion in enzyme replacement therapy. The liposome may be a good choice for the delivery system in this case, as it is biocompatible, non-toxic, and protects the enzyme from degradation. Thus, the development of a delivery system surface-modified with a lysosomotropic ligand to target lysosomes should be a good option for this enzyme. With this in mind, a lysosome-targeting liposomal delivery system where the surface of a liposomal delivery is modified with a lysosomotropic ligand, octadecyl rhodamine B, was developed. Thus, the second approach deals with the use of liposomal system for specific delivery to lysosomes by surface modification of liposomes with lysosomotropic ligands to enhance the delivery of the APIs/enzymes to the lysosomes.
The overall purpose of this thesis project is to show that surface modification of lipid-based delivery systems with targeting ligands enhances the cell-specific and organelle-specific delivery of the encapsulated guest molecules, using pro-apoptotic small molecule and lysosomal enzyme as examples.

The three specific aims of the project are:

**Section (a)**

1. To develop, characterize and test *in vitro* micellar drug delivery systems loaded with pro-apoptotic small molecule and conjugated with TRAIL or mAb2C5, with this in mind:
   a. Prepare PEG-PE-based micelles loaded with DMPIT-1 and conjugated with TRAIL and characterize them for size and stability;
   b. Investigate the cytotoxicity of these PEG-PE-based micelles loaded with DMPIT-1 and modified with TRAIL against cancer cells *in vitro*;
   c. Develop PEG-PE-based micelles loaded with DMPIT-1, conjugated with mAb2C5 antibody and characterize them for their sizes and stability;
   d. Investigate the cytotoxicity of these PEG-PE-based micelles loaded with DMPIT-1 and modified with mAb2C5 against cancer cells *in vitro*. 
Section (b)

2. To develop and characterize an intracellular lysosome-specific drug delivery system based on liposomes surface-modified with octadecyl-rhodamine B (RhB). With this in mind:
   a. Prepare liposomes surface-modified with octadecyl rhodamine B and characterize them for their size and stability;
   b. Prepare liposomes surface-modified with octadecyl rhodamine B and loaded with Fluoresceinisothiocyanato-dextran (FITC-dextran) and study their intracellular distribution;
   c. Prepare liposomes surface-modified with octadecyl rhodamine B and loaded with FITC-dextran (Fluoresceinisothiocyanato-dextran) and confirm the association with lysosomal fraction.

3. To provide efficient enzyme delivery into lysosomes using liposomes surface-modified with octadecyl-rhodamine B (RhB) (from specific Aim 2) and loaded with FITC-dextran VPRIV and investigate the ability of lysosome-targeted liposomes to deliver enzymes to the lysosomes of a Gaucher cell model (developed by maturation of monocytes) and Gaucher fibroblasts in vitro. With this in mind:
   a. Encapsulate VPRIV into octadecyl rhodamine-modified liposomes (lysosomotropic liposomes and then characterize them for size and stability.
   b. Evaluate the efficiency of liposomal delivery of VPRIV into lysosomes using a Gaucher’s disease cell models in vitro.
Chapter 4. Materials and Methods

Section a
[1] Materials

1,2-Disteratoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)-2000] (PEG-DSPE), 1,2- dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and phosphatidyl-ethanolamine lissamine rhodamine B (Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Triethylamine (TEA) was from Sigma-Aldrich Chemical Co. (St. Louis, MO). Polyethyleneglycol-diphenyl carbonate (PEG-(NPC)$_2$, MW 3400, was from New SunBio (Orinda, CA). Purified monoclonal antibody 2C5 (mAb 2C5) was prepared by Harlan Bioproducts for Science (Indianapolis, IN) using the hybridoma cell line from our laboratory. Recombinant His6-tagged human TRAIL was produced in E.coli and purified using Ni2+ chromatography as previously described [117]. The bacterial expression vector was a generous gift of Dr V. Dixit (Genentech). Polyvinylidene difluoride dialysis membranes with molecular weight cut-off sizes of 100 and 250 kDa were from Spectrum Laboratories Inc. (USA). Cell culture media and supplements were from CellGro (Kansas City, MO). All other reagents and buffer solution components were analytical grade preparations. Reverse osmosis purified and deionized water was used in all experiments. Human glioblastoma cell line U-97 MG (ATCC, HTB-14, TRAIL-resistant), murine melanoma cell line B-16, F1 (ATCC, CRL-6323), and murine metastatic breast cancer cells 4T1 (ATCC, CRL-2539) were purchased from the American Type Culture Collection (Manassas, VA).

pNP-PEG-PE was synthesized according to the earlier published procedure [34]. Briefly, 800 mg of PEG-(NPC)$_2$ was dissolved in chloroform to obtain a 200 mg/ml solution. A further 1.3 ml DOPE (25 mg/ml) was added to the solution. After further addition of 25 µl of TEA, the sample was incubated overnight at room temperature (RT) with stirring under argon. A rotary evaporator was used for the removal of organic solvents. A water bath at RT was used for the formation of pNP-PEG-PE micelles in 0.001 M HCl. Subsequently, a Sepharose CL-4B column (2.5cm x 50 cm) was used to separate the micelles from the unbound PEG and free pNP using 0.001 M HCl as an eluent. Pooled fractions containing pNP-PEGPE were freeze-dried. Finally, pNP-PEG-PE micelles were extracted with chloroform and stored as a chloroform solution at −80 °C.

[3] Preparation of PEG-PE micelles loaded with DM-PIT-1

PEG-PE was dissolved in chloroform at 20 mg/ml. A DM-PIT-1 stock solution at 0.5 mg/ml was obtained by dissolving the DM-PIT-1 in acetonitrile. Two milliliters of DM-PIT-1 stock solution was added to 1.25 ml of a chloroform solution of PEG-PE. The organic solvents were removed by the rotary evaporation to form a thin film of drug/micelle material mixture. This film was further dried under high vacuum overnight to remove any traces of remaining solvent. To form the micelles, the film was re-hydrated in a 10 mM HEPES buffer saline, pH 7.4, and sonicated for 10 min. The non-entrapped, precipitated DM-PIT-1 was removed by filtration through a 0.22 µm filter (Fisher Scientific, USA).
[4] Preparation of surface-modified PEG-PE micelles

Two milliliters of stock solution of DM-PIT-1 (0.5 mg/ml) were added to a mixture of PEG-PE and pNP-PEG-PE (95:5 molar ratio) in chloroform and DM-PIT-1-loaded PEG-PE-based micelles prepared as above. One milliliter of a 12 µM solution of mAb 2C5 or TRAIL (1 mg/ml) in PBS buffer, pH 7.4, was added to 1 ml (for the attachment of mAb 2C5 antibody) or 2 ml (for the attachment of TRAIL) of drug-loaded pNP-PEG-PE containing micelles at the total PEG-PE concentration of 2.2 mM. The pH of the final mixture was adjusted to 8.5–8.8. Incubation for 3 hr at RT resulted in a sufficient mAb 2C5 or TRAIL binding and simultaneous hydrolysis of non-reacted pNP groups. The mAb2C5- antibody- or TRAIL-containing micelles were separated from the unbound mAb2C5 or TRAIL by the dialysis against 10 mM HEPES-buffered saline, pH 7.4, using polyvinylidene difluoride dialysis membranes (MWCO 250 and 100 kDa, respectively, Spectrum Laboratories, Inc. CA). Control micelles were prepared using the same lipid components and in the same concentration as DM-PIT-1-loaded micelles but contained no DM-PIT-1.

The immunological activity of the micelle-attached mAb 2C5 was estimated by the standard enzyme-linked immunosorbent assay (ELISA) using the water-soluble fraction of calf thymus nucleohistone (Worthington Biochemical, Lakewood, USA) as an antigen and horseradish peroxidase/anti-mouse IgG conjugate (ICN Biomedical, Aurora, USA) as a secondary antibody. Anti-mouse IgG/peroxidase conjugate was quantified by the degradation of diaminobenzidine supplied as a ready-for-use solution (Neogen, Lexington, USA). The intensity of the color developed was analyzed by an ELISA.
reader, Labsystems Multiscan MCC/340 (Labsystems and Life Sciences International, Helsinki, Finland). The association of TRAIL with the surface of polymeric micelles was confirmed by dissolving extensively dialyzed micelles in SDS loading buffer (50 mM Tris HCl, pH 6.8, 100 mM DTT, 2% SDS, 12.5% glycerol, 0.1% bromophenol blue), and SDS gel-electrophoresis, followed by Coomassie Blue staining (GelCode Blue reagent, Pierce, Rockford, USA).


Stock solutions stability, freeze-thaw stability, short-term temperature stability, and stability of the DM-PIT-1 extracted from biological samples was monitored as changes in the chromatographic profile of DM-PIT-1. Briefly, stability of stock solutions of DM-PIT-1 (in acetonitrile) was checked in DM-PIT-1 solutions kept at room temperature (RT) for 6 hrs and compared for the peak area (AUC) against freshly prepared stock solution. The freeze-thaw stability was characterized at −80 °C, by subjecting low, medium and high concentrations of DM-PIT-1 to three consecutive freeze–thaw cycles. Part of the samples were stored at −80 °C for 24 hrs and then thawed at RT. The short-term stability of samples was checked on samples kept at RT for 6 h. The low, medium and high concentrations of DM-PIT-1 extracted from plasma were checked for stability after storage of extracts for 1 month at +4 °C.

[6] Quantification of DM-PIT-1 in micelles by HPLC

The amount of DM-PIT-1 in the micellar preparations was determined by the isocratic reverse-phase HPLC (Hitachi, Elite La Chrome, Tokyo, Japan). Before HPLC analyses,
the DM-PIT-1-loaded micelles were destroyed by acetonitrile addition, centrifuged at 14,000 g for 5 min and the amount of DM-PIT-1 determined. The analysis was performed on a C-18 column (Richard Scientific, Novato, USA) with the photodiode-array detection (L-2455, Hitachi, Elite La Chrome, Tokyo, Japan). The elution was performed with an isocratic mobile phase consisting of acetonitrile and water (70:30) with 0.1% formic acid (v/v). The flow rate was 1 ml/min and the total run time was 10 min. DM-PIT-1 were detected by the UV absorbance (320 nm). Detection and quantification limits of DM-PIT-1 in acetonitrile were below 5 and 10 ng/mL, respectively. The calibration curve studied in the range of 10 to 1000 ng/mL was linear, $R^2 = 0.9994$. The retention time for DM-PIT-1 was 6.3 min. All samples were analyzed in duplicate. Drug fraction in micelles (wt%) was calculated as the amount of DM-PIT-1 in micelles divided by the amount of the polymer used in micelle preparation.

[7] Physicochemical characterization and stability

The mean size of different micelles was measured by the dynamic light scattering (DLS) with a scattering angle of 90° at 25 °C using a N4 Plus Submicron Particle System (Coulter Corporation, Miami, FL). The micelle suspensions were diluted with a 10 mM HEPES buffer saline, pH 7.4, until a concentration providing a light scattering intensity of $5 \times 10^4$ to $1 \times 10^6$ counts was achieved. The measurements were done in triplicate. Micelle surface charge analysis was performed using a Zeta Phase Analysis Light Scattering (PALS) UltraSensitive Zeta Potential Analyzer instrument (Brookhaven Instruments, Holtsville, USA). Each sample of micelle suspension was diluted with
deionized distilled water to optimize signal intensity. The measurements were done in triplicate.

The stability of the micelles was monitored by the changes in particle size of the micellar samples during the storage period at 72 hr and 1 week at 4 °C. To test the stability of micelles in the presence of blood serum components, DM-PIT-1-loaded micelles were incubated with 0.2% bovine serum albumin (BSA) at the PEG-PE concentration of 4 mM for 96 hr at RT. The samples were diluted with HEPES-buffered saline, pH 7.4, and analyzed for changes in the micelle size as above.

[8] Cell viability experiments

Human U87MG glioblastoma cells were plated at a density of 1X 10^4 cells/well in 96-well plates (Corning, Inc., Corning, USA). Cells were cultured in DMEM10 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 °C in a 5% CO2 atmosphere. After the overnight incubation, the medium was replaced with medium containing formulated DM-PIT-1 at concentrations ranging from 1 to 200 µg/ml of DM-PIT-1. Cells were incubated at 37 °C, 5% CO2 for an additional 24, 48, or 72 hr. The cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (CellTiter96 (R)AQeuous One Solution Cell Proliferation Assay, Promega, Madison, USA), or with the CellTiter-Glo ATP Viability assay (Promega, Madison, USA) according to manufacturer’s instructions.
[9] Statistical analysis

Statistical analysis was performed using the Student’s t-test, with a p-value of less than 0.05 considered statistically significant.
Section b Part 1
[1] Materials

Egg phosphatidylcholine (ePC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Fluorescein isothiocyanate–dextran (FD, 4000 MW), octadecyl-rhodamine B (Rh), β-galactosidase enzyme (product number G0413), and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO, USA). Bio-Gel A-1.5m was purchased from Bio-Rad (Hercules, CA, USA). Lysosome Enrichment Kit, Coomassie-based protein assay kit, and DyLightTM 350-conjugated goat anti-mouse IgG were obtained from Pierce Biotechnology (Rockford, IL, USA). Lysotracker Red, fluorescein di-β-D-galactopyranoside (FDG), 5- dodecanoylamino fluorescein di-β-D-galactopyranoside (C12FDG) was purchased from Invitrogen/Molecular Probes, Inc. (Eugene, OR, USA). Mouse monoclonal (H4B4) anti-lysosome associated membrane protein antibody (anti-Lamp2) was purchased from Abcam (Cambridge, MA, USA). Mounting medium Fluoromount-G was from SouthernBiotech (Birmingham, AL, USA). Human epithelial cervical cancer cells CCL-2 (HeLa) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell culture media and supplements were from CellGro (Kansas City, MO, USA). All other chemicals and buffer components were analytical grade preparations. Reverse osmosis purified and deionized water was used in all experiments.

[2] Preparation of liposomes

Liposomal formulations used in this study are listed in Table 6. For all preparations, the lipid films were first obtained from a mixture of ePC and CHOL (7:3 molar ratio) in
chloroform. Rh dissolved in ethanol was added to the lipid mixture in chloroform at desired concentrations (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 thin film formed was hydrated with vigorous vortexing with PBS (pH 7.4) or PBS supplemented with FITCD (45 mg/ml). To produce liposomes with a narrow size distribution, the hydrated lipid films were extruded 20 times through double-stacked 200 nm pore size Nuclepore polycarbonate membranes (Whatman, Clifton, NJ, USA) using an Avanti hand extrusion device (Avanti Polar Lipids, Alabaster, AL, USA). The liposomes and non-incorporated FITC-dextran were separated on a BioGel 1.5M. Alternatively, liposomes were loaded with C12FDG [118] a 12-carbon lipophilic variant of fluorescein di-β-D-galactopyranoside (FDG). C12FDG solubilized in DMSO was added to ePC, CHOL, and Rh (7:3:0.1 molar ratio) dissolved in chloroform, and after evaporation of the solvents, the lipid film was re-dissolved in chloroform. After a second chloroform evaporation and freeze-drying of the film, C12FDG-loaded liposomes were prepared by hydration of the lipid film in PBS with subsequent extrusion as described above. Finally, the C12FDG-Rh-liposomes were purified from the non-incorporated Rh and C12FDG by gel-filtration on a BioGel 1.5M column. To estimate C12FDG loading, C12FDG-liposomes were re-suspended in PBS at 150 µg/ml and incubated with or without recombinant β-galactosidase (0.635 µg/ml) for 24 h at 37C. After the liposome dissolution with 0.2% Triton-X100 (to avoid a possible fluorescence resonance energy transfer (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.
[3] **Physicochemical characterization of liposomes**

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

[4] **In vitro stability of liposomes in culture medium**

To measure the stability of liposomes during the prolonged incubation with the culture medium, plain and Rh-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 0.7x25 cm, BioGel A1.5M, PBS). *In vitro* liposomal stability was examined by the incubation of calcein-loaded plain or Rh-modified liposomes (50 µg/ml) with either PBS or DMEM supplemented with fetal bovine serum (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 hours and then solubilized by Triton-X100 treatment (50 µl of 10% Triton-X100 to 2 ml of the liposomal suspension). The percent of calcein release was calculated as the increase in the fluorescent intensity after Triton-X100 solubilization over the background fluorescence intensity.

[5] **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
cells 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.

[6] 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 FACS experiments. When required, cells treated with liposomes for 4 h and washed with DMEM, were additionally incubated for 20 h at 37°C in 5% CO2 in complete liposome-free DMEM.

[7] FACS analysis

The binding of Rh-modified or plain liposomes to cultured HeLa cells was tested by flow cytometry. Control (untreated) HeLa cells or cells treated with liposomes were washed twice with DMEM, trypsinized, re-suspended in 1 ml of ice-cold PBS, and their fluorescence was determined in a fluorescence-activated cell sorter (FACS). Data acquisition was performed on 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 FL2). To eliminate possible overlap of Rh fluorescence with channel FL-1, the compensation between FL-1 and FL-2 channels (1-2 %) was
applied using the cells treated with Rh-modified FD-free liposomes as an additional control. A total of 10,000 events was acquired for each sample.

[8] **Confocal immunofluorescence microscopy**

Intracellular trafficking and localization of Rh-modified liposomes were tested using an upright confocal microscope Zeiss LSM 510 META (Thornwood, NY) equipped with UV (351 and 364 nm), Ar/Kr (458, 477, 488 and 514 nm) and 2°—He/Ne (543 and 633 nm) lasers. 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 PBS wash, quenching with NaBH4 in PBS for 5 min, and another PBS wash. The cells were then permeabilized by incubation with 0.2% saponin and 1% 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 anti-mouse 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 complete 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.
equipped with a 63°Ø, 1.4-numerical aperture plan-apochromat 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).

[9] Preparation of lysosome-enriched fractions

The lysosomal cell fraction was isolated using a Lysosomal Enrichment Kit from Pierce Biotechnology according to the manufacturer’s protocol. HeLa cells were grown to 90% confluence at the conditions described above. The 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 (~0.7-1X10^8 cells per experimental treatment) were collected by trypsinization, washed once with ice-cold PBS, and the cell’s 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’s 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 at the top of a 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 from the top of the tube, washed with PBS to completely
remove traces of the gradient medium, pelleted at 30,000g for 30 min at 4°C (TLA-100.3, Beckman Coulter Optima TLX Tabletop), and then resuspended in equal volumes of cold PBS. Protein concentration in each fraction was measured using a Coomassie-based protein assay [119].

To measure FITC-dextran delivery to lysosomes and Rh distribution through the subcellular fractions, the fluorescent intensity of equal volumes of each fraction was measured in triplicate with a Synergy™ microplate reader (BioTek Instruments, Winooski, VT, USA) at 488/520 nm (ex/em) for FITC-dextran or at 530/590 nm (ex/em) for Rh and normalized to the protein content. The fractional distribution of Rh was calculated as the percent of the fluorescence in each fraction to the total fluorescent intensity of all fractions. The quantity of FITC-dextran in each subcellular fraction was calculated using the calibration curve of standard FITC-dextran solutions in PBS.

In order to identify the lysosomal fraction, β-galactosidase activity was evaluated using fluorescein-di-β-D-galactopyranoside (FDG), a specific fluorescent substrate for the lysosomal β-galactosidase. The lysate obtained from the control (untreated) HeLa cells was subjected to subcellular fractionation at the same conditions as described above. Fifty microliters of each of five fractions was dispersed in 150 µl water supplemented with 15 µM of FDG and incubated for 18 h at 37°C. The fluorescence intensity of the resultant 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 μM) 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.

[10] Statistical analysis
The data were tested for statistical significance using the Student's t-test. P values, calculated with the SPSS 10.00 software package, were considered significant at p < 0.05.
[1] Materials

Egg phosphatidylcholine (ePC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. VPRIV® (velaglucerase alfa for injection), a lysosomal glucocerebroside-specific enzyme indicated for long-term enzyme replacement therapy (ERT) for pediatric and adult patients with type 1 Gaucher disease, was a generous gift from Shire (Lexington, MA, USA). Fluorescein isothiocyanate–dextran (FD, 40,000 MW), octadecyl-rhodamine B (Rh), β-galactosidase enzyme (product number G0413), were purchased from Sigma (St. Louis, MO, USA). Bio-Gel A-1.5m was purchased from Bio-Rad (Hercules, CA, USA). DyLightTM 350-conjugated goat anti-mouse IgG were obtained from Pierce Biotechnology (Rockford, IL, USA). Lysotracker Red, 5-(pentafluorobenzyolamino) fluorescein diglucoside (PFB-FDGlu, P-11947) were purchased from Invitrogen/Molecular Probes, Inc. (Eugene, OR, USA). Mouse monoclonal (H4B4) anti-lysosome associated membrane protein antibody (anti-Lamp2) was purchased from Abcam (Cambridge, MA, USA). Mounting medium Fluoromount-G was from SouthernBiotech (Birmingham, AL, USA). Human histiocytic lymphoma cells (U-937), human fibroblasts (neonates) (PCS 201-010) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Phorbol-12-myristate 13-acetate (P 8139), conduritol-β-epoxide (CBE) (C5424) were obtained from Sigma Aldrich (Saint Louis, MO, USA). Gaucher Type 1 fibroblast (GM00372) was purchased from Corriell Cell Repository, (CCR, Camden, NJ, USA). Cell culture media and supplements were from CellGro (Kansas City, MO, USA). All other chemicals and buffer components were analytical
grade preparations. Reverse osmosis purified and deionized water was used in all experiments.

[2] Preparation of liposomes

For all preparations, the lipid films were first obtained from a mixture of ePC and CHOL (6.9:3 molar ratio) in chloroform. Rh dissolved in ethanol was added to the lipid mixture in chloroform at desired concentrations (1 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 thin film formed was hydrated with vigorous vortexing with PBS (pH 7.4) or PBS supplemented with FITCD 40k (45 mg/ml). Additionally, liposomal formulations with transferrin or β-galactosidase or β-glucocerebrosidase supplemented in PBS were also prepared. To produce liposomes with a narrow size distribution, the hydrated lipid films were extruded 20 times through double-stacked 200 nm pore size Nuclepore polycarbonate membranes (Whatman, Clifton, NJ, USA) using an Avanti hand extrusion device (Avanti Polar Lipids, Alabaster, AL, USA). The liposomes and non-incorporated FITC-dextran 40k were separated on a BioGel 1.5M.

To estimate loading of guest molecules in liposomes, liposomal lysis was carried out with 0.1% CHAPS (v/v). For transferrin loaded liposomes, released transferrin was measured by Coomassie blue at 562 nm. For β-glucocerebrosidase (VPRIV) and β-galactosidase loaded liposomes, the fluorescent intensity of the released enzyme was measured by incubating the enzyme loaded liposomes with 5-(pentafluorobenzoylamino)-fluorescein diglucoside (PFB-FDGlu) and fluorescein di-β-D-galactopyranoside (FDG) respectively and fluorescence was measured on a microplate reader SynergyTM HT (ex/em: 488/520 nm) and normalized for lipid content.
[3] **Physicochemical characterization of liposomes**

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

[4] **Cell culture**

U937 monocytes cells were grown at 37°C in 5% CO2 and 95% humidity in RPMI supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1X MEM non-essential amino acids. Gaucher fibroblasts were grown at 37°C in 5% CO2 and 95% humidity in RPMI supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 1X MEM non-essential amino acids. The fibroblasts were detached by trypsinization with 0.25% trypsin in PBS containing 0.025% EDTA. The cells were used for all experiments up to passage 9.

[5] **Monocyte derived macrophages (MDM) development**

Monocytes were matured into macrophages by culturing it in presence of 10nM phorbol myristate acetate (PMA) in RPMI for 48 hrs at 37°C. The differentiated cells were washed off PMA and incubated with 200 uM CBE in RPMI for 72 hrs to cause β-glucocerebrosidase inhibition [120, 121].
[6] Enzyme recovery in monocyte derived macrophages (MDMs)

Enzyme recovery in MDMs were measured with flow cytometry. The differentiated cells were washed off CBE and were replaced with fresh RPMI. At 0 hrs, the MDMs were detached with 1ml of 4mg/ml Lidocaine and 1 ml 55 uM EDTA and incubated at 37 C for 5 min; remaining adhered cells were detached by spraying it out gently with Lidocaine and EDTA solutions from inside the wells. The differentiated cells were supplemented with RPMI followed by centrifugation at 1000g for 5 min to wash off lidocaine and EDTA. Centrifugation was carried out twice. Later, cells were resuspended in 300 ul of staining medium (serum-free RPMI). Then 300 ul fraction were divided into 3 separate 100 ul fractions. To stain cells for glucocerebrosidase (GC) activity, the cell suspensions were mixed with equal volume of PFBFDGlu to obtain final concentration of 75 uM and incubated it at 37°C for one hour [122]. To terminate substrate loading, the tubes were transferred onto ice and then analyzed by flow cytometry. The flasks were tested, likewise, at 24 hrs, 48 hrs and 72 hrs for enzyme recovery, post-CBE removal.

[7] Interaction of liposomes with cells *in vitro*

Cells grown to 60-80% confluence were incubated with liposomes (at 100 µg/ml lipid concentration) in complete RPMI (15% FBS, 1% antibiotics and 1X MEM non-essential amino acids) for 4 h, washed three times with RPMI to remove non-bound liposomes, and used for microscopy or FACS experiments. When required, cells treated with liposomes for 4 h and washed with RPMI, were additionally incubated for 20 h at 37°C in 5% CO₂ in complete liposome-free RPMI.
[8] FACS analysis

The binding of Rh-modified or plain liposomes to MDMs was tested by flow cytometry. Control (untreated) MDMs or MDMs treated with liposomes were washed twice with RPMI, detached, re-suspended in 1 ml of ice-cold PBS, and their fluorescence was determined in a fluorescence-activated cell sorter (FACS). Data acquisition was performed on 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). To eliminate possible overlap of Rh fluorescence with channel FL-1, the compensation between FL-1 and FL-2 channels (1-2 %) was applied using the cells treated with Rh-modified enzyme-free liposomes as an additional control. A total of 5,000 events was acquired for each sample.

[9] Confocal immunofluorescence microscopy

Intracellular trafficking and localization of Rh-modified liposomes were tested using an upright confocal microscope Zeiss LSM 700 (Thornwood, NY) equipped with UV (351 and 364 nm), Ar/Kr (458, 477, 488 and 514 nm) and 2°—He/Ne (543 and 633 nm) lasers. MDMs grown on glass coverslips 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 PBS wash, quenching with NaBH₄ in PBS for 5 min, and another PBS wash. The cells were then permeabilized by incubation with 0.2% saponin and 1% 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 anti-mouse IgG /A (1:100 dilution) for 60 min at RT followed with five washes with the blocking solution. As an alternative to staining with the antibodies, MDMs treated with liposomes were incubated with 100 nM Lysotracker Red (Ex/Em: 577/590 nm) for 60 min in complete RPMI. 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 700 confocal microscope equipped with a 63°ø, 1.4-numerical aperture plan-apochromat 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).

[10] FACS analysis for enzyme delivery into lysosomes

The delivery of enzymes by Rh-modified or plain liposomes to MDMs and Gaucher’s fibroblasts was tested by flow cytometry. Control (untreated) MDMs, Gaucher’s or MDMs and Gaucher’s cells were treated with liposomes and incubated for 4 hrs; later they were additionally incubated for 20 h at 37°C in 5% CO₂ in complete liposome-free RPMI or MEM. The cells were detached with 1 ml of 4 mg/ml Lidocaine and 1 ml 5 mM EDTA and incubated at 37 C for 5 min; remaining adhered cells were detached by spraying it out gently with Lidocaine and EDTA solutions from inside the wells. The differentiated cells were resuspended with RPMI followed by centrifugation at 1000g for
5 min to wash off lidocaine and EDTA. Later, cells were resuspended in 300 ul of staining medium (serum-free RPMI or MEM). To stain cells for glucocerebrosidase (GC) activity, the cell suspension were mixed with equal volume of PFB-FDGlue to obtain final concentration of 75 uM and incubated it at 37º C for one hour [122]. To terminate substrate loading, the tubes were transferred onto ice and then analyzed by a fluorescence-activated cell sorter (FACS). A total of 5,000 events was acquired for each sample. Same methodology was carried out to measure the enzyme levels in Gaucher's cells and human fibroblasts except the cells were not treated with VPRIV loaded liposomes.


The data were tested for statistical significance using the Student's t-test. P values, calculated with the SPSS 10.00 software package, were considered significant at p < 0.05.
Section a
[1] Preparation and characterization of micellar DM-PIT-1

DM-PIT-1-loaded PEG-PE micelles were prepared using the thin-film hydration method. The incorporation of DM-PIT-1 into these polymeric micelles significantly increased its solubility. Free DM-PIT-1 precipitates from the aqueous solution at ~ 30 µM but micellar drug stays in solution even at a concentration >4 mM. For the detection and quantification of DM-PIT-1 in solubilized/micellar forms we used the reversed phase HPLC method. The chromatographic determination of DM-PIT-1 is represented in Figure 7. DM-PIT-1 resulted in a single symmetric peak without any endogenous sources of interference, at the retention time of 6.3 ± 0.02 min. The resulting calibration curve was linear in the range of 10-1000 ng/ml. The regression equation was $y = 120.17x - 745.38$ ($R^2 = 0.9984$, n=7) where y is the peak area (arbitrary unit) and x is the concentration (ng/ml). The limit of detection was 5 ng/ml at a signal-to-noise ratio of 3:1. The limit of quantitation for DM-PIT-1 was found to be 10 ng/ml with a (relative standard deviation) RSD value of 12.0 % (n=5). This method showed good accuracy of 99.6 ±4.37 %. This HPLC method is a simple and straightforward way to determine DM-PIT-1 concentration in micellar formulations.
Figure 7. (A) HPLC chromatogram and calibration curve of (250 ng DM-PIT-1 in acetonitrile. (B) The calibration curve was determined for the range of 10–1000 ng/ml. Data represents the mean ± SD from 3 independent experiments at seven different concentrations, repeated in triplicate.
Table 1. The stability of stock solutions of DM-PIT-1 expressed as per cent of accuracy\(^1\).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nominal DM-PIT-1 concentration ng/ml</th>
<th>Freeze-thaw stability (n=6)</th>
<th>Short-term stability (RT °C (n=4))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Temperature -80 °C (A)</td>
<td>Temperature -80 °C (B)</td>
</tr>
<tr>
<td>Low QC</td>
<td>50</td>
<td>99.2±5.3</td>
<td>99.8±1.3</td>
</tr>
<tr>
<td>Medium QC</td>
<td>250</td>
<td>100.0±2.9</td>
<td>97.5±2.6</td>
</tr>
<tr>
<td>High QC</td>
<td>1000</td>
<td>100.6±1.6</td>
<td>99.7±5.0</td>
</tr>
</tbody>
</table>

The stability of the stock solutions of DM-PIT-1 was studied (Table. 1) at storage conditions [freeze-thaw -80 °C for 6 hrs (a) or 24 hrs (b)] and at working conditions (RT for 6 hrs) as described in Materials and Methods. DM-PIT-1 solutions in acetonitrile were stable in storage at -80 °C and at RT with average per cent of accuracy from 97.5±5.0 to 101.6±4.7 for low, medium and high QCs.

\(^1\) Accuracy is usually determined by measuring a known amount of standard material under a variety of conditions to ensure that other components do not interfere with the analytical method.
Table 2. Some properties of micellar formulation

<table>
<thead>
<tr>
<th>PEG-PE micellar formulation</th>
<th>Size (nm)</th>
<th>Zeta Potential (-mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain PEG micelles</td>
<td>11.0 ±0.4</td>
<td>4.4 ± 1.6</td>
</tr>
<tr>
<td>DM-PIT-1 loaded micelles</td>
<td>14.4 ±1.8</td>
<td>3.9 ± 4.5</td>
</tr>
<tr>
<td>DM-PIT-1 loaded micelles with mAB 2C5 antibody</td>
<td>15.5 ±0.1</td>
<td>3.6 ± 4.2</td>
</tr>
<tr>
<td>DM-PIT-1 loaded micelles with TRAIL</td>
<td>13.5 ±0.6</td>
<td>4.9 ± 1.5</td>
</tr>
</tbody>
</table>

Characterization of DM-PIT-1-loaded PEG-PE-based micelles including those modified with mAb 2C5 or TRAIL have been provided in Table 2. As one can see from the Table 2, loading with DM-PIT-1 slightly increases the size of plain PEG-PE micelles, while the further attachment of mAb 2C5 or TRAIL to drug-loaded micelles did not have any effect on the micelle size: all DM-PIT-1-loaded micelles had a size of ca. 14 to 16 nm and a narrow size distribution. All PEG-PE-based micelles have a slightly negative surface zeta potential of 4 to 5 mV.
### Table 3. Stability of DM-PIT-1-loaded micelles upon storage

<table>
<thead>
<tr>
<th>Micelle and storage condition</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM-PIT-1 loaded micelles (fresh micelles in presence of BSA)</td>
<td>15.0 ±2.5</td>
</tr>
<tr>
<td>DM-PIT-1 loaded micelles (stored at 4°C for 96 hrs in presence of BSA)</td>
<td>15.9 ±1.6</td>
</tr>
<tr>
<td>DM-PIT-1 loaded micelles (stored at 4°C for 1 week in presence of BSA)</td>
<td>14.6 ±2.9</td>
</tr>
</tbody>
</table>

DM-PIT-1-loaded PEG-PE micelles were stable in aqueous dispersions exhibiting no aggregation for up to 1 week. Micelle incubation in 0.2% BSA at 4°C for 96 hr also did not lead to any changes in micelle size, i.e. no micelle aggregation/destabilization occurs (Table 3).
[2] mAb 2C5- and TRAIL-modified DM-PIT-1-loaded micelles

![Graph showing ELISA results](image)

**Figure 8.** ELISA results for different micellar preparations compared to the non-modified mAb 2C5. Data represent the mean ± SD of triplicate samples.

The ELISA experiments clearly demonstrate that mAb 2C5 attached to the surface of DM-PIT-1-loaded micelles, to a major extent, retains the specific activity required for the successful targeting of drug-loaded micelles to cancer cells (Figure 8). The coupling chemistry used for attaching mAb 2C5 to PEG-PE micelles via pNP groups allowed for the successful coupling of TRAIL with drug-loaded micelles. This association of TRAIL with PEG-PE micelles was determined by standard gel-electrophoresis procedures. The amount of TRAIL attached to the surface of polymeric micelles was approximately 7% of initially added. As shown in Figure 9, the intense TRAIL band in TRAIL-modified PEG-PE micelles confirmed a good association of the protein with micelles. The bands were taken from the gel just to show the presence of TRAIL in both samples and not to compare the relative mobility.
Figure 9. Gel-electrophoresis of TRAIL preparations. 1 – TRAIL attached to micelles via pNP-PEG-PE moieties, 2 – soluble TRAIL (control). Ten microliters of TRAIL-modified PEG-PE micelles were loaded on 12% SDS-PAGE and stained using GelCode® Blue Stain Reagent. Control lane (2) contains 50 ng of the recombinant TRAIL used for conjugation. Gel selection were taken out just to confirm the presence of TRAIL, not to reflect actual mobility.
[3] Cytotoxicity of micellar DM-PIT-1

Figure 10. In vitro cytotoxicity of micellar DM-PIT-1 against murine B-16 melanoma (left) and 4T1 metastatic breast cancer cells (right). Cancer cells were incubated with micellar DM-PIT-1 for 24, 48 and 72 hrs. Data represent the mean ± SD of three experiments and are expressed relative to untreated controls.

The cytotoxic action of DM-PIT-1-loaded PEG-PE micelles on murine B-16 melanoma and 4T1 metastatic breast carcinoma cells after different incubation times with different concentrations of micellar preparations (as DM-PIT-1) is shown in Figure 10. It was seen that micellar DM-PIT-1 effectively kills both types of cancer cells at concentrations around 100 uM. Taking into account the fact that the aqueous solubility of free DM-PIT-1 is under 30 uM, only the micellar form of the drug can be this effective. It was observed that the efficacy of drug action increases with an increase in the incubation time.

Figure 11. Toxicity of the micellar DM-PIT-1 and its combination with TRAIL against TRAIL-resistant U87MG cells. Cells were treated with indicated amounts (in µM) of plain micelles modified with TRAIL (1); micellar DM-PIT-1 (2), and micellar DM-PIT-1 modified with TRAIL (3). The amount of lipid in all wells was kept constant at 2.5 mg/ml. Cell viability was determined after 24 hr using the MTT assay. Data represent the mean ± SD of three independent experiments and are expressed relative to the untreated control (**P<0.01).

In order to check if the cytotoxicity of DM-PIT-1 in PEG-PE micelles can be further improved by combining it with TRAIL, TRAIL-conjugated DM-PIT-1-loaded PEG-PE micelles were prepared and its cytotoxicity was evaluated against TRAIL-resistant U87MG cells. The data presented in Figure 11 show that, while drug-free PEG-PE micelles modified with TRAIL are not cytotoxic towards U87MG cells and DM-PIT-1-loaded PEG-PE micelles demonstrate cytotoxicity within the earlier established range, the additional attachment of TRAIL to drug-loaded micelles results in the synergistic effect and leads
to significantly increased cell death. The viability of U87MG cells was significantly lower in all groups treated with TRAIL-conjugated DM-PIT-1-loaded PEG-PE micelles than in groups treated with the plain DM-PIT-1-loaded micelles groups (p < 0.01).

These data show that the combination of DM-PIT-1 and TRAIL in one micellar preparation provides a convenient method for their simultaneous delivery to cancer cells. These results offer an approach that should be beneficial for a broad range of both TRAIL-sensitive and TRAIL-resistant cancers.
[5] Drug-loaded micelles conjugated with mAb 2C5

Figure 12. The cytotoxicity towards U87MG cells of mAb2C5-conjugated DM-PIT-1-loaded PEG-PE micelles (solid black bars) compared to DM-PIT-1-loaded plain PEG-PE micelles (white bars). Cell viability was determined using the MTT assay. Viability of cells, in the presence of plain micelles and immunomicelles, was observed at various concentrations of DM-PIT-1 loaded in micellar delivery system. Data represent the mean ± SD of three independent experiments and are expressed relative to the untreated control (* p < 0.01).

We have investigated whether or not the modification of DM-PIT-1-loaded PEG-PE micelles with cancer cell-specific mAb 2C5 can further enhance the delivery and consequent toxicity. The data presented in Figure 12 show that the attachment of mAb 2C5 to DM-PIT-1-loaded micelles significantly increased the cytotoxicity of the micellar DM-PIT-1 over the whole investigated DM-PIT-1 dose range (p < 0.01). The increase in the efficacy of immunomicellar DM-PIT-1 may be due to the higher quantity of DM-
PIT-1-containing micelles associated with the cells surface because of the specific interaction of mAb 2C5 immunomicelles with cancer cells.
Drug-loaded micelles conjugated with mAb 2C5 in the presence of TRAIL

Figure 13. The cytotoxicity towards U87MG cells of mAb2C5-conjugated PEG-PE micelles loaded with DM-PIT-1 (solid black bars) compared to DM-PIT-1-loaded plain PEG-PE micelles (white bars), in the presence of the soluble TRAIL at 50 ng/ml. Cell viability was determined using the MTT assay. Viability of cells in the presence of plain micelles, immunomicelles, was observed at various concentrations of DMPIT-1 loaded in micellar delivery system. TRAIL presenting in the system was not conjugated on the micellar surface. Data represent the mean ± SD of three independent experiments and are expressed relative to the untreated control (** p < 0.01, * p<0.05).

The cytotoxicity mAb2C5-conjugated PEG-PE micelles loaded with DM-PIT-1 was compared to DM-PIT-1-loaded plain PEG-PE micelles. Additionally, TRAIL was added into the system separately. TRAIL, in this system, is not conjugated on the micellar surface. Interestingly, it seems like DM-PIT-1 in micelles can overcome TRAIL.
resistance in resistant cells (U87MG cells), since the addition of a soluble TRAIL into the system quite significantly increased the cytotoxicity of both mAb 2C5-modified and non-modified DM-PIT-1-loaded PEG-PE micelles. The maximum effect was achieved with mAb 2C5 conjugated micelles (Figure 13).

[7] Conclusion

In summary, lipid-core polymeric micelles increase the solubility of a novel proapoptotic agent, DM-PIT-1, which has a low aqueous solubility. DM-PIT-1-loaded PEG-PE micelles demonstrated high in vitro cytotoxicity against B-16 melanoma and 4T1 metastatic breast cancer cell lines. This cytotoxicity was further enhanced by additional modification of DM-PIT-1-loaded micelles with TRAIL or with cancer-specific monoclonal antibody. Such preparations have potential usefulness for targeted combination therapy of TRAIL-resistant cancers. Testing these preparations in vivo is the subject of our current research.
Section b Part 1
**[1] Preparation and characterization of liposomes**

### Table 4. Formulations and their abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Formulation</th>
</tr>
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<tbody>
<tr>
<td>Lip-FD</td>
<td>ePC:CHOL (7:3) + FITC-Dextran</td>
</tr>
<tr>
<td>Lip-FD-Rh1%</td>
<td>ePC:CHOL :Rh (7:3:0.1) + FITC-Dextran</td>
</tr>
<tr>
<td>Lip-FD-Rh3%</td>
<td>ePC:CHOL :Rh (7:3:0.3) + FITC-Dextran</td>
</tr>
<tr>
<td>Lip-Rh1%</td>
<td>ePC:CHOL:Rh (7:3:0.1)</td>
</tr>
<tr>
<td>Lip-Rh3%</td>
<td>ePC:CHOL:Rh (7:3:0.3)</td>
</tr>
</tbody>
</table>

### Table 5. Characterization of Liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size (nm)</th>
<th>Zeta Potential ±SD (mV)</th>
<th>FITC-dextran/lipids (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip-FD</td>
<td>189 ± 23</td>
<td>-9.3 ± 3.3 (n = 8)</td>
<td>0.48 ± 0.13 (n = 3)</td>
</tr>
<tr>
<td>Lip-FD-Rh1%</td>
<td>187 ± 16</td>
<td>-8.5 ± 2.9 (n = 8)</td>
<td>0.49 ± 0.10 (n = 3)</td>
</tr>
<tr>
<td>Lip-FD-Rh3%</td>
<td>193 ± 15</td>
<td>-5.7 ± 2.3 (n = 5)</td>
<td>0.47 ± 0.11 (n = 3)</td>
</tr>
<tr>
<td>Lip-Rh1%</td>
<td>233 ± 29</td>
<td>17.0 ± 4.9 (n = 3)</td>
<td>_</td>
</tr>
<tr>
<td>Lip-Rh3%</td>
<td>209 ± 13</td>
<td>21.9 ± 5.9 (n = 3)</td>
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</tr>
</tbody>
</table>

All liposomal formulations (Table 4) were characterized in terms of size, zeta-potential and FITC-dextran content (Table 5). The presence of Rh did not influence the liposome size and the amount of FITC dextran loaded into the liposomes. The presence of positively charged Rh in the membrane of FITC dextran-free liposomes increased their zeta-potential (Table 5). However, Rh-modified and FITC-dextran-loaded liposomes
demonstrated a slightly negative charge that can be explained by the charge neutralization upon association of the negatively charged FITC-dextran [123] with the positively charged liposomal surface.
Figure 14. HeLa cell uptake of liposomes. Flow cytometry fluorescence intensity (channel FL1) of HeLa cells treated with equal amounts of Lip-FD, Lip-FD-Rh1% or Lip-FD-Rh3% for 4 h (grey bars) followed by washing and additional incubation for 20 h (dashed bars). The data are a mean of four different experiments ± SEM. Significant differences from control (untreated) cells: *, p < 0.05; #, p < 0.05; &e, p < 0.05; †, p < 0.05; §, p < 0.01; σ, p < 0.005.

The interaction of Rh-modified liposomes with cells was evaluated by flow cytometry (Figure 14) in order to observe the presence of FITC dextran in cytoplasm. Based on the results of the liposomal stability in complete culture medium, two different protocols for *in vitro* cells-liposomes interaction were used (see Methods). The cells treated with
FITC-dextran-loaded plain (Lip-FD) or Rh-modified liposomes (Lip-FD-Rh1% and Lip-FD-Rh3%) demonstrated a similar total level of FITC fluorescence irrespective of their intracellular distribution. Additional incubation for 20 h in complete liposome-free DMEM after 4 h of the treatment with liposomes led to the decrease in cell fluorescence in all cases that can be explained by the partial quenching of FITC fluorescence and catabolism and elimination of FITC-dextran. Nevertheless, the fluorescence of the cells incubated for 24 h remained significantly increased compared to control (untreated) cells.
**[3] Intracellular localization of Rh-modified liposomes**

**Figure 15. Confocal microscopy of liposome-treated cells.** HeLa cells were treated with Lip-FD (A to C) or Lip-FD-Rh1% (D to 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) FITC-dextran-loaded plain liposomes (green). (B) Lysotracker Red-stained lysosomes (red). (C) Overlay of A and B images with their respective DIC image. (D) Rh (red). (E) anti-
Lamp2 mAb stained lysosomes (blue). (F) Overlay of D and E images with their respective DIC image. Bar = 10 µm.

Figure 15 shows representative confocal fluorescence micrographs of HeLa cells treated with Lip-FD or Lip-FD-Rh1% for 4 h, and after 24 h with further incubation for 20 h. Lysotracker Red or anti-Lamp-2 mAb were used to visualize the lysosomes. The colocalization of FITC-dextran (green channel) and Lysotracker Red (red channel) was followed for the Lip-FD-treated cells, while the colocalization of Rh (red channel) and anti-Lamp2 mAb (blue channel) was used in the case of the Lip-FD-Rh1% treated cells. The results of confocal microscopy (Figure 15, upper panel D to F) showed that 4 h incubation of the cells with Lip-FD-Rh1% led to the localization of Rh mostly in the lysosomes with a high rate of the colocalization with anti-Lamp2 mAb (Pearson’s correlation coefficient, PCC = 0.7; Mander’s overlap coefficient, MOP = 0.8). The cells treated with the same concentration of plain Lip-FD (50 µg/ml) showed much lower localization of FITC-dextran in the lysosomes (PCC = -0.1; MOP = 0.2). It should be noted that, in contrast to Rh-modified liposomes, most of the plain liposomes were not internalized after 4 h incubation with cells and remain on the cell surface (Figure 15A). An additional incubation of Lip-FD-treated cells for 20 h led to an increased co-localization of FITC-dextran with Lysotracker Red labeled lysosomes (PCC = 0.3, MOC = 0.4). However, it was still far less than the co-localization of Rh-modified liposomes and 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 Rh and FITC-dextran components (PCC = 0.7, MOC = 0.8) in the Lip-FD-Rh1%-treated
cells (not shown), indicating that both the lipophilic (Rh) and the hydrophilic (FITC-dextran) components of the liposomal preparation were preferentially concentrated in lysosomes.
Lysosomal Delivery of FITC-dextran by Liposomes

Figure 16. 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 fractions were isolated from the nuclear-free cell lysates by subcellular fractionation and characterized for: (A) β-galactosidase activity (n = 3); (B) fractional distribution of Lysotracker Red (n = 3); (C) fractional distribution of liposomal Rh (n = 8); (D) FITCdextran content (n = 8).
The data are means of n experiments ± SEM. The results of the paired Student’s t-test:

* p < 0.02; # p < 0.002.

In order to evaluate the efficiency of FITC-dextran delivery into lysosomes, we compared the fluorescence intensity of the lysosome-enriched fractions isolated by ultracentrifugation from cell lysates loaded on a discontinuous density gradient of Iodixanol (see Methods). The lysosomal fraction was identified by the β-galactosidase activity (Figure 16A), or by fractional distribution of Lysotracker Red (Figure 16B). Most of the lysosomes were concentrated in the fraction #1 (about 70%) with a minor part (about 15-20%) in the fraction #2. The fractional distribution of liposomal Rh (Figure 16B) correlated well with the fractional distribution of Lysotracker Red with preferential Rh accumulation (60%) in the lysosome-enriched fraction (Figure 16C). As seen in Figure 16D, the efficiency of FITC-dextran delivery into lysosomes provided by Rh-modified liposomes, evaluated by FITC fluorescence intensity of the lysosomal fractions, was 2-fold higher as compared to plain liposomes (Figure 16D). The observed increase was not due to spectral overlap of Rh and FITC channels because the comparison of the liposomal fractions from the cells treated with Rh-modified but FITC-dextran-free liposomes revealed the same level of the autofluorescence as in control, untreated cells. Moreover, the elevation of Rh content in the liposomal membrane from 1 to 3 mol % did not affect the FITC fluorescence of the liposomal fraction and thus eliminated a possible ambiguity of the results due to an overlap of Rh/FITC fluorescence.
Flow cytometric detection of lysosomal targeting using Rh-modified liposomes loaded with C12FDG

To additionally confirm the ability of Rh-modified liposomes to specifically target lysosomes, we developed a new method of evaluation of the lysosomal targeting by flow cytometric analysis of live cells using C12FDG, a lipophilic substrate for the lysosomal β-galactosidase [124]. We proposed that, as presented in the Figure 17, upon undergoing endocytosis, C12FDG-loaded liposomes would eventually fuse with the lysosomes followed by hydrolysis of lysosomal β-galactosidase the non-fluorescent C12FDG to form the fluorescent C12FITC. This fluorescent product should be retained in the lysosomes because of its lipophilic moiety.

Figure 17. Scheme of lysosomal targeting of a cell by C12FDG-loaded liposomes.

Using a standard flow cytometry procedure, the lysosomal targeting was quantified by the fluorescence intensity of live intact cells. We prepared plain and Rh-modified
liposomes loaded with C12FDG and characterized their size, zeta-potential, and the percentage of C12FDG incorporation (Table 8). The loading of C12FDG into the Rh-modified liposomes (as measured by β-galactosidase cleavage of C12FDG localized on the surface of the intact liposomes) was lower than into the plain liposomes and therefore both Lip-Rh liposomes (1 and 3 mol % of Rh) produced approximately ten times less fluorescence than the plain liposomes.
Table 6. Composition and characterization of liposomes loaded with 5-dodecanoylamino fluorescein di-β-D-galactopyranoside (C12FDG).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition (mol %)</th>
<th>Size ± SD (nm)</th>
<th>Zeta potential ± SD (mV)</th>
<th>C12FDG loading Fluorescence Intensity (488/520 nm)</th>
<th>% to plain Lip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip-C12FDG</td>
<td>ePC 70 CHO 30 —</td>
<td>1.5 189 ± 60</td>
<td>-10.1 ± 1.2</td>
<td>3185 ± 383</td>
<td>100</td>
</tr>
<tr>
<td>Lip-C12FDG-Rh1%</td>
<td>ePC 70 CHO 30 1</td>
<td>1.5 190 ± 65</td>
<td>-10.2 ± 1.0</td>
<td>514 ± 53</td>
<td>16</td>
</tr>
<tr>
<td>Lip-C12FDG-Rh3%</td>
<td>ePC 70 CHO 30 3</td>
<td>1.5 191 ± 64</td>
<td>21.6 ± 5.6</td>
<td>346 ± 51</td>
<td>11</td>
</tr>
<tr>
<td>Lip-Rh1%</td>
<td>ePC 70 CHO 30 —</td>
<td>— 192 ± 66</td>
<td>11.5 ± 2.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lip-Rh3%</td>
<td>ePC 70 CHO 30 —</td>
<td>— 192 ± 66</td>
<td>20.4 ± 2.4</td>
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<td>—</td>
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</tbody>
</table>

Figure 18. Flow cytometry of lysosomal targeting by liposomes loaded with C12FDG. HeLa cells were incubated with Lip-FD (20 and 200 µg/ml), Lip-Rh1% (A) or with Lip-Rh3% (B) (200 µg/ml). The liposomes were loaded with C12FDG (1.5 % mol/
mol), a fluorescent substrate for the lysosomal 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 FL1) was determined by the flow cytometry. Each value is the mean ± SD of 2 different experiments.

Such a decrease in the C12FDG loading may be attributed to a stoichiometric competition between C12FDG and Rh 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 µg/ml and 200 µg/ml) were used for cell treatment to achieve the same amount of C12FDG as with Rh-modified liposomes (200 µg/ml). Untreated cells showed the level of the cell autofluorescence. Cells treated with Rh-modified, but C12FDG-free liposomes, were used as an additional control to eliminate any effect of Rh fluorescence due to the possible overlap of channels FL-2 and FL-1. In particular, 0.5% compensation of the FL-1 channel was used for Lip-Rh1% (Figure 18A) and 1% for Lip-Rh3% (Figure 18B). The results presented show 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 can deliver C12FDG to lysosomes in amounts sufficient to be detectable by a flow cytometric analysis. As seen, the cells treated with 200 µg/ml of Rh-modified liposomes demonstrated significantly increased C12FITC fluorescence compared to the cells treated with both 20 and 200 µg/ml of the plain liposomes. These findings on intact cells indicate that the
modification of the liposomal membrane with Rh increases lysosomal targeting compared to plain non-modified liposomes.
Discussion

Earlier, Huth et al. noted that pH-sensitive liposomes loaded with FITC-dextran or labeled with the lipophilic membrane marker rhodamine-B-phosphoethanolamine (Rh-PE) have a different intracellular distribution pattern after being endocytosed by cells. The highly lipophilic Rh-PE was colocalized with the lysosomotropic dye LysoTracker Red, whereas liposomal FITC-dextran was not accompanied by LysoTracker Red in all cases[125]. The authors had concluded that Rh-PE might influence the uptake of particles and their intracellular distribution due to its surface-modifying properties.

In this study, we attempted to demonstrate that liposomes modified with octadecyl-rhodamine B, a lysosomotropic targeting ligand, acquire the ability to specifically target lysosomes and allow for increased delivery of liposome-entrapped substances to these organelles. FITC-dextran was chosen as a model compound (to mimic an enzyme load for ERT) because of its relatively high molecular mass (MW 4400) and stability against digestion in the hydrolytic intra-lysosomal compartment. We prepared liposomes from the mixture of phosphatidylcholine and cholesterol (70:30 mol %) and modified them with the lipophilic octadecyl-rhodamine B (1 and 3 mol %). The Rh-modified and plain non-modified liposomes were loaded with FITC-dextran and their interaction with HeLa cells and the ability to deliver FITC-dextran to lysosomes were investigated using flow cytometry, confocal microscopy, and subcellular fractionation.

By comparison of the fluorescence of intact lysosome-enriched fractions, we have clearly demonstrated an increased (2-fold) lysosomal delivery of FITC-dextran in cells treated with Rh modified liposomes compared to the cells treated with control non-modified liposomes. At the same time, the results of flow cytometry revealed that the
treatment of cells with the equal amounts of plain or Rh-modified liposomes led to the same level of their total FITC fluorescence, which clearly indicates that the increased lysosomal accumulation of FITC-dextran in the case of the cell treated with Rh modified liposomes cannot be explained by different amounts of cell-introduced FITC-dextran due to the enhanced cell-liposome interaction.

It is known that the charge and lipid composition of liposomal membrane may strongly affect the endocytosis of liposomes. Thus, it was shown that HeLa cells internalize positively charged liposomes better than either neutral or negatively charged liposomes [126]. As shown in Table 5, the introduction of octadecyl-rhodamine B into the membrane of negatively charged liposomes increased their zeta potential to moderately positive (17.0 ± 4.9 mV). However, loading of the Rh-modified liposomes with FITC-dextran reversed their positive charge to slightly negative (-8.5 ± .9 mV). This means that there should be no charge-mediated differences between plain and Rh-modified liposomes in their interaction with cells.

The lysosomal targeting of Rh-modified liposomes was also confirmed by the results of confocal microscopy showing an elevated co-localization of the liposomal Rh and specific lysosomal marker Lamp-2. It is important to mention the presence of good co-localization of Rh and FITC-dextran, suggesting a simultaneous accumulation of both the lipophilic (Rh) and hydrophilic (FITC-dextran) liposomal markers (i.e. intact loaded liposomes) within lysosomes. Confocal microscopy also revealed that after 4 h of cell-liposome interaction, a significant part of plain liposomes remained associated with the cell surface indicating a slow rate of their internalization. Although, following an additional 20h incubation, the lysosomal accumulation of the plain liposomes increased,
it remained significantly below that for Rh-modified liposomes. Since such methods as immunostaining for microscopy or subcellular fractionation of cell lysates are quite disruptive, it is important to confirm the results in intact live cells.

Therefore, we employed a new method which allowed us to track the lysosomal accumulation of the internalized liposomes in intact live cells by flow cytometry (Figures 14-15). Using C12FDG, a substrate for the intra-lysosomal β-galactosidase, we clearly showed its increased delivery into the lysosomes by Rh-modified liposomes, confirmed 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, caveolae-mediated endocytosis, phagocytosis and macropinocytosis, and non-clathrin–non-caveolae-dependent endocytosis[127-129]. Thus, it has been shown that HUVEC and COS-7 cells internalize liposomes via both, caveolae- and clathrin-mediated endocytosis [130]. It is also known that the endocytosis by caveolae-dependent mechanism ends up in caveosomes, intracellular vesicles which has a neutral pH compartment which lack markers of endosomes or lysosomes, and can be transported directly to smooth endoplasmic reticulum [131]. Because lysosomes are highly dynamic organelles that can fuse with different cellular compartments, the use of lysosomotropic ligands may allow avoidance of the trafficking pathway from endosomes to the Golgi apparatus or endoplasmic reticulum and increase the lysosomal accumulation. We assumed that if liposomes are internalized by caveolae-mediated endocytosis, then a lysosomotropic ligand such as Rh 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 lysosomal storage disease.

**CONCLUSION**

The modification of the liposomal surface with the lysosomotropic octadecyl-rhodamine B significantly increases the delivery of liposome-loaded model marker, FITC-dextran to lysosomes. Such lysosome-targeted liposomes could be of interest as pharmaceutical carriers for enzyme replacement therapy.
Section b Part 2
Evaluation of enzyme recovery in monocyte derived macrophages (MDMs)

Fluorescence intensity of MDMs pre-incubated with CBE for 72 hrs was measured at 0 hr, 24 hrs, 48 hrs and 72 hrs time periods, post-CBE removal, using 5-(pentafluorobenzoylamino) fluorescein di-ß-D-glucopyranoside (PFB-FDGlu)- a specific ß-glucocerebrosidase substrate (n=3).

It is known that phorbol myristate acetate (PMA) matures the monocytes into macrophages, and conduritol-ß-epoxide (CBE) acts as an glucocerebrosidase inhibitor. Therefore, to develop a disease model of macrophages from monocytes, we treated U937 monocytes with PMA (conc. 10 nM) and later with CBE (200 uM) for 72 hrs. We evaluated the reappearance of enzyme glucocerebrosidase in MDMs by comparing the fluorescence intensity obtained by incubating the MDMs with (pentafluorobenzoylamino) fluorescein di-ß-D-glucopyranoside (PFB-FDGlu) - a lysosomal specific ß-glucocerebrosidase substrate. It can be seen in Figure 19 that after the removal of the
inhibitor, the enzyme levels rose to around 30% within the first hour (macrophages were incubated PFB-FDGlut for 1 hour), 46% after 24 hrs, remained around 50% even after 48 hrs, and rose to 62% after 72 hrs.
[2] Preparation and characterization of liposomal formulation

Table 7. Formulations and their abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip-FD</td>
<td>ePC:CHOL (7:3) + FITC-Dextran</td>
</tr>
<tr>
<td>Lip-FD-Rh</td>
<td>ePC:CHOL :Rh (7:3:0.1) + FITC-Dextran</td>
</tr>
<tr>
<td>Lip-Rh</td>
<td>ePC:CHOL:Rh (7:3:0.1)</td>
</tr>
</tbody>
</table>

Table 8. Characterization of Liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition</th>
<th>Size ± SD (nm)</th>
<th>Zeta-potential ± SD (mV)</th>
<th>FITC-dextran/ lipids (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ePC</td>
<td>Chol</td>
<td>Rh</td>
<td></td>
</tr>
<tr>
<td>Lip-FD</td>
<td>70</td>
<td>30</td>
<td>-</td>
<td>170 ±23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=2</td>
</tr>
<tr>
<td>Lip-FD-Rh</td>
<td>69</td>
<td>30</td>
<td>1</td>
<td>185 ±11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>n=2</td>
</tr>
<tr>
<td>Lip-Rh</td>
<td>69</td>
<td>30</td>
<td>1</td>
<td>207 ±16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n=2</td>
</tr>
</tbody>
</table>

All liposomal formulations (Table 7) were characterized in terms of size, zeta-potential and FITC-dextran content (Table 8). The presence of Rh did not influence the liposome size and the amount of FITC dextran 40 K loaded into the liposomes. The presence of positively charged Rh in the membrane of FITC dextran-free liposomes increased their zeta-potential (Table 8). However, Rh-modified and FITC-dextran-loaded liposomes demonstrated a slight negative charge that can be explained by the charge neutralization upon association of the negatively charged FITC-dextran with the positively charged liposomal surface.
[3] Cell-liposome interaction study by flow cytometry

Figure. 19 Interaction of liposomes with MDM (Monocytes derived macrophages)

Fluorescence intensity (channel FL1) of MDMs-(A)Non-CBE; (B)CBE treated; treated with equal amounts of Lip-FD, Lip-Rh or Lip-FD-Rh for 4 h (white bars) followed by washing and additional incubation for 20 h (black bars) as determined by flow cytometry. The data are a mean of three different experiments ± SEM.

The interaction of Rh-liposomes and plain liposomes with cells was evaluated by flow cytometry in order to follow the extent of FITC-dextran accumulation in the cytoplasm when MDMs were treated with CBE and not treated with CBE. Both, treated and non-treated MDMs showed a similar level of FITC fluorescence irrespective of its distribution in the cells. A decrease in fluorescence was observed in case of additional incubation for 20 hrs in liposome-free medium. This may be attributed to the partial quenching of FITC fluorescence. Despite this decrease in fluorescence, the fluorescence from the 24 hrs incubation group was significantly higher than the control cells.
[4] Intracellular localization of Rh-modified liposomes

Fig 20. Co-localization of liposomal formulations with lysosomal markers
MDMs (CBE treated) were treated with Lip-FD or Lip-FD-Rh for 4 h, followed by washing or by washing and additional incubation for 20 h in liposome-free RPMI. The treated cells were stained with lysosomal markers and analyzed by confocal microscopy. Bar = 10 µm.

Figure 20 shows representative confocal fluorescence micrographs of MDMs treated with Lip-FD or Lip-FD-Rh for 4 h, and after 24 h with further incubation for 20 h. Lysotracker Red or anti-Lamp-2 mAb were used to visualize the lysosomes. The co-localization of FITC-dextran (green channel) and Lysotracker Red (red channel) was followed for the Lip-FD-treated cells, while the co-localization of Rh (red channel) and anti-Lamp2 mAb (blue channel) was used in the case of the Lip-FD-Rh treated cells. The results of confocal microscopy showed that 4h incubation of the cells with Lip-FD-Rh1% led to the localization of Rh mostly in the lysosomes with a high rate of the co-localization with anti-Lamp2 mAb (Pearson’s correlation coefficient, PCC = 0.8; Mander’s overlap coefficient, MOP = 0.9). The cells treated with the same concentration of plain Lip-FD (100 µg/ml) showed much lower localization of FITC-dextran in the lysosomes (PCC = 0.2; MOP = 0.3). Most of the plain liposomes were not internalized after 4 h incubation with cells and remain on the cell surface in contrast to Rh-modified liposomes which were found close to the nucleus. An additional incubation of Lip-FD-treated cells for 20 h led to an increased co-localization of FITC-dextran with Lysotracker Red labeled lysosomes (PCC = 0.3, MOC = 0.4). However, it was still far less than the co-localization of Rh-modified liposomes and anti-Lamp2 mAb-labeled lysosomes (PCC = 0.7, MOC = 0.8).
These experiments were performed to evaluate the feasibility of the MDMs as a cell model for Gaucher’s disease. We observed that the use of CBE and PMA in the development of MDMs did not cause any interference with the fluorescence of FITC-dextran which was used as a model compound in the liposomes. The results obtained were similar to that seen with HeLa cells and therefore we concluded that MDMs can be used as a cell model for evaluating the efficiency of lysosomotropic liposomes in the delivery of enzymes to the lysosomes in vitro.
Preparation and characterization of liposomes loaded with transferrin and β-galactosidase

Table 9. Formulations and their abbreviations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Abbreviation</th>
</tr>
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<tbody>
<tr>
<td>ePC:CHOL (7:3) + Transferrin</td>
<td>Lip-Tr</td>
</tr>
<tr>
<td>ePC:CHOL:Rh (6.9:3:0.1) + Transferrin</td>
<td>Lip-Rh-Tr</td>
</tr>
<tr>
<td>ePC:CHOL (7:3) + β-galactosidase</td>
<td>Lip-β-gal</td>
</tr>
<tr>
<td>ePC:CHOL:Rh (6.9:3:0.1) + β-galactosidase</td>
<td>Lip-Rh-β-gal</td>
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Table 10. Characterization of Liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition (mol %)</th>
<th>Size ± SD (nm)</th>
<th>Enzyme/lipids (ug/mg)</th>
<th>Enzyme/ml of formulation (ug/ml)</th>
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<tr>
<td>Lip-Tr</td>
<td>ePC 70  CHOL 30  Rh —</td>
<td>237 ± 45</td>
<td>5.12 ± 0.54</td>
<td>103.63</td>
</tr>
<tr>
<td>Lip-Rh-Tr</td>
<td>ePC 69  CHOL 30  Rh 1</td>
<td>177 ± 16</td>
<td>9.02 ± 0.23</td>
<td>180.41</td>
</tr>
<tr>
<td>Lip-β-gal</td>
<td>ePC 70  CHOL 30  Rh 1</td>
<td>177 ± 15</td>
<td>2.11 ± 0.38</td>
<td>88.73</td>
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<td>Lip-Rh-β-gal</td>
<td>ePC 69  CHOL 30  Rh 1</td>
<td>177 ± 29</td>
<td>2.4 ± 0.23</td>
<td>119.83</td>
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All liposomal formulations (Table 9) were characterized in terms of size, zeta-potential and respective protein content (Table 10). The presence of Rh did not influence the liposome size and the amount of protein loaded into the liposomes. These studies were carried out to confirm whether lysosomal proteins can be loaded inside liposomes. Transferrin was used as a model load it has a molecular weight close to the
glucocerebrosidase, and β-galactosidase was used for the reason that it is a lysosomal enzyme like glucocerebrosidase and has similar properties.
Preparation and characterization of liposomes loaded with VPRIV

Table 11. Formulations and their abbreviations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>ePC:CHOL (7:3) + VPRIV</td>
<td>Plain-Lip</td>
</tr>
<tr>
<td>ePC:CHOL:Rh (6.9:3:0.1) + VPRIV</td>
<td>Rh 1% Lip</td>
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Table 12. Characterization of Liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition (mol %)</th>
<th>Size ± SD (nm)</th>
<th>Zeta-potential ± SD (mV)</th>
<th>Enzyme/ml of formulation (Units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ePC</td>
<td>CHOL</td>
<td>Rh</td>
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<tr>
<td>Plain Lip</td>
<td>70</td>
<td>30</td>
<td>—</td>
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<td></td>
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<td>(n=4)</td>
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</table>

Both liposomal formulations (Table 11) were characterized in terms of size, zeta-potential and protein content (Table 12). The presence of Rh did not influence the liposome size. In our previous experiments, we had observed that the presence of positively charged Rh in the membrane created a positively charged delivery system, while Rh-modified FITC-dextran-loaded liposomes demonstrated a slight negative charge. We observed a similar trend in this case too. It seems like there might be a charge neutralization upon VPRIV association with the liposomal surface.
Enzyme level determination

Figure 21 Enzyme level determination

Enzyme levels were determined for two cell lines (Gaucher’s and Normal Human fibroblasts). Cells were treated with PFB-FDG glu, a commercially available substrate that interacts with glucocerebrosidase inside lysosomes, and resultant fluorescence intensity (channel FL1) was determined with flow cytometry. Each value is the mean ± SD of 3 different experiments.

The levels of enzymes in Gaucher’s fibroblasts and normal human fibroblasts were determined by measuring the fluorescence intensity (channel FL1) with flow cytometry after cells were treated with PFB-FDG glu, commercially available substrate that interacts with glucocerebrosidase inside lysosomes (See Methods). As expected, the fluorescence intensity measured in normal fibroblasts was 4-folds higher than in Gaucher’s fibroblasts, which indicates the absence/lower levels of the enzyme in the latter.
[8] Lysosomal delivery of VPRIV rhodamine modified liposomes

Fig 22 Flow cytometry of lysosomal targeting by liposomes loaded with VPRIV. Two different cell lines, MDMs [A] and Gaucher’s [B], were incubated with Plain Lip (L) [100 ug/ml lipid], Rh1% Lip [100 ug/ml lipid], Plain Lip (E) [enz. equivalent of Rh1% Lip]
or with free VPRIV. After 4 h incubation with liposomes, the cells were washed with and additionally incubated for 20 h with liposome-free MEM or RPMI respectively. The fluorescence intensity (channel FL1) was determined by the flow cytometry after treating the cells with PFB-FDGGlu. Each value is the mean ± SD of 3 different experiments. (* \( p<0.01 \); ## \( p<0.01 \); ** \( p<0.05 \))

In order to evaluate the efficiency of enzyme delivery into lysosomes, we compared the fluorescence intensity of the cells after the liposomal treatment and subsequent incubation with commercially available lysosome-specific substrate 5-(pentafluorobenzoylamino) fluorescein diglucoside (PFB-FDGluc). Two different cell lines, Gaucher’s fibroblasts and MDMs were incubated with Plain Lip (L) [100 ug/ml lipid], Rh1%-Lip [100 ug/ml lipid], Plain Lip (E) [enz. equivalent of Rh1%-Lip] or with free VPRIV [4.2 mUnits/ml]. Plain Lip (L) represents the lipid equivalent to that present in Rh 1% Lip while Plain Lip (E) represents the enzyme equivalent to that present in Rh 1% Lip. After 4 h incubation with liposomes, the cells were washed with and additionally incubated for 20 h with liposome-free MEM or RPMI and subsequent steps were carried out before measuring with FACS, as mentioned (See Methods). As seen in Figure 22 A and B, the efficiency of enzyme delivery into lysosomes provided by Rh-modified liposomes was significantly higher as compared to plain liposomes.
Discussion

In our earlier experiments, it has been proved that liposomes modified with octadecyl-rhodamine B, a lysosomotropic targeting ligand, acquire the ability to specifically target lysosomes and allow for increased delivery of liposome-entrapped substances to these organelles. We had used FITC-dextran as the model compound (to mimic the API). The experiments were performed using HeLa cells. In this study, we have attempted to demonstrate that liposomes modified with octadecyl-rhodamine B, a lysosomotropic targeting ligand, delivers the encapsulated load into lysosomes of Gaucher’s cells and monocyte derived macrophages (MDMs). We used FITC-dextran (MW: 40 KDa) as the model compound in the initial studies; we also prepared VPRIV-loaded liposomes and tested them on Gaucher’s cells and MDMs. The Rh-modified and plain non-modified liposomes were loaded with FITC-dextran and their interaction with MDMs and the ability to deliver FITC-dextran to lysosomes were investigated using flow cytometry and confocal microscopy, the Rh-modified and plain non-modified liposomes loaded with VPRIV, their ability to deliver VPRIV to lysosomes of Gaucher’s fibroblasts and MDMs were investigated using flow cytometry.

As mentioned, previous experiments were carried out in HeLa cells. The results, though conclusive, did not apply to lysosomal storage disease conditions. Studies performed with lysosomal storage disease cell models were therefore required to demonstrate that these delivery systems can be used for potential therapy of lysosomal storage disease. Since macrophages are the main cell type affected in Gaucher disease, we developed monocyte derived macrophages (MDMs), which resemble macrophages of Gaucher’s Type 1. We used monocyte U937 for the development of MDMs. It is known that
addition of PMA (10 nM) in complete medium causes complete stoppage of cell proliferation and initiates the maturation of monocytes into macrophages, and the monocytes start adhering to and completely spread on the bottom of culture flasks. Daigneault et al. 2010 have reported that PMA stimulation followed by resting in the absence of PMA increases the number of lysosomes and other organelles in THP-1 cells [132]. The cells were incubated with PMA for 48 hrs after which PMA was removed and replaced with RPMI containing CBE (200uM) and incubated for 72 hrs. Das et al have already showed complete inhibition of glucocerebrosidase when rat peritoneal macrophages are treated with CBE at 100 uM for 2hrs. These developed macrophages were treated with PFBFDG-Glu, an enzyme-specific substrate (see Method), and the fluorescence was measured with flow cytometry to evaluate the enzyme levels. We noticed that enzyme levels bounced back to 25-30% (Figure 19) within an hour of CBE removal. This closely resembled Gaucher's macrophages which typically have 5-25% enzyme activity as compared to wild type.

We carried out flow cytometry and confocal microscopy experiments to check whether the developed model could be used for studies with liposomal formulation containing enzyme. With this mind, the Rh-modified and plain non-modified liposomes loaded with FITC-dextran (MW 40,000) were prepared because the molecular weight of FITC-dextran was closer to the VPRIV (63 kDa). The interaction of Rh liposomes and plain liposomes with MDMs were evaluated by flow cytometry in order to follow the extent of FITC-dextran accumulation in the cytoplasm when MDMs were treated with CBE and not treated with CBE (Figure 19). We also carried out confocal microscopy experiments
with MDMs. The developed model proved to be robust and therefore was used for further studies with VPRIV loaded liposomes.

We also prepared transferrin-loaded liposomes and β-galactosidase-loaded liposomes. Transferrin was used as a model load. It has a molecular weight close to the glucocerebrosidase, and β-galactosidase was used because it is a lysosomal enzyme like glucocerebrosidase and has similar properties. These studies confirmed that lysosomal proteins can be loaded inside liposomes. Finally, we prepared VPRIV-loaded Rh modified liposomes and compared the cellular delivery by VPRIV-loaded liposomes with that to non-modified liposomes. The comparison of the fluorescence determined by flow cytometry clearly demonstrated an increased (1.5-fold) lysosomal delivery of VPRIV in cells treated with Rh-modified liposomes, compared to the cells treated with control non-modified liposomes indicating that Rh-modified liposomes increased the delivery of enzymes into these organelles. It is known that nanoparticles (10-200 nm) are endocytosed through clathrin-mediated endocytosis and caveolae-mediated endocytosis while the free enzyme uptake and lysosomal delivery is usually by clathrin-mediated endocytosis which may be impaired in some cases [77]. The use of lysosomotropic ligand-modified liposomes may therefore aid delivery despite an impaired clathrin-mediated endocytosis and allow the avoidance of the trafficking pathway from endosomes to the Golgi apparatus or endoplasmic reticulum due to the presence of a lysosomotropic ligand and thus further increase the lysosomal accumulation. Liposomes modified with octadecyl rhodamine B could improve the lysosomal targeting due to more effective fusion of the endocytosed liposomes with lysosomes.
Conclusion

The modification of the liposome surface with the lysosomotropic octadecyl-rhodamine B significantly increases the delivery of lysosomal enzyme VPRIV, to lysosomes in Type 1 Gaucher’s fibroblasts and MDMs.
Chapter 6. Conclusions

Section a
Lipid-core polymeric micelles were successfully used to increase the solubility of a novel proapoptotic agent, DM-PIT-1, which has an intrinsic low solubility. Its cytotoxicity was further enhanced by additional modification of DM-PIT-1-loaded micelles with TRAIL or with cancer-specific monoclonal antibody. Such preparations could be useful for targeted combination therapy of TRAIL-resistant cancers. Testing these preparations in vivo is the subject of our current research.

Section b Part 1
The modification of the liposome surface with the lysosomotropic octadecyl-rhodamine B significantly increased the delivery of liposome-loaded model marker, FITC-dextran to lysosomes. Such lysosome-targeted liposomes are of clear interest as pharmaceutical carriers for enzyme replacement therapy.

Section b Part 2
The modification of the liposome surface with the lysosomotropic octadecyl-rhodamine B significantly increased the delivery of the therapeutic enzyme, VPRIV to lysosomes in Type 1 Gaucher's fibroblasts and MDMs.
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