Enriching lipid nanovesicles with short-chain glucosylceramide improves doxorubicin delivery and efficacy in solid tumors

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ABSTRACT For amphiphilic anticancer drugs, such as the anthracyclin doxorubicin (Dox), uptake by tumor cells involves slow diffusion across the plasma membrane, a limiting factor in clinical oncology. Previously, we discovered that preinsertion of short-chain sphingolipids such as N-octanoyl-glucosylceramide (GC) in the tumor cell membrane enhances cellular Dox uptake. In the present study, we apply this strategy in vitro and in vivo by coadministering GC and Dox in a lipid nanovesicle (LNV). GC enrichment of Dox-LNVs strongly enhanced in vitro cytotoxicity toward B16 melanoma and A431 carcinoma, as evidenced by 6-fold decreased IC₅₀ values compared with Dox-LNVs. This correlated with enhanced cellular Dox uptake observed by confocal microscopy. Intravital optical imaging in window chamber-bearing mice with orthotopically implanted B16 melanoma demonstrated enhanced GC-mediated Dox delivery to tumor cells. Treatment of nude mice bearing human A431 xenografts with 6 mg/kg GC-Dox-LNVs almost doubled the tumor growth delay compared with Dox-LNVs. A second administration of 5 mg/kg after 3 d induced even 3-fold delay in tumor growth, while no systemic toxicity was found. GC-enriched Dox-LNVs displayed superior in vitro and in vivo antitumor activity, without systemic toxicity. This new drug delivery concept, aiming at increased membrane permeability for amphiphilic drugs, provides an opportunity to improve cancer chemotherapy.—Van Lummel, M., van Blitterswijk, W. J., Vink, S. R., Veldman, R. J., van der Valk, M. A., Schipper, D., Dicheva, B. M., Eggermont, A. M. M., ten Hagen, T. L. M., Verheij, M., Koning, G. A. Enriching lipid nanovesicles with short-chain glucosylceramide improves doxorubicin delivery and efficacy in solid tumors. FASEB J. 25, 280–289 (2011). www.fasebj.org

Key Words: chemotherapy • liposome • nanomedicine • short-chain sphingolipid • cell membrane permeability

Insufficient delivery of chemotherapeutic agents to their intended cellular and molecular targets within the tumor cell remains a major obstacle in clinical oncology. Most of these drugs are amphiphilic, and their (passive) diffusion through the plasma membrane is therefore slow and a major limitation for sufficient uptake within the tumor cell. While extensive work has been devoted to multidrug transporters, i.e., transmembrane P-glycoproteins that (actively) pump drugs out of the cell (1), no successful attempts have yet been made to modulate the lipid composition of the plasma membrane to enhance the cellular uptake of these amphiphilic anticancer agents. This novel approach is addressed here.

The lipid distribution in the plasma membrane is heterogeneous and also differs between the inner and outer leaflet (2). The latter is highly enriched in sphingolipids, i.e., sphingomyelin (SM) and glycosphingolipids, and consists of microdomains (lipid rafts and caveolae) that are formed on the basis of the auto-organizing properties of these sphingolipids, both with themselves and in association with cholesterol (3, 4). Due to microdomain occurrence, the plasma membrane should be considered as a mosaic-like patchwork with a differentiated lipid distribution (5). Microdomain formation may create local differences in lipophility, membrane fluidity, and lipid packing, which determines transmembrane diffusion of amphiphilic drugs. We hypothesized that modifying the tumor cell membrane with short-chain sphingolipids, (SCSs) could cause imperfect lipid packing or affect auto-organization.

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of sphingolipids and might therefore facilitate the transmembrane diffusion of doxorubicin (Dox) and other amphiphilic drugs.

Indeed, we identified a series of SCSs that, when preinserted in the plasma membrane, greatly and specifically enhanced the uptake and action of various anticancer drugs in vitro, without causing membrane leakage, toxicity, or other trivial effects by itself (6). The drug-uptake enhancing effect was related to the lipophilicity of the drug, with Dox showing the highest effect (up to 4-fold) in a panel of tumor cell lines and in endothelial cells. This finding is suggestive of an improved therapeutic index for combinations of SCS and Dox compared with Dox alone.

Dox has become one of the most often used drugs for treatment of cancer. Its antitumor efficacy is primarily attributed to direct interactions with DNA or DNA topoisomerase (7). To deliver Dox and the SCS to tumors simultaneously, lipid nanovesicles (LNVs), or liposomes carrying the SCS in their bilayer and Dox in the aqueous phase, were developed (Fig. 1). Formulation of drugs in liposomes provides an attractive improvement for anticancer drug administration (8–11). Liposomes are generally considered nontoxic, biodegradable, and nonimmunogenic. Hydrophobic drugs can be entrapped in the aqueous compartment (11, 12), while the lipid bilayer can be utilized to incorporate hydrophobic drugs (13, 14). Associating a drug with liposomes markedly changes its pharmacokinetic properties and lowers systemic toxicity; furthermore, the drug is prevented from early degradation and/or inactivation in circulation (12). In normal vasculature, these nanovesicles remain confined to the vascular compartment. Tumor sites are characterized by immature and leaky vasculature due to angiogenesis (15, 16). The favorable circulation properties of long-circulating liposomes in combination with the leaky vasculature results in an enhanced accumulation of entrapped drugs in the tumor area both in animal tumor models (17–19) as well as in cancer patients (20, 21).

Nowadays, several liposomal products merely entrap

![Figure 1. Schematic representation of GC-enriched Dox-LNVs. Dox-containing LNVs (consisting of HSPC, cholesterol, and DSPE-PEG2000) are modified by incorporating the short-chain sphingolipid GC in the lipid bilayer. Dox is remotely loaded into the liposomes via an ammonium sulfate gradient.](image-url)

GLUCOSYLCERAMIDE IMPROVES EFFICACY OF DOXORUBICIN

MATERIALS AND METHODS

Reagents

Hydrogenated soy phosphatidylcholine (HSPC) and distearoylphosphatidylethanolamine (DSPE)-PEG2000 were from Lipoid (Ludwigshafen, Germany), and C8-glucosylceramide (GC) was from Avanti Polar Lipids (Alabaster, AL, USA). Polycarbonate filters were from Northern Lipids (Vancouver, BC, Canada), and PD-10 Sephadex columns were from GE Healthcare (Diegem, Belgium). Doxorubicin-HCl (Dox) was from Pharmachemie (Haarlem, The Netherlands). Cholesterol, sodium 3’-[1-phenylaminocarbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT), sulforhodamine B (SRB), and the fixatives formaldehde and ethanol/acetic acid/formol saline (EAF) were from Sigma-Aldrich (Zwijndrecht, The Netherlands). Hematoxylin and eosin (HE) staining was from VWR, Inc. (Amsterdam, The Netherlands).

Preparation of GC-enriched LNVs

LNVs were prepared by lipid film hydration and extrusion and subsequent loading of Dox using an ammonium sulfate method (30, 31). Mixtures of HSPC, cholesterol, and PEG2000-DSPE (molar ratio 1.85:1:0.15) were prepared. To these lipids, 0.05, 0.1, or 0.15 mol GC was added per mole of lipid. Lipids dissolved in chloroform/methanol (9:1 v/v) were mixed, and a lipid film was created under reduced pressure on a rotary evaporator and subsequently dried under a stream of nitrogen. LNVs were formed by addition of a 250 mM (NH4)2SO4 solution to the lipid film. The hydrated lipid
dispersion was sized, successively, by repeated extrusion through sequentially 200-, 100-, and 50-nm polycarbonate filters. Nonencapsulated (NH₄)₂SO₄ was removed by gel permeation chromatography using a PD-10 Sephadex column, eluted with 123 mM citrate buffer, pH 5.5. Dox was added in a ratio of 0.2:1 (w/w) Dox to total lipid and incubated for 1 h at 65°C. Nonencapsulated Dox was removed by ultracentrifugation at 200,000 g in a Beckman ultracentrifuge (Beckman Coulter, Woerden, The Netherlands). The LNV pellet was resuspended in buffer containing 135 mM NaCl and 10 mM HEPES, pH 7.4.

LNV formulations with 0, 5, 10, or 15 mol% of GC all had similar characteristics. Particle size, as measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), was ~20 ± 0.5 nm, and polydispersity index was always <0.1, demonstrating a homogeneous particle population. Dox concentration was 1.25 ± 0.13 mg/ml, as measured spectrophotometrically after destruction of the LNV bilayer using a 1% Triton X-100 solution, and remained constant during storage.

Cell culture

B16 cells (murine melanoma) and A431 cells (human squamous carcinoma) were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in DMEM, supplemented with 10% (v/v) FCS, and 4 mM l-glutamine. Cells were subcultured weekly by trypsinization and maintained in a water-saturated atmosphere of 5% CO₂ at 37°C. Cells used for experiments were obtained from cultures with a confluency of 80–90%.

Cell toxicity

B16 cells and A431 cells were plated in flat-bottom 96-well plates at a density of 5000 cells/well in culture medium and allowed to adhere overnight. Cells were incubated with the various Dox-LNV formulations in culture medium for 24 h. Cell survival was determined by measurement of the mitochondrial-dependent conversion of XTT into a water soluble orange formazan dye (32). Before use, 100 μl electron coupling reagent 3-amino-phenyl dibenzopyrazine methysulfate (1.25 mM in PBS; Sigma) was added to 5 ml XTT solution (1 mg/ml in RPMI 1640). After incubation of the cells, drug-containing medium was replaced by 150 μl fresh culture medium to which 50 μl XTT solution was added. Next, cells were incubated for 1 h at 37°C, after which XTT conversion was detected by measuring absorbance at 490 nm in a PerkinElmer Victor Wallac plate reader (Perkin Elmer, Groningen, The Netherlands). Alternatively, cell survival was determined by measuring total cellular protein levels using the SRB assay (33). For both assays, cell survival was calculated as a percentage relative to nontreated control cells, which were set at 100%. The drug concentration reducing the survival to 50% of the control (IC₅₀) was calculated from the growth curves after fitting the data points according to the Richard’s equation (34) using GraphPad Prism software (GraphPad, San Diego, CA, USA).

Confocal microscopy

B16 and A431 cells were cultured on a cover glass coated with 0.1% gelatin. Dox formulated in LNVs with or without GC was added at a concentration of 0.1 or 1.0 μg/ml. Cells were incubated for the indicated time, after which they were washed with PBS and fixed for 20 min with a 4% paraformaldehyde solution in PBS. Cover glasses were mounted on glass slides using Vectashield (Bruschwig, Amsterdam, The Netherlands). Dox uptake and distribution were visualized using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Sliedrecht, The Netherlands) using the 543-nm laser and 560- to 615-nm bandpass filter. Images of the different incubations were taken using identical settings for laser power, pinhole, and photomultipliers.

Intravitral optical imaging

Intratumoral Dox distribution and cellular uptake were studied by intravitral optical imaging. Specific pathogen-free C57BL/6 mice (Harlan-CPB, Zeist, The Netherlands) were used, which received a standard laboratory diet ad libitum (Hope Farms, Woerden, The Netherlands). The syngeneic B16 melanoma tumor model was used for this study. All intravitral studies were done in accordance with protocols approved by the Committee on Animal Research of the Erasmus Medical Center (Rotterdam, The Netherlands). Preparation of the dorsal skin-fold chamber implanted orthotopically with a small piece of tumor (0.1 mm³) obtained from in vivo-maintained B16 melanoma tumor was done as described previously (29). Experiments began 10–14 d after implantation of the dorsal skin-fold chamber. Mice were injected intravenously with 5 mg/kg Dox formulated in LNVs with or without 10 mol% GC. Images were taken 24 h after injection. Mice were anesthetized using isoflurane and fixed to the heated microscope stage. Dox uptake and distribution were studied using a Zeiss LSM 510 Meta confocal microscope using the 543-nm laser and 560- to 615-nm bandpass filter. Images of the 2 treatment groups were taken using identical settings for laser power, pinhole, and photomultiplier using a ×10 or ×20 lens.

In vivo therapeutic efficacy of Dox-LNVs

Male BALB/c nude (nu/nu) mice, with ages between 6 and 10 wk (18–30 g) were obtained from the animal department of the Netherlands Cancer Institute. Animals were kept and handled according to institutional guidelines complying with Dutch legislation and received a standard diet and acidified water ad libitum. Mice were anesthetized by intraperitoneal injection of a 1:1:2 (v/v) mixture of Hypnorm (VetaPharma, Leeds, UK), Dormicum (Roche, Woerden, The Netherlands), and water. Next, mice were injected subcutaneously at the lower back with 1 × 10⁶ A431 cells in 50 μl physiological NaCl solution, and tumor volume was measured regularly, using a caliper (palpation). Tumor size was calculated using the following formula: volume = π/6 × length × width × height, where tumor volume at the start of treatment was set at 100%. When the tumor reached a mean diameter of ~5 mm (measured in 3 orthogonal directions), treatment was started. To determine the optimal Dox treatment, various concentrations of Dox-LNVs (6, 8, and 10 mg Dox/kg body weight) were administered. For each treatment, 3 different groups (5 mice/group) were distinguished: control (physiological NaCl solution), standard Dox-LNVs, and GC-enriched Dox-LNVs. Vehicle or Dox-LNV preparations were injected intravenously via the tail vein in mice placed in custom-designed jigs. Tumor growth and body weight measurements were conducted for a period of 14–20 d. Tumor growth delay for reaching 200% initial volume was calculated using GraphPad Prism 4.00 for Windows.

Toxicity and histopathology

To evaluate acute Dox-induced toxicity, mice were killed by CO₂ inhalation 8 d after treatment, and full necropsy was
performed. Tissues were fixed in 4% buffered formaldehyde or EAF (40:5:10:45, v/v), embedded in paraffin, sectioned to 3-μm slices, stained with HE, and examined by an experienced mouse pathologist. Tissues examined include testis, ovary, kidney, liver, spleen, intestines, thymus, heart, lungs, extremities, salivary gland, whole head (brain, pituitary gland, and incisors), and sternal and femoral bone marrow. As controls, nontreated tumor-bearing mice were examined.

Hematology

At d 2, 4, 6, and 8, blood was collected to assess Dox-induced cytotoxicity as defined by hematological parameters. Blood was collected by heart puncture, with EDTA as an anticoagulant. Measurements of red blood cell counts, hemoglobin and hematocrit levels, the derived mean platelet volume, and white blood cell counts (including differentiation) were performed on a Sysmex K-4500 machine (Goffin-Meyvis, Tiel, The Netherlands).

Statistics

The tumor sizes in the in vivo experiments were indicated as mean ± se tumor size (weight or volume). IC50 calculations were done using GraphPad Prism 4.00 for Windows. Student’s t tests were performed with SPSS 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

GC enhances cytotoxicity of Dox-LNVs in vitro

Dox-LNVs coformulated with 5, 10, or 15 mol% GC and Dox-LNVs lacking GC were prepared and characterized (see Materials and Methods). Treatment of B16 melanoma and A431 carcinoma cells with these Dox-LNV formulations for 24 h reduced the cell viability in a dose-dependent fashion (Fig. 2). In both cell lines, the toxicity of Dox-LNVs increased with increasing mole percentage of GC, with an optimum of 10 mol% (15 mol% GC showed no further toxicity increase). The IC50 value of Dox formulated in 10 mol% GC-Dox-LNVs was >6-fold lower than that of Dox-LNVs without GC (Table 1) and similar to 15 mol% GC-Dox-LNVs. Dox-LNVs with 5 mol% GC caused intermediate IC50 values. Data shown were obtained by XTT viability assays and were similar when using SRB viability assays (not shown). GC itself, at the concentrations used here, did not affect cell viability (30). Empty LNVs containing 10% GC also did not have any effect on cell survival (not shown). Thus, the increased efficacy of the GC-modified Dox-LNVs is most likely due to enhanced intracellular Dox delivery.

Increased intracellular delivery of Dox by GC-enriched LNVs in vitro

To show the enhanced intracellular delivery of Dox by GC-enriched LNVs, B16 and A431 cells were incubated for various periods with Dox (1 µg/ml) encapsulated in LNVs with or without 10 mol% GC.
on tumor growth in vivo

To investigate the effect of GC-enriched Dox-LNVs

Enhanced antitumor efficacy of GC-enriched
vitro

Because uptake in tumor cells, the image shown in Fig. 4

distances from tumor-feeding blood vessels (Fig. 4

distribution within the tumor tissue, also at greater
intensities and showed a much more homogeneous
delivery in the tumor (Fig. 4). Dox from standard
LNVs (Fig. 4A–D) appeared at low intensities and
located in proximity of tumor blood vessels, whereas
Dox delivered from GC-LNVs appeared at higher
intensities and showed a much more homogeneous
distribution within the tumor tissue, also at greater
distances from tumor-feeding blood vessels (Fig.
4E–H). Higher-magnification images demonstrated
significantly more Dox accumulation in tumor cell
nuclei after treatment with GC-Dox-LNVs (Fig.
4G, H), than with standard Dox-LNVs (Fig. 4C, D). Bes-
sides uptake in tumor cells, the image shown in Fig.
4H is suggestive of Dox uptake by vascular endothelial
cells. These intravital imaging data clearly con-
firm the enhanced intracellular delivery observed in
vitro.

Enhanced antitumor efficacy of GC-enriched
Dox-LNVs

To investigate the effect of GC-enriched Dox-LNVs
on tumor growth in vivo, BALB/c nude mice bearing
A431 tumor xenografts were treated with increasing
concentrations of Dox-LNVs with or without 10 mol% GC,
after which tumor size was followed in time. Two
different treatment schedules, single or multiple dose,
were applied, and the results are shown in Fig. 5. In the
single-dose study, Dox formulated in LNVs with or with-
ot 10 mol% GC was administered at 6, 8, or 10 mg/kg. At
a dose of 6 mg/kg, GC greatly improved the efficacy of
Dox-LNVs, resulting in reduced tumor sizes for a period
of 14 d (Fig. 5A), and delayed tumor outgrowth (from 6.0
to 10.7 d; Table 2) compared with standard Dox-LNVs. At
single doses of 8 or 10 mg/kg Dox-LNVs, tumor out-
growth was further delayed in mice treated with GC-
enriched Dox-LNVs compared with mice treated with
standard Dox-LNVs (Fig. 5B, C). Thus, the effect of GC
was most pronounced at the highest dose. However, at
these higher doses, mice in both treatment groups suf-
ered from body weight loss, indicative of systemic toxicity
(see below). At the 10 mg/kg dose (with or without GC),
body weight losses were sometimes >10% and hence led
to exclusion of these mice from the experiment. Tumor
growth delay values (Table 2) could therefore not be
calculated in the groups receiving 10 mg/kg Dox-LNVs.
At 8 mg/kg, GC-Dox-LNVs delayed tumor growth from an
average of 7.9–10.4 d and reduced tumor mass by 65% (at
d 6; Fig. 5B).

Considering systemic toxicity, 6 mg/kg seems the max-
imum tolerable dose for a single administration of Dox-
LNVs. To prevent toxicity, yet to enhance efficacy, a
second administration of 5 mg/kg Dox-LNVs was applied
3 d after the initial administration of 6 mg/kg (Fig. 5D) in
a new group of mice. For this experiment, a new control
group was implemented. Mice treated with GC-Dox-LNVs
showed a more impressive tumor regression and reduced
tumor size than those treated with standard Dox-LNVs.
Tumor size measurements could be continued much
longer in this group, due to slower expansion of tumor
mass. GC-Dox-LNVs significantly increased tumor growth
delay from 5.0 to 14.6 d (Table 2). Strikingly, this re-
peated injection with a cumulative dose of 11 mg/kg Dox
was not toxic to the mice (see below).

Systemic toxicity of standard vs. GC-modified Dox-
LNVs

Body weight loss was monitored and used as an index
for systemic toxicity of the Dox-LNV treatments. A

TABLE 1. IC50 values

<table>
<thead>
<tr>
<th>GC (mol%)</th>
<th>B16 melanoma (µg/ml)</th>
<th>A431 carcinoma (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>4.90 ± 0.56</td>
<td>10.04 ± 1.35</td>
</tr>
<tr>
<td>5</td>
<td>2.46 ± 0.49</td>
<td>5.10 ± 0.84</td>
</tr>
<tr>
<td>10</td>
<td>0.75 ± 0.04</td>
<td>1.45 ± 0.19</td>
</tr>
<tr>
<td>15</td>
<td>0.81 ± 0.04</td>
<td>1.71 ± 0.15</td>
</tr>
</tbody>
</table>

B16 and A431 cells were incubated with Dox-LNVs enriched with
0, 5, 10, or 15 mol% GC. IC50 values represent the concentration
required to inhibit tumor cell growth to 50% (mean±se) and were
calculated from Fig. 2 as indicated in Materials and Methods.

Intravital optical imaging shows enhanced Dox
delivery by GC-LNVs

The enhanced delivery of Dox observed in vitro was
confirmed in an in vivo model. To this end, B16
melanoma tumors were implanted and grown ortho-
topically in the skin of C57BL/6 mice using a dorsal
skin-fold window chamber. Mice were intravenously
injected with Dox-LNVs formulated with or without 10
mol% GC, and after 24 h the intratumoral fate of Dox
was visualized by intravital optical imaging. The pre-

cence of GC in the Dox-LNVs strongly enhanced Dox
delivery in the tumor (Fig. 4). Dox from standard
LNVs (Fig. 4A–D) appeared at low intensities and
located in proximity of tumor blood vessels, whereas
Dox delivered from GC-LNVs appeared at higher
intensities and showed a much more homogeneous
distribution within the tumor tissue, also at greater
distances from tumor-feeding blood vessels (Fig.
4E–H). Higher-magnification images demonstrated
significantly more Dox accumulation in tumor cell
nuclei after treatment with GC-Dox-LNVs (Fig. 4G,
H), than with standard Dox-LNVs (Fig. 4C, D). Bes-
sides uptake in tumor cells, the image shown in Fig.
4H is suggestive of Dox uptake by vascular endothelial
cells. These intravital imaging data clearly con-
firm the enhanced intracellular delivery observed in
vitro.

Figure 3. Increased in vitro intracellular Dox delivery by
GC-enriched Dox-LNVs. B16 or A431 cells were incubated
with Dox-LNVs with or without 10 mol% GC at a Dox
concentration of 1 µg/ml. At the indicated time points,
intracellular Dox was visualized by confocal microscopy using
the intrinsic fluorescence of the drug. GC enrichment of
Dox-LNVs significantly enhanced the appearance of Dox in
the nucleus of both cell lines.
single dose of 6 mg/kg Dox-LNVs did not affect body weight significantly (Fig. 6A). However, in the group receiving a dose of 8 mg/kg, 2 mice treated with GC-Dox-LNVs and 1 mouse with standard Dox-LNVs developed >10% body weight loss after 8–10 d (Fig. 6B). These mice had to be excluded from the experiment. Therefore, for this group, the calculated tumor growth delay values (Table 2) were based on treatment of 3 and 4 mice with GC-LNVs vs. standard Dox-LNVs, respectively. In the 10 mg/kg group, 3 mice treated with GC-Dox-LNVs and 2 mice treated with standard Dox-LNVs developed >10% body weight loss (after 5–6 d; Fig. 6C) and therefore had to be excluded from the experiment. Considering this toxicity, the maximum tolerated single dose of Dox-LNVs was set at 6 mg/kg. Notably, 2 consecutive doses of 6 and 5 mg/kg, respectively, at 3 d interval did not induce decreases in body weight (Fig. 6D), contrary to the single doses of 8 and 10 mg/kg, yet increased antitumor efficacy, especially when the Dox-LNVs were enriched in GC (Fig. 5).

**Histopathology**

Detailed histopathological analysis of possible toxic effects of Dox-LNVs was performed on full necropsy of animals 8 d after administration of 2 consecutive doses, 6 and 5 mg/kg of Dox-LNVs, at 3 d interval. Many tissues were examined after HE staining. Organs and tissues of particular interest included liver, spleen and bone marrow (in view of LNV biodistribution) and heart (most sensitive to Dox toxicity). In addition, several other organs were investigated. No signs of toxicity of Dox were observed in the heart, lungs, jejunum, liver, pancreas, spleen, stomach, duodenum, colon, kidneys, secondary sex glands, and bladder in both groups. In the bases of the incisors, no effects of a recent treatment with cytostatics were observed (data not shown). Both sternal and femoral bone marrow did not show signs of depletion (Supplemental Fig. 1A–C). In some cases, irrespective of treatment group, a slight increase in apoptosis was observed in some tubuli seminiferi of the testis. In one case, increased pigmentation in some
glands in the nasal mucosa was observed, but without clear signs of necrosis. In summary, there were no apparent abnormalities indicative for drug-related toxicity up to 8 d after treatment.

**Hematology**

Clinical side effects of Dox treatment include bone marrow depression, such as leucopenia, neutropenia, anemia and thrombocytopenia, inflammation (mainly upper air ducts), and fever. We therefore performed hematological measurements of mice treated with a multiple dose of either standard or GC-modified Dox-LNVs (6 mg/kg plus additional dose of 5 mg/kg at d 3). Mice were killed on d 2, 4, 6, and 8 after start of treatment. Blood was collected and hematological parameters were analyzed. For normal reference values, 8 healthy mice (similar sex, age, weight, and diet) were killed, hematological parameters were analyzed, and mean values were calculated. Mice treated with Dox-LNVs with or without GC showed no signs of obvious spontaneous anemia and no significant differences in hemoglobin (Supplemental Fig. 1, bottom panel) and hematocrit values (not shown), red blood cells, mean platelet volume, platelet counts, and total white blood cells over the course of time (Supplementary Fig. 1D–G). There was no indication of inflammation, since numbers of white blood cells, lymphocytes, monocytes, and granulocytes remained close to the control reference values. We conclude that treatment of mice with standard or GC-modified Dox-LNVs did not cause any significant hematological abnormalities.

**TABLE 2. In vivo antitumor efficacy of standard or GC-modified Dox-LNV**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Standard</th>
<th>GC-modified</th>
<th>P value</th>
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<tbody>
<tr>
<td>1 × 6 mg/kg</td>
<td>6.0 ± 1.0</td>
<td>10.7 ± 1.3</td>
<td>≤0.005</td>
</tr>
<tr>
<td>1 × 8 mg/kg</td>
<td>7.9 ± 1.7</td>
<td>10.4 ± 1.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>1 × 10 mg/kg</td>
<td>10.4 ± 2.1</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>1 × 6 mg/kg plus 1 × 5 mg/kg</td>
<td>5.0 ± 1.4</td>
<td>14.6 ± 1.5</td>
<td>≤0.0005</td>
</tr>
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Normalized tumor growth delay for animals treated with standard or GC-modified Dox-LNVs is expressed as the number of days necessary to reach 200% of the initial tumor volume minus the number of days necessary to reach 200% of the initial tumor volume for untreated animals (mean±se). Initial dosage of Dox-LNV was administrated at d 0. N.D., not determined; N.S., not significant.

**DISCUSSION**

Side effects of Dox include acute and chronic cardio-toxicity, bone marrow depletion (myelosuppression), and gastrointestinal toxicities. Commercially available liposomal drug delivery systems, such as Caelyx or Doxil and Myocet, have improved the toxicity profile of Dox, particularly by reducing its acute and chronic cardio-toxicity. Due to their prolonged residence in circulation in combination with immature leaky vasculature
in tumors, extravasation from circulation into the tumor tissue can occur, resulting in increased tumor drug levels (17–21). However, due to the slow and incomplete release from liposomes, bioavailability of liposomal Dox was only half of the total amount of liposomal Dox arriving in the tumor, which limits its therapeutic activity (28). Attempts to improve drug release rates by adapting the liposomal lipid composition did not further improve drug bioavailability, but in some instances did increase toxic side effects (35).

Our strategy here was fundamentally different. We focused on modifying the permeability of the tumor cell membrane rather than the liposome. For this purpose, we exploited the short-chain sphingolipid GC, of which we discovered strong Dox-uptake enhancing properties in vitro (6, 30). We modified a Dox-LNV formulation that closely mimics the commercial formulation Caelyx/Doxil by enriching the lipid bilayer with this sphingolipid. From previous work (30), it was known that GC transfers from the LNVs onto the tumor cell plasma membrane to exert its Dox-uptake enhancing effect.

In the present study, we demonstrated both in vitro and in vivo a strong enhancement of Dox delivery into tumor cells on enriching Dox-LNVs with GC. The enhancement of Dox delivery resulted in strong antitumor activity in vitro and caused a strong tumor reduction and inhibition of tumor progression in vivo. These promising results were obtained without an increase in overall toxicity, resulting in a net increase in the therapeutic index of Dox-LNVs on GC modification. In vitro, Dox-LNVs coformulated with increasing GC content showed enhanced antiproliferative activity. A GC content of 10 mol% proved optimal in terms of cytotoxicity in A431 carcinoma and B16 melanoma cells. Since empty LNVs with 10 mol% GC but without Dox did not inhibit proliferation, we can conclude that the addition of the short-chain sphingolipid GC results in an increased Dox bioavailability from GC-modified Dox-LNVs. This conclusion was confirmed by confocal microscopy experiments, which demonstrated a rapid and enhanced delivery of Dox into tumor cells when Dox-LNVs were enriched with GC. It is noteworthy that the GC-enhanced accumulation of Dox persists for prolonged times, which is important for the successful application in vivo.

Enhanced Dox bioavailability through GC-enriched LNVs was also confirmed by intravital optical imaging in experimental animals equipped with a dorsal skinfold window chamber with orthotopically implanted B16 melanoma. Dox from GC-LNVs distributed deeper and more homogeneously into the tumor tissue and showed much higher levels of nuclear uptake 24 h after administration than Dox from standard LNVs. In some instances, the Dox fluorescence pattern after GC-Dox-LNV treatment was suggestive of Dox uptake by endothelial cells, a phenomenon that was not seen with standard Dox-LNVs. Since tumor vasculature represents an interesting additional target in tumor drug delivery (36–38), the relevance of the increased delivery to this cell type and the involved mechanism require further attention. Of note in this regard, also in

Figure 6. Body weight changes after administration of standard or GC-modified Dox-LNVs. After treatment of A431 xenograft mice with Dox-LNV preparations [standard (□) or GC-modified (■)], measurements of body weights were performed as an indicator for Dox-induced toxicity. Control group (△) received saline. Note that body weights in the control group show a strong increase due to significant increase of tumor mass. A–C) Mice treated with 6 mg/kg (A), 8 mg/kg (B), or 10 mg/kg (C) at d 0. D) Treatment of mice with 6 and 5 mg/kg at d 0 and d 3. Data represent means ± se (n=5). At doses of 8 and 10 mg/kg, mice in both treatment groups suffered from body weight loss, indicative of systemic toxicity. However, 2 consecutive doses of 6 and 5 mg/kg, respectively, at 3 d interval did not induce decreases in body weight.
vitro endothelial cells display enhanced Dox uptake on exposure to short-chain sphingolipids (6).

GC-modified Dox-LNVs greatly enhanced the antitumor effect in an A431 xenograft mouse model, suggesting a significant increase in the bioavailability of Dox from the GC-modified Dox-LNVs compared with standard Dox-LNVs. By changing single-dose application of Dox-LNVs to multiple injections, we were able to enhance the cumulative dose of Dox-LNVs without unacceptable body weight loss (as measured for systemic toxicity). This resulted in a net increase in the therapeutic index of GC-enriched Dox-LNVs.

We extensively studied possible adverse side effects of the GC-Dox-LNVs, an issue that is neglected in most studies dealing with carrier-based drug delivery. While animal studies usually are mainly focused on the activity of novel formulations toward tumors, our study includes thorough histopathological and hematological examinations. Notably, we found no significant toxic side effects in the tumor-bearing nude mice treated with 2 doses of standard or GC-modified Dox-LNVs up to 8 d after treatment.

The mechanism by which GC incorporated in Dox-LNVs exerts its antitumor effect in vivo remains unclear. We have determined the molecular requirements of GC and related sphingolipids to display this uptake-enhancing effect. A short N-linked acyl chain (between 2 and 10 C atoms) and a small polar headgroup (phosphocholine or 1–2 hexose units) are required. We excluded possible involvement of endocytosis, multidrug transporters, lipid rafts, and trivial membrane leakage (6). Of particular interest is the recent recognition of a tendency for self-association of certain sphingolipids leading to membrane channel formation (39–41). Such membrane channels, or perhaps imperfect lipid packing (due to the short acyl chain), might well underlie the enhanced membrane permeability toward amphiphilic drugs. Obviously, the mechanism of short-chain sphingolipid-induced cellular uptake of anthracyclines remains to be further investigated.

In summary, we have introduced a new concept of drug delivery to cancer cells and proved its therapeutic value in vitro and in vivo. The novelty of this concept is the introduction of a permeability change of the tumor cell membrane imposed by a specific short-chain sphingolipid (GC) from a GC-modified lipid nanovesicle that facilitates enhanced and persistent accumulation and bioavailability of doxorubicin in the tumor. This advanced drug delivery technology provides great opportunities to improve anticancer chemotherapy.

REFERENCES


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