Co-formulated N-octanoyl-glucosylceramide improves cellular delivery and cytotoxicity of liposomal doxorubicin

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ABBREVIATIONS: C6-SM, N-hexanoyl-sphingomyelin; C8-GlcCer, N-octanoyl-glucosylceramide; DPPC, dipalmitoylphosphatidylcholine; DSPE-PEG2000, distearylphosphatidyl-ethanolamine-PEG2000; HSPC, hydrogenated soy bean phosphatidylcholine; LDH, lactate dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PEG, polyethyleneglycol 2000; TLC, thin layer chromatography

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ABSTRACT

The anti-cancer agent doxorubicin is in certain cases administered as a long-circulating liposomal formulation. Due to angiogenesis-related structural abnormalities in the endothelial lining of many neoplasms, these complexes tend to extravasate and accumulate in the tumor stroma. However, delivery of doxorubicin is still not optimal since liposomes are not taken up directly by tumor cells. Instead, doxorubicin is gradually released into the interstitial space, and the subsequent uptake by surrounding cells is a limiting step in the delivery process. We recently demonstrated that plasma membrane-inserted short-chain sphingomyelin facilitates the cellular uptake of free doxorubicin (Veldman RJ, Zerp S, van Blitterswijk WJ and Verheij M (2004) N-hexanoyl-sphingomyelin potentiates in vitro doxorubicin cytotoxicity by enhancing its cellular influx. Br J Cancer 90:917–925). Here we report that N-octanoyl-glucosylceramide acts equally potent but is itself less toxic. When co-formulated with liposomal doxorubicin, this short-chain glycosphingolipid administered to cultured A431 epidermoid carcinoma cells led to superior (up to 4-fold) cellular doxorubicin accumulation and cytotoxicity, as compared to control doxorubicin liposomes. These results were fully reproducible when N-octanoyl-glucosylceramide was post-inserted into Caelyx®, a commercial liposomal doxorubicin preparation. The doxorubicin-potentiating effect of N-octanoyl-glucosylceramide-enriched liposomes proved relatively insensitive to high serum concentrations, indicating that in vivo application is a feasible option. N-octanoyl-glucosylceramide-enrichment might thus represent a major improvement of conventional liposomal doxorubicin formulations.
INTRODUCTION

The anthracycline doxorubicin has been in clinical use for several decades, and is still among the most widely used chemotherapeutic agents for treatment of a variety of neoplasms (Weiss, 1992; Zagotto et al., 2001; Lothstein et al., 2001). Despite many years of research in developing new and better anthracyclines, little or no change in the molecular structure of doxorubicin made it to the clinic. However, with the development of liposomal formulations, its delivery to at least certain types of tumors underwent a major improvement (Tardi et al., 1996; Gabizon, 2001). Compared to systemic application of free doxorubicin, liposomal doxorubicin exhibits significant advantages, such as reduced normal tissue toxicities (Orditura et al., 2004). Improved loading procedures, resulting in high doxorubicin encapsulation efficiencies, further increased the therapeutic index of entrapped doxorubicin (Papahadjopoulos et al., 1991; Horowitz et al., 1992; Haran et al., 1993). Another major step forward was the development of polyethyleneglycol (PEG)-coated liposomes. This coating prevents opsonization and reduces the uptake by macrophages from the reticulo-endothelial system, in turn resulting in prolonged circulation times, as compared to free doxorubicin or to non-coated liposomes (Papahadjopoulos et al., 1991; Vaage et al., 1992; Robert and Gianni, 1993; Gabizon et al., 1996; Uster et al., 1996). PEG-liposome-encapsulated doxorubicin (commercially available as Caelyx® and Doxil®) is now in clinical use for the treatment of various types of neoplasms, and innovations such as covalent attachment of tumor-specific antibodies or ligands will further enhance drug targeting and therapeutic value (Park et al., 2002; Pan et al., 2003).

The endothelial lining of healthy blood vessels effectively prevents escape of liposomes from the circulation. In contrast, angiogenesis-associated vascular abnormalities of many solid tumors do allow extravasation of long-circulating PEG-liposomes into the tumor stroma (Yuan et al., 1994). Despite the resulting accumulation in tumor tissue, the liposomes are not taken up by the tumor cells. Instead, their doxorubicin load is gradually released into the interstitial space (Horowitz et al., 1992; Harasym et al., 1997). Given the intracellular localization of its molecular targets, sufficient cellular uptake of doxorubicin is required for its action (Speth et al., 1988; Lothstein et al., 2001). However, doxorubicin does not possess the optimal degree of lipophilicity
for efficient plasma membrane traversal, which limits its efficacy (Heijn et al., 1999; Washington et al., 2001).

We recently demonstrated that the cellular uptake of free doxorubicin, and with that its cytotoxic action, is greatly enhanced when the short-chain sphingolipid \( N \)-hexanoyl-sphingomyelin (C\(_6\)-SM) is co-administered with the drug \textit{in vitro} (Veldman et al., 2004). \textit{In vivo}, however, doxorubicin and the sphingolipid will most likely show differences in biodistribution, metabolic kinetics, and clearance, and will thus not be delivered at the same site at the same time, which is a prerequisite for the drug-uptake enhancing effect.

In the present study, we investigated the \textit{in vitro} properties and efficacy of PEG-liposomal doxorubicin that was co-formulated with \( N \)-octanoyl-glucosylceramide (C\(_8\)-GlcCer), another doxorubicin uptake-enhancing sphingolipid that we identified. We choose C\(_8\)-GlcCer because it exhibits lower cellular toxicity than C\(_6\)-SM. Incorporation of this sphingolipid into the liposome bilayer would ensure co-delivery of doxorubicin and the lipid, as required. We report here that this adaptation greatly enhances doxorubicin transfer to tumor cells, in turn leading to increased cytotoxicity. Importantly, this was also the case at high serum concentrations, which holds promise for \textit{in vivo} application.
METHODS

Materials. Dipalmitoyl-phosphatidylcholine (DPPC) and distearyl-phosphatidylethanolamine (DSPE)-PEG2000 were from Lipoid (Ludwigshafen, Germany) and C₈-GlcCer was from Avanti Polar Lipids (Alabastar AL, USA). Polycarbonate filters were from GE Osmonics (Minnetonka MN, USA) and PD-10 Sephadex columns from Pharmacia (Upsala, Sweden). Doxorubicin.HCl was obtained from Pharmachemie (Haarlem, The Netherlands) and Caelyx® from Schering-Plough (Heist-op-den-Berg, Belgium). Cholesterol, bicinechonic acid protein kit, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Dowex® 50WX4-400 resin (Dowex) were from Sigma (St. Louis MO, USA). LK6D silica TLC plates were from Whatman (Maidstone, UK) and Vectashield mounting medium from Vector Laboratories (Burlingame CA, USA). CytoTox 96 lactate dehydrogenase (LDH) activity kit was from Promega (Madison WI, USA).

Liposome preparation and analysis. Liposomes were prepared by lipid film hydration and extrusion, and subsequent remote loading using an ammonium sulfate method (Haran et al., 1993). Mixtures of DPPC, cholesterol, DSPE-PEG2000 and C₈-GlcCer were prepared in various molar ratios (Table 1). Lipids, dissolved in ethanol, were mixed and a lipid film was created under reduced pressure on a rotary evaporator and then dried under a stream of nitrogen. Liposomes were then formed by addition of a 250 mM (NH₄)₂SO₄ solution to the lipid film. The hydrated lipid dispersion was sized by extrusion through, successively, 200 nm (3 passes), 100 nm (3 passes), and double 50 nm (6 passes) polycarbonate filters. This standardized procedure combines easy production (at low pressure), high lipid recovery and the production of a homogeneous population of small (100 nm) unilamellar vesicles. Non-encapsulated (NH₄)₂SO₄ was removed by gel permeation chromatography using a PD-10 Sephadex column (Pharmacia, Sweden), eluted with 123 mM citrate buffer, pH 5.5. For liposomal doxorubicin loading, the drug was added in a molar ratio of doxorubicin to total lipid of 1 to 5, and incubated for 1 hour at 55°C (Haran et al., 1993). Non-encapsulated doxorubicin was removed by gel permeation chromatography using a PD-10 Sephadex column, eluted with 135 mM NaCl in 10 mM Hepes, pH 7.4. This entire procedure was used for all liposome formulations.
For post-insertion of C₈-GlcCer into Caelyx®, the liposomes were incubated (30 min at 37°C) with various concentrations of the sphingolipid (Fig. 5), which was added as an ethanolic solution (never exceeding 2%, v/v). Liposomes were then subjected to ultracentrifugation in a Kontron TFT 80.2 rotor (70,000 rpm, 1 h at 20°C), and pellets were washed with 135 mM NaCl in 10 mM Hepes, pH 7.4.

Phospholipid phosphorus of each liposome preparation was determined by a phosphate assay after perchloric acid destruction (Rouser et al., 1970). To confirm C₈-GlcCer incorporation and post-insertion, lipids were extracted from the liposomes (Bligh and Dyer, 1959). Extracts were then applied to a 60 Å silica gel TLC plate, which was developed in chloroform/methanol/water (60:30:8, v/v/v). Lipids were visualized by iodine vapor staining, and C₈-GlcCer was identified by co-running a standard on the same plate. Liposomal doxorubicin was measured by fluorescence, after solubilization of the liposomes in 1% (w/v) Triton X-100, by comparison to standard amounts. Particle size and size distribution were determined by dynamic laser light scattering using a Malvern 4700 system equipped with a 75 mW Argon laser (Uniphase, San Jose, USA), with data analysis by Automeasure software, version 3.2 (Malvern, UK). The mean diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. The liposomes used in the present study had a mean particle size of 0.1 µm and a polydispersity <0.1. Polydispersity is a measure for variation in particle size within a liposome population, and varies from 0 (complete monodispersity) to 1 (large variations in particle size). The extent of doxorubicin leakage was measured after storing the liposomes for various periods of time and at various temperatures (Table 1). Samples were then subjected to ultracentrifugation as described. Released doxorubicin was measured fluorometrically in aliquouts of the supernatants using a Perkin Elmer Victor Wallac II fluorescence plate reader. Excitation wavelength was 485 nm and emission wavelength was 535 nm. Background fluorescence was subtracted and doxorubicin leakage was expressed relative to the total liposomal fluorescence measured after solubilization in 1% Triton X-100.

Cell culture. A431 cells (adherent human epidermoid carcinoma cells), purchased from the American Type Culture Collection (Manassas VA, USA), were cultured in Dulbecco's modified Eagle medium, supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 µg/ml
Cells were sub-cultured once a week by trypsinization and maintained in a water-saturated atmosphere containing 5% CO₂ at 37°C. All experiments were performed at a confluency of 80 - 90%.

**Cellular doxorubicin uptake.** A431 cells were cultured in flat-bottom 96-well plates. At confluency, cells were changed to serum-free medium and exposed to 50 µM free or to 20 µM liposomal doxorubicin. These concentrations were chosen because they closely match the actual plasma concentrations that are reached upon intravenous bolus administrations into average sized persons. (Note that clinically used standard dosages differ for free doxorubicin and liposomal doxorubicin (50-75 mg/m² and 20-40 mg/m², respectively)). After extensive washing, cells were lysed in 100 µl of 1% (w/v) Triton X-100. Doxorubicin fluorescence was then measured by a Perkin Elmer Victor Wallac II fluorescence microplate reader, using 485 nm and 535 nm filters for excitation and emission, respectively. All values were corrected for background fluorescence. Cellular doxorubicin contents were calculated with the aid of standard amounts, and corrected for any differences in protein content, as determined with the bicinchoninic acid assay (Smith et al., 1985).

**Fluorescence microscopy.** For microscopic studies, A431 cells were cultured on 0.5% (w/v) gelatin-coated glass coverslips. After exposure to liposomal doxorubicin, cells were washed, fixed (10 min in 4% (w/v) paraformaldehyde in PBS) and mounted in Vectashield on glass slides. All samples were examined with a Zeiss Axiovert S 100 inverted fluorescence microscope, employing a mercury lamp in combination with a filter set consisting of a 450-490 nm band pass excitation filter, a 510 nm beam splitter and a 520 nm long pass emission filter. Specimens were photographed through a 40x oil immersion objective by a Zeiss AxioCam CCD camera, using equal exposure times (1350 ms).

**Plasma membrane leakage assay.** To investigate (loss of) plasma membrane integrity, the release of the cytosolic enzyme LDH into phenol red-free cell culture medium was monitored. Flat-bottom 96-well plates containing (liposome-treated) A431 cells were centrifuged (2000 rpm, 5 min at 20°C) and 50 µl aliquots of the supernatants were assessed for LDH activity with the CytoTox 96 kit, according to the manufacturer's instructions. Values obtained from cells that were
lysed in 0.8% Triton X-100 served as positive control. All data were corrected for protein contents, as determined with the bicinchoninic acid assay (Smith et al., 1985).

**Cell viability.** For viability assessment, A431 cells were cultured in flat-bottom 96-well plates. After experimental treatments, 100 µg of the mitochondrial dehydrogenase substrate MTT was added to each well (Carmichael et al., 1987). Cells were then incubated for 60 min at 37°C. After centrifugation (3000 rpm, 10 min at 4°C) supernatants were removed. The precipitated blue formazan products were then dissolved in 100 µl DMSO, and absorbencies were read in a Bio-Tek Instruments EL 340 photospectrometric microplate reader at 540 nm. Background absorbencies were subtracted and values from wells containing untreated control cells were set at 100% viability.
RESULTS

C₆-SM enhances cellular doxorubicin uptake but is cytotoxic at high concentrations, while C₈-GlcCer shows the same uptake enhancement but is less toxic

We previously reported that the cellular uptake of free doxorubicin could be greatly (more than 3-fold) enhanced in vitro, by co-addition of C₆-SM, a semi-synthetic short-chain sphingolipid (Veldman et al., 2004). At its effective low micromolar concentrations, and during short-term incubations (serum-free), C₆-SM displayed no toxic effects by itself. However, higher concentrations (Fig. 1) and/or longer incubation periods (data not shown) with this lipid were clearly toxic. Overall viability of A431 cells dramatically decreased at C₆-SM concentrations that exceed 10 µM (Fig. 1A). This is partly the result of loss of plasma membrane integrity by intrusion of this lipid, as demonstrated by the dose-dependent leakage of the cytosolic enzyme LDH into the extracellular medium (Fig. 1B).

In a search for less toxic alternatives, we now identified C₈-GlcCer as another sphingolipid with equally potent doxorubicin uptake-enhancing properties (335.8 ± 88.5% at 10 µM in A431 cells) as C₆-SM. In contrast to C₆-SM, however, C₈-GlcCer clearly exhibited less toxicity at high concentrations (Fig. 1A). In addition, C₈-GlcCer caused no leakage of LDH, even at concentrations as high as 100 µM (Fig. 1B), indicating that the plasma membrane remained completely intact.

Characterization of C₈-GlcCer-enriched PEG-liposomes

To investigate whether C₈-GlcCer retains its doxorubicin uptake-enhancing properties when co-formulated with liposomal doxorubicin, we prepared a series of PEG-liposomes, containing increasing mol% of C₈-GlcCer (Table 1). Whereas the relative amount of PEG-containing phospholipid (DSPE-PEG 2000) was kept constant, DPPC and cholesterol contents slightly decreased in this series, to compensate for increasing mol% of C₈-GlcCer. Liposomes were loaded with doxorubicin as described (Haran et al., 1993). The lipid composition, doxorubicin loading efficiencies, size, and stability of the liposome preparations fully matched those of commercial PEG-liposomal doxorubicin (Caelyx®). Since truncated lipid analogues are known to
destabilize the lipid bilayer, we limited the C₈-GlcCer content to 17 mol%. At this C₈-GlcCer content, the liposomes were still very stable, as shown by the minimal doxorubicin leakage (at 37°C or during prolonged storage at 4°C) (Table 1).

Enhanced cellular doxorubicin uptake from C₈-GlcCer-enriched liposomes

When cultured A431 cells were incubated for 24 h in the presence of normal (conventional) PEG-liposomal doxorubicin, 2.6 ± 0.1 nmol drug per mg of cellular protein accumulated in the cells. When using liposomes that were enriched with C₈-GlcCer (up to 17%), however, this uptake increased up to 4-fold (Fig. 2). This observation was confirmed by fluorescence microscopy: Doxorubicin accumulation in cell nuclei during 24 h incubation with C₈-GlcCer-enriched liposomes (Fig. 3C) was much higher than with conventional liposomes (Fig. 3B).

Improved doxorubicin delivery to cells results in enhanced cytotoxicity

The in vitro efficacy of the modified liposomes was tested by the MTT viability assay. This assay relies on the fact that only viable cells have the required mitochondrial metabolic capacity to convert MTT into a detectable product, whereas dead or dying cells have not (Carmichael et al., 1987). Compared to a control treatment (empty liposomes; set at 100%), conventional doxorubicin-loaded PEG liposomes exhibited only modest cytotoxicity (58.4% of A431 cells was still viable after 24 h; Fig. 4). At increasing mol% of C₈-GlcCer however, this viability decreased dramatically, and correlated well with increasing doxorubicin uptake of the cells (Figs 2 and 3). The increased potency of the adapted liposomes can be fully attributed to doxorubicin, since control liposomes (without doxorubicin and without C₈-GlcCer; or without doxorubicin, but with C₈-GlcCer) had no significant effect on cell viability. These results were confirmed by viability assessment through phase contrast microscopy (Fig. 5).

An alternative preparation procedure: post-insertion of C₈-GlcCer into Caelyx®

We tested whether post-insertion of C₈-GlcCer into existing (commercial) doxorubicin liposomes (Caelyx®) would also result in improved cytotoxic efficacy. For this, Caelyx® was incubated for 30 min in buffer supplemented with various concentrations of C₈-GlcCer. After
washing the liposomes, TLC analysis revealed that this simple procedure sufficed to incorporate substantial amounts of C₈-GlcCer into Caelyx®, in a concentration-dependent manner (Fig. 6 - insert). However, due to the presence of the PEG shielding, it is difficult to assess on theoretical grounds, whether the lipid actually inserted into the liposomal bilayer, or merely remained associated to the liposomal surface.

Fig. 6 shows that C₈-GlcCer-loaded Caelyx® resulted in a similar improved doxorubicin transfer to cells as the newly prepared liposomes (compare with Fig. 2). Accordingly, this was likewise accompanied by an increased cytotoxicity, as determined by the MTT viability assay: While 76.2 ± 18.2% of A431 cells were still viable after 24 h incubation with normal Caelyx®, C₈-GlcCer-enriched Caelyx® dropped this viability value down to 19.2 ± 11.8%. Since liposome production involves tedious and time-consuming procedures, post-insertion thus provides a convenient alternative for quick preparation of C₈-GlcCer-enriched batches of liposomal doxorubicin, with improved cytotoxicity.

C₈-GlcCer-enriched liposomes are relative insensitive to serum components

As described, under serum-free conditions, C₈-GlcCer greatly improves the cellular uptake of free doxorubicin. In the presence of serum, however, both the doxorubicin uptake and the enhancing effect of the sphingolipid hereupon, were strongly reduced (Fig. 7A). Most likely, this is due to binding of the amphiphilic doxorubicin and C₈-GlcCer to, and sequestration by albumin and lipoproteins, thus preventing interaction of these molecules with cells. Although less pronounced, also the uptake of doxorubicin from conventional PEG-liposomes was diminished by the presence of serum (Fig. 7B). Interestingly, however, the relative effect of co-formulated C₈-GlcCer was fully retained under high-serum conditions. In the presence of 50% (v/v) serum for example, 8.6 ± 0.4 nmol/mg doxorubicin was taken up from C₈-GlcCer-enriched liposomes, whereas this was only 1.6 ± 0.3 nmol/mg from an equal amount of C₈-GlcCer-free liposomes. Comparable results were obtained with (post-inserted) C₈-GlcCer-Caelyx® (data not shown).

As expected, high serum concentrations had a strong protective effect on the viability of doxorubicin-treated A431 tumor cells (not shown in figure). In the presence of 50% serum, for example, 85.4 ± 9.1% of the cells survived a 24 h-treatment with conventional liposomal
doxorubicin. When employing C₈-GlcCer-enriched doxorubicin liposomes, however, only 35.7 ± 6.2% of the cells survived under these conditions. Again, the increased drug uptake correlated well with an increased toxicity. It can be concluded that, under high serum conditions (like *in vivo*), C₈-GlcCer-enriched doxorubicin liposomes retain higher cytotoxicity than conventional liposomes (Caelyx®) or free doxorubicin.

**Liposome-cell contact is required for C₈-GlcCer action but not for doxorubicin transfer**

To address the question whether or not doxorubicin transfer from liposomes to cells requires the direct interaction of the liposome with the cell surface (plasma membrane), we used Dowex, a non-toxic ion-exchange resin that binds to free doxorubicin with high affinity, but not to liposome-encapsulated doxorubicin (Storm et al., 1985; Druckmann et al., 1989). Supplementation of cell culture media with 5 mg/ml Dowex, which is itself not taken up by cells, prevented death of A431 cells, as induced by a 24 h-treatment with conventional PEG-liposomal doxorubicin (90.4 ± 24.9% surviving cells). Similarly, cell death induced by 17 mol% C₈-GlcCer-containing liposomal doxorubicin could also be largely prevented by Dowex co-treatment (81.9 ± 6.3% survival). These findings suggest that doxorubicin is delivered to cells via the aqueous extracellular environment, rather than by direct membrane-membrane contact.

We next questioned how C₈-GlcCer in the liposome improves the cytotoxicity of liposomal doxorubicin. Is there any interaction or complex formation between the lipid and the drug prior to interaction with the cell membrane? To address this question, we pre-incubated A431 cells with the sphingolipid and subsequently removed non-bound lipid by washing. This treatment sufficed to improve the cellular uptake of subsequently added doxorubicin (data not shown). Similarly, when A431 cells were treated with a combination of free doxorubicin together with empty 17 mol% C₈-GlcCer liposomes, drug uptake increased to 260.4 ± 28.2%, compared to empty liposomes without C₈-GlcCer (set at 100% uptake). Conversely, doxorubicin uptake from normal liposomes (0 mol% C₈-GlcCer) improved, when C₈-GlcCer was added separately, rather than co-formulated in the liposome (data not shown). Taken together, these results indicate that the sphingolipid acts at the level of the plasma membrane, and not within the liposome or the aqueous environment. Furthermore, the doxorubicin-uptake enhancing effect does neither require
simultaneous introduction of C₈-GlcCer and doxorubicin into the plasma membrane, nor complex formation between the lipid and the drug prior to interaction with the cell membrane.
In this paper, we have shown that the cellular uptake of doxorubicin from C₈-GlcCer-enriched doxorubicin liposomes (either newly prepared or by post-insertion into Caelyx\textsuperscript{®}) is superior both to free doxorubicin and to conventional liposomal doxorubicin (normal Caelyx\textsuperscript{®}), even under high serum conditions \textit{in vitro}. These results suggest the feasibility of \textit{in vivo} applications of C₈-GlcCer modified liposomes.

We have addressed the mechanism by which liposomal C₈-GlcCer enhances the cellular uptake of doxorubicin. We know that PEG-liposomes, due to their hydrophilic coating, are not taken up by cells and do not fuse with the plasma membrane (Koning et al., 1999, 2003; Everts et al., 2003). Instead, their doxorubicin content is gradually released free into the surrounding medium, from where it is then taken up by neighboring cells (Horowitz et al., 1992; Harasym et al., 1997). Incorporation of C₈-GlcCer into the liposomal bilayer did not lead to enhanced doxorubicin leakage, and neither did it result in improved doxorubicin loading of liposomes (Table 1). Apparently, the sphingolipid does not affect doxorubicin diffusion through the liposomal bilayer, and therefore does not enhance its release rate. There is no direct liposome-cell contact required for the transfer of doxorubicin to the plasma membrane. This was demonstrated by the prevention of doxorubicin-induced cell death by using the ion-exchanger Dowex that binds to free doxorubicin but not to liposome-encapsulated doxorubicin. These data together with the drug-sequestration effect of serum (Fig. 7B), indicate that doxorubicin is delivered to cells via the aqueous extracellular environment, rather than by direct membrane-membrane contact.

We previously demonstrated that plasma membrane-inserted short-chain sphingolipids, such as C₈-GlcCer, improve the uptake of free (non-liposomal) doxorubicin, by facilitating its trans-membrane diffusion (Veldman et al., 2004). The molecular and cellular mechanisms involved are not fully understood, and are currently under investigation. Importantly, simultaneous presence of free drug and the lipid in the culture media was not required for the effect, and no drug-lipid complex formation, prior to interaction with the cell membrane, was involved. In fact, a pre-incubation of cells with the sphingolipid (in free or liposomal form) and subsequent removal of non-bound lipid, was sufficient to improve the cellular uptake of subsequently added doxorubicin.
These results indicate that the sphingolipid acts at the level of the cell (membrane), and not within the liposome or the aqueous environment.

An important question is how C8-GlcCer moves from the liposomes to the plasma membrane. Interestingly, serum does not inhibit the relative effect of C8-GlcCer on liposomal doxorubicin uptake, despite the presence of large amounts of lipid-binding entities (Fig. 7B). This suggests that, contrary to doxorubicin, C8-GlcCer could (partly) relocate via direct contact between cell-associated liposomes and the plasma membrane. Such a (spontaneous) redistribution, quite common for short-chain lipid analogues, would eventually result in an equilibrium in C8-GlcCer distribution between cells and liposomes (Jeckel and Wieland, 1993; Bai and Pagano, 1997). A similar transfer to tumor cell membranes was described for the liposomal bilayer-incorporated prodrug, 5-fluorodeoxyuridine-dipalmitate (Koning et al. 1999).

In summary, we may explain the improved cell delivery of C8-GlcCer-enriched liposomal doxorubicin by the following sequence of events: Upon addition of the liposomes, doxorubicin is gradually released free into the cell culture medium, just like from normal liposomes. From those liposomes that (transiently) come into physical contact with cells, C8-GlcCer spontaneously undergoes inter-bilayer movement towards the plasma membrane, whereas the other liposome components do not. Future studies should confirm that C8-GlcCer is indeed taken up by the cells, in the same way as C6-SM (Veldman et al., 2004). We hypothesize that C8-GlcCer, after lateral diffusion in the plane of the plasma membrane, creates local areas that exhibit an increased permeability towards amphiphilic drugs. This would result in an enhanced cellular influx and accumulation of doxorubicin, in turn explaining the improved cytotoxic action of C8-GlcCer-enriched liposomes.

**Concluding remarks**

The anthracycline doxorubicin has been in widespread clinical use for several decades. Nevertheless, its delivery to tumor cells and therewith its efficacy, is far from optimal. Even currently used liposomal formulations do not fully exhibit the desired pharmacological properties. By C8-GlcCer enrichment, we now introduce a simple but very effective modification, which significantly improves doxorubicin transfer from liposomes into cells, as compared to existing
formulations. This finding might be a next step forward in the evolution of doxorubicin pharmacology, and holds promise for clinical application.
References


Footnotes:

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FIGURE LEGENDS

Fig. 1 - C₈-GlcCer is less toxic than C₆-SM. (A) A431 cells were exposed for 24 h to the indicated concentrations of free C₆-SM (●) or free C₈-GlcCer (○) under serum-free conditions. Viability of the cells was then established by the MTT assay, as described under Methods. (B) A431 cells were incubated for 1 h with free C₆-SM (●) or free C₈-GlcCer (○) under serum-free conditions. Plasma membrane integrity was assessed by measuring the leakage of cytosolic LDH into the culture medium, as described under Methods. Media from cells that were lysed with 0.8% Triton X-100 served as the positive control (set at 100% leakage). Data are means ± S.D (n=5).

Fig. 2 - Quantification of C₈-GlcCer-enhanced cellular uptake of doxorubicin from liposomes. A431 cells (in serum-free medium) were treated for 24 h with doxorubicin-loaded PEG-liposomes, containing increasing mol% of C₈-GlcCer. The liposomal doxorubicin concentration in the culture medium was 20 µM. Unbound liposomes were washed away and cells were lysed. Doxorubicin fluorescence was quantified, corrected for cellular protein content and expressed as mean ± SD (n=3 independent experiments, each performed in 6-fold).

Fig. 3 - Fluorescence microscopy showing C₈-GlcCer-enhanced doxorubicin uptake from liposomes. A431 cells were cultured on gelatin-coated coverslips and treated for 24 h, under serum-free conditions, with (A) buffer; (B) conventional PEG-liposomes; (C), C₈-GlcCer-enriched (17 mol%) PEG-liposomes. Final liposomal doxorubicin concentrations were 20 µM. Cells were washed, fixed in 4% (w/v) paraformaldehyde and mounted. Specimens were examined by fluorescence microscopy, using a filter set consisting of a 450-490 nm band pass excitation filter, a 510 nm beam splitter and a 520 nm long pass emission filter. Cells were photographed through a 40x oil immersion objective, using equal exposure times (1350 ms).

Fig. 4 - C₈-GlcCer co-formulation improves the cytotoxic efficacy of liposomal doxorubicin. Under serum-free conditions, A431 cells were treated for 24 h with doxorubicin liposomes that were enriched with the indicated mol% of C₈-GlcCer. Liposomal doxorubicin concentrations in the...
medium were 0 µM (solid bars; empty liposome controls) or 50 µM (hatched bars). Cell viability was assessed with the MTT assay, and expressed as mean % ± SD (n=3, each experiment performed in 4-fold). Untreated control cells were set at 100% viability.

**Fig. 5** - C<sub>8</sub>-GlcCer-enriched doxorubicin liposomes show superior cytotoxicity. Confluent A431 cells were treated under serum-free conditions with: (A) control liposomes (no C<sub>8</sub>-GlcCer, no doxorubicin); (B) empty C<sub>8</sub>-GlcCer-enriched liposomes (17 mol% C<sub>8</sub>-GlcCer, no doxorubicin); (C) normal doxorubicin liposomes (no C<sub>8</sub>-GlcCer, 50 µM doxorubicin); (D) C<sub>8</sub>-GlcCer-enriched doxorubicin liposomes (17 mol% C<sub>8</sub>-GlcCer, 50 µM doxorubicin). After 48 hours cells were examined by phase contrast microscopy and photographs were taken through a 20x objective.

**Fig. 6** - C<sub>8</sub>-GlcCer post-inserted into Caelyx® dose-dependently enhances doxorubicin transfer to cells. Commercial liposomal doxorubicin (Caelyx®) was loaded with C<sub>8</sub>-GlcCer (concentrations indicated), using the procedure described in Methods. C<sub>8</sub>-GlcCer insertion was confirmed by TLC analysis (insert; iodine vapor staining). A431 cells were exposed (24 h; serum-free) to 20 µM doxorubicin in modified Caelyx®. After washing the cells, cellular doxorubicin was quantified and corrected for total cellular protein. Data are means ± SD (n=3).

**Fig. 7** C<sub>8</sub>-GlcCer in liposomes, but not in free form, retains its doxorubicin-uptake enhancing effect in the presence of serum. (A) In the presence of various concentrations of fetal calf serum, A431 cells were treated for 24 h with 50 µM of free doxorubicin, in the absence (open bars) or presence (hatched bars) of 10 µM C<sub>8</sub>-GlcCer (not in liposomes). (B) Cells were treated with 50 µM doxorubicin formulated in liposomes, containing either 0 mol% C<sub>8</sub>-GlcCer (open bars) or 17 mol% of C<sub>8</sub>-GlcCer (hatched bars). (Liposomal) doxorubicin that was not taken up by the cells was washed away, and the cells were lysed. Cellular doxorubicin was quantified and corrected for protein contents (mean ± SD, n=3).
Table 1 - Composition and characteristics of liposome preparations used in this study.

Liposomes with various lipid compositions were prepared, as described in the Methods section, and loaded with doxorubicin, or not (empty liposomes). For comparison, a commercially available liposomal doxorubicin preparation (Caelyx®) was analyzed.

<table>
<thead>
<tr>
<th></th>
<th>C8-GlcCer-enriched liposomes</th>
<th>Caelyx®</th>
<th>empty liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC (mol%) (HSPC in Caelyx)</td>
<td>62 60 58 56 53 50</td>
<td>55</td>
<td>62 50</td>
</tr>
<tr>
<td>cholesterol (mol%)</td>
<td>33 32 31 30 29 28</td>
<td>40</td>
<td>33 28</td>
</tr>
<tr>
<td>DSPE-PEG2000 (mol%)</td>
<td>5 5 5 5 5 5</td>
<td>5</td>
<td>5 5</td>
</tr>
<tr>
<td>C8-GlcCer (mol%)</td>
<td>0 3 6 9 13 17</td>
<td>0</td>
<td>0 17</td>
</tr>
<tr>
<td>doxorubicin (µmol/µmol phospholipid)</td>
<td>0.23 0.27 0.28 0.26 0.28 0.26</td>
<td>0.29  -  -</td>
<td></td>
</tr>
<tr>
<td>mean size (nm)²</td>
<td>107 104 104 107 108 99</td>
<td>85</td>
<td>106 99</td>
</tr>
<tr>
<td>leakage (% loss after 1 day at 37°C)</td>
<td>0.35 0.14 0.25 0.33 0.37 0.24</td>
<td>0.57 - -</td>
<td></td>
</tr>
<tr>
<td>leakage (% loss after 60 days at 4°C)</td>
<td>0.46 0.66 0.56 0.49 0.44 0.48</td>
<td>n.d. - -</td>
<td></td>
</tr>
</tbody>
</table>

1Doxorubicin content of the preparations was measured and expressed as µmol per µmol of liposomal phospholipid.

2Mean liposome diameters were obtained from volume distribution curves, produced by a dynamic laser light scattering-based particle analyzer. Polydispersity, a measure for variation in particle size within the liposome population, varies between 0 (complete monodispersity) and 1 (maximal variation). With polydispersities below 0.065, all present preparations fell well within acceptable limits of variation.
Figure 1

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Figure 2

The graph shows the doxorubicin uptake (nmol/mg cellular protein) in relation to the percentage of C₈-GlcCer (mol%). The x-axis represents the percentage of C₈-GlcCer, while the y-axis represents the doxorubicin uptake. The data points are indicated with error bars, showing the variability in uptake. The uptake increases as the percentage of C₈-GlcCer increases.
Figure 4

![Bar graph showing viability (% of control) vs. C$_8$-GlcCer (mol%)](image-url)
Figure 5

control  17 mol% C₈-GlcCer

control

50 µM doxorubicin

A

B

C

D
Figure 6

The graph shows the doxorubicin uptake (nmol/mg cellular protein) in relation to the C₈-GlcCer loading concentration (µM). The concentrations tested were 0, 66, 132, 220, 330, and 440 µM. The data indicates an increase in doxorubicin uptake as the concentration of C₈-GlcCer increases.
Figure 7

- **Free doxorubicin (A)**
  - Y-axis: doxorubicin uptake (nmol/mg cellular protein)
  - X-axis: fetal calf serum (v/v)

- **Liposomal doxorubicin (B)**
  - Y-axis: doxorubicin uptake (nmol/mg cellular protein)
  - X-axis: fetal calf serum (v/v)