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Intracellular delivery of ceramide lipids via liposomes enhances apoptosis in vitro

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Abstract

Ceramide lipids have emerged as important intracellular signalling molecules that mediate diverse cellular effects, of which programmed cell death, or apoptosis, has attracted significant interest. Although the exact mechanism(s) by which ceramides trigger apoptosis is not fully understood, there is considerable evidence that they are key mediators of this response. Exogenously applied, cell-permeable ceramides have been shown to induce apoptosis when incubated with cells in culture. We examined here the cytotoxicity of ceramides with varying acyl chain lengths in order to determine whether acyl chain length affects pro-apoptotic activity within the concentration range of 0–100 μM. We found that for C_6-, C_8-, C_{10}-, C_{14}- and C_{16}-ceramide, the chain length was inversely proportional to cytotoxic activity, with C_6-ceramide being most active (IC_{50} values in the 3–14 μM range) and C_{16}-ceramide being least active (IC_{50} values in excess of 100 μM) in the MDA435/LCC6 human breast cancer and J774 mouse macrophage cell lines investigated. Using these two ceramide forms we were able to correlate the observed cytotoxicity with cellular uptake, and we observed that a lack of intracellular delivery may be responsible for the weak activity of C_{16}-ceramide. We therefore investigated the possibility of incorporating ceramide lipids into liposome bilayers to enhance this delivery. We demonstrate that stable, ceramide-containing liposomes can be formulated, and that they are cytotoxic when taken up by cells in vitro. These results provide an increased understanding of the differences in cytotoxic activity of exogenous short- and long-chain ceramide lipids, and their incorporation into biologically active liposomal formulations opens new avenues for apoptosis induction.

Keywords: Sphingolipid; Ceramide; Apoptosis; Liposome; Bioactive lipid

1. Introduction

Sphingolipids are a complex and diverse group of lipids that has gained much attention as intracellular signalling molecules involved in cell differentiation, cell cycle arrest, senescence and apoptosis [1–3]. The sphingolipid *ceramide* has been identified as an important second messenger that mediates these effects, and its specific involvement in apoptosis has attracted significant interest. Intracellular ceramide may be generated by hydrolysis of the membrane lipid sphingomyelin via plasma membrane and cytosolic forms of neutral sphingomyelinase enzymes (nSMases), via endosomal/lysosomal acid sphingomyelinases (aSMases), or via de novo synthesis from serine and palmitoyl CoA by ceramide synthase. The exact mechanism by which ceramide elicits apoptosis has not been fully elucidated, and the pathways appear to be both stimulus- and cell type-specific. However, ceramide lipids are known to partic-

Abbreviations: C_2-ceramide, *N*-acetoyl-D-erythrosphingosine; C_6-ceramide, *N*-hexanoyl-D-erythrosphingosine; C_8-ceramide, *N*-octanoyl-D-erythrosphingosine; C_{10}-ceramide, *N*-decanoyl-D-erythrosphingosine; C_{14}-ceramide, *N*-myristoyl-D-erythrosphingosine; C_{16}-ceramide, *N*-palmitoyl-D-erythrosphingosine; IC_{50}, 50% inhibitory concentration; nSMase, neutral sphingomyelinase; aSMase, acid sphingomyelinase; GlcCer, glucosylceramide; MDR, multidrug resistance; GCS, glucosylceramide synthase; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide]; CHE, cholesterylhexadecylether; DMEM, Dulbecco's Modified Eagle's Medium; Hanks, Hanks Balanced Salt Solution; EDTA, ethylenediaminetetraacetic acid; ATCC, American Type Culture Collection; Abs, absorbance; DPPC, dipalmitoyl phosphatidylcholine; DSPC, disteroyl phosphatidylcholine; CHEMS, cholesteryl hemisuccinate; Chol, cholesterol; BCA, bicinchoninic acid; BSA, bovine serum albumin; Cer, ceramide

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ipate in signal transduction cascades by activating serine/threonine kinases and by stimulating protein phosphatases. Emerging evidence also suggests that sphingomyelin-enriched lipid domains or "rafts" may serve as substrate pools for SMase-induced formation of ceramide microdomains that act as platforms from which these signal transduction cascades originate [4]. Ceramides have also been shown to exert direct effects on mitochondria [5,6], and one mechanism that has been proposed for ceramide-mediated apoptosis is via channel or pore formation in mitochondrial membranes [7].

The emerging role for ceramide in apoptosis makes it an attractive intracellular target from a therapeutic perspective. Exogenous, cell-permeable ceramides have been shown to result in tumor cell apoptosis in a number of cell lines. Many cancer chemotherapy modalities, including doxorubicin, daunorubicin, etoposide and radiation, act in part by inducing ceramide generation, and synergistic effects between exogenous ceramide and anticancer treatments have been reported [8]. These observations suggest that stimuli which result in increased intracellular ceramide levels have pro-apoptotic effects. The involvement of ceramide in apoptosis was further demonstrated by the finding that alterations in ceramide metabolism, whereby pro-apoptotic ceramide is converted to its non-cytotoxic glucosylceramide (GlcCer) metabolite, contribute to the emergence of multidrug resistance (MDR) [9]. Several tumor cell lines and clinical samples have been shown to overexpress the glucosylceramide synthase (GCS) enzyme which converts ceramide to GlcCer [10]. This has been correlated with resistance to cytotoxic agents in vitro and poor response to chemotherapy in cancer patients. Several studies have since demonstrated that inhibitors of ceramide metabolism can be used to chemosensitize resistant cells in vitro [11–15].

The above observations indicate that therapeutic approaches aimed at increasing intracellular ceramide levels should lead to an enhanced apoptotic response. In this paper we used the MTT cytotoxicity assay to investigate the effect of adding exogenous ceramides of varying acyl chain lengths to tumor cells in vitro. We demonstrate that cytotoxicity is dependent on chain length, and these results were correlated with cellular uptake of radiolabeled ceramide lipids. We also investigated the feasibility of using liposomal delivery systems to enhance intracellular ceramide accumulation and cytotoxicity. Our aim was to exploit the ability of liposomes to deliver therapeutic lipids in order to achieve intracellular delivery of ceramide lipids. The results of these studies provide an increased understanding of the basis for the differences in cytotoxic activity of exogenous short- and long-chain ceramide lipids, and open opportunities for therapeutic strategies based on controlled ceramide delivery.

2. Materials and methods

2.1. Materials

All phospholipids and ceramides were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol, cholesteryl hemisuccinate and MTT reagent were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). [^{3}H]Cholesterylhexadecyl ether (CHE) was purchased from Perkin-Elmer (Boston, MA). [^{14}C]C_6- and [^{14}C]C_{16}-ceramide were purchased from American Radiolabeled Chemicals (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) and Hanks Balanced Salt Solution (without pH indicator; Hanks) were obtained from Stem Cell Technologies (Vancouver, BC, Canada). Fetal bovine serum was purchased from Hyclone (Logan, UT). L-Glutamine and trypsin-EDTA were obtained from Gibco BRL (Burlington, ON, Canada). The Micro BCA Protein Assay kit was purchased from Pierce (Rockford, IL). Tissue culture flasks, incubation plates and cell scrapers were obtained from Falcon (Becton Dickinson, Franklin Lakes, NJ).

2.2. Cell lines and culture

Human estrogen receptor negative MDA435/LCC6 wild-type and MDR-1 gene-transfected MDA435/LCC6^{MDR1} multidrug resistant breast cancer cell lines were a generous gift from Dr. Robert Clark, Georgetown University, Washington, DC. J774 murine macrophage cells were obtained from ATCC (Rockville, MD). All cells were grown as adherent monolayer cultures in 25-cm^2 Falcon flasks in DMEM supplemented with 10% fetal bovine serum and 1% L-glutamine. Cells were maintained at 37 °C in humidified air with 5% CO_2. Cells were subcultured weekly using 0.25% trypsin with 1 mM EDTA (MDA435/LCC6) or gentle cell scraping (J774).

2.3. Preparation of liposomes

Lipids were weighed into individual test tubes and dissolved in 1 ml of chloroform (DPPC, DSPC, CHEMS, Chol), ethanol (C_2-, C_6-, C_8-, C_{10}-, C_{14}-ceramide) or chloroform/methanol (2:1, v/v; C_{16}-ceramide). C_{16}-ceramide required brief heating at 65 °C to achieve complete dissolution. Appropriate volumes of each lipid were transferred to a single tube in order to achieve the desired ratio of each lipid component. All ratios indicated in this research are on a mole/mole basis. [^{3}H]CHE was incorporated at 1 μCi/mg lipid as a nonexchangeable, non-metabolizable lipid marker [16] to facilitate liposome quantitation. For the preparation of ceramide-containing liposomes, [^{14}C]C_6-ceramide or [^{14}C]C_{16}-ceramide was incorporated into the formulation at 0.5 μCi/mg ceramide. The mixtures were evaporated with vortexing and heating under a stream of nitrogen gas and subjected to vacuum drying for a minimum of 4 h to produce a homogenous lipid film. The lipid film was

hydrated in 1 ml of warm HEPES buffered saline (HBS; 20 mM HEPES/150 mM NaCl; pH 7.4) with vortexing. Homogeneously sized liposomes were then produced following a 10-cycle extrusion through three stacked 100-nm polycarbonate filters (Nucleopore, Canada) at 65 °C for non-ceramide formulations and 95 °C for ceramide formulations, using an extrusion apparatus (Lipex Biomembranes, Vancouver, BC, Canada). The resulting mean liposome diameter obtained following extrusion was within a range of 91–132 nm, depending on lipid composition, as determined by quazi-elastic light scattering using the Nicomp 270 submicron particle sizer model 370/270 [17,18]. Liposome and ceramide concentrations were determined by liquid scintillation counting.

2.4. Cytotoxicity assays

Cell suspensions were diluted 1:1 with trypan blue, counted with a hemocytometer and seeded into 96-well microtiter plates at 1.5×10^6 cells/well in 0.2-ml complete medium. The perimeter wells were not used and contained 0.2-ml sterile water. The cells were allowed to adhere for 24 h at 37 °C, after which the medium was aspirated and replaced with 0.1-ml fresh medium. Free ceramide, control liposome or ceramide-liposome stocks were diluted into complete medium and added to cells in 0.1 ml to achieve the desired final concentration. The C_{16}-ceramide stock was kept warm and diluted into warm medium prior to addition to the cells, and remained in solution at all times. After 72 h the cell viability was assessed using a conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay. Fifty microliters of 5 mg/ml MTT reagent in phosphate buffered saline (PBS) was added to each well. Viable cells with active mitochondria reduce the MTT to an insoluble purple formazan precipitate that is solubilized by the subsequent addition of 150-μl dimethyl sulfoxide. The formazan dye was measured spectrophotometrically using a Dynex plate reader (570 nm). All assays were performed in triplicate. The cytotoxic effect of each treatment was expressed as percent cell viability relative to untreated control cells (% control) and is defined as: $[(Abs_{570}$ treated cells)/$(Abs_{570}$ control cells)$] \times 100$.

2.5. Lipid uptake studies

Cell suspensions were diluted 1:1 with trypan blue, counted with a hemocytometer and seeded into six-well Falcon plates at 2.5×10^5 cells/well in 2-ml complete medium. The cells were allowed to adhere for 24 h at 37 °C, after which the medium was aspirated and replaced with 1-ml complete medium. Free ceramide, control liposome or ceramide-liposome stocks were diluted in 1-ml complete medium and added to each well to give the desired final concentration. Cells were incubated with the treatments for 1, 4 and 24 h at 37 °C. The incubation medium was then aspirated and cells were washed twice with 2-ml Hanks. Cells were gently scraped into 0.5-ml Hanks and collected into glass scintillation vials using glass pipettes. Each well was rinsed with an additional 0.5-ml Hanks to remove residual cells. An aliquot of cells was removed for protein quantification and the remainder was counted for radioactivity by scintillation counting.

2.6. Spectrophotometric protein quantification

The protein content of each cell aliquot was determined using the Pierce Micro BCA Protein Assay according to the method included with the assay kit. Briefly, a standard curve was prepared using the supplied purified bovine serum albumin (BSA) diluted in distilled water to a final volume of 0.5 ml. Samples were prepared using 5 μl cell suspension+ 495 μl dH2O. Micro BCA reagents A, B and C were added in the specified ratios. All samples and standards were prepared in glass test tubes. which were heated in a 65 °C water bath for 1 h and cooled to room temperature. The absorbance at 562 nm of each sample was read against a dH_2O reference. The protein concentration for each cell sample was determined using a standard curve prepared from the known BSA samples.

3. Results

3.1. Chain length dependence of free ceramide cytotoxicity in the MDA435/LCC6 human breast cancer cell line

The cytotoxic activity of exogenously applied ceramides with increasing acyl chain length was evaluated by incubating the MDA435/LCC6 and MDA435/LCC6^{MDR1} cells with C_2- C_6- C_8- C_{10}- C_{14}- and C_{16}-ceramide over a range of 0–100 μM final ceramide concentration. The 72 h MTT cytotoxicity results shown in Fig. 1 demonstrate that cytotoxic activity is dependent on ceramide acyl chain-length. With the exception of C_2-ceramide, as acyl chain length increased the cytotoxic activity decreased. This trend may be explained by the cell-permeability characteristics of the various acyl chain lengths. In order to be active, the exogenous ceramide must transfer from the tissue culture medium in which it is dissolved, cross the plasma membrane of the cell and then exchange into cellular membranes from which it can interact with it intracellular target(s). The C_2-ceramide, being very hydrophillic, likely remains dispersed in the tissue culture media, which is consistent with its IC_{50} values of 74 and 79 μM for the wild-type and MDR cell lines, respectively (Table 1). The C_6- and C_8-ceramides are the most cytotoxic, with IC_{50} values in the 3–14 μM range. As the chain length increases to C_{10}-, C_{14}- and C_{16}-ceramide the hydrophobic nature also increases, and IC_{50} values of approximately 45 μM are observed for C_{10}-ceramide and in excess of 100 μM are observed for C_{14}- and C_{16}-ceramides. From these results we identified C_6-ceramide as the most potent exogenous ceramide form.

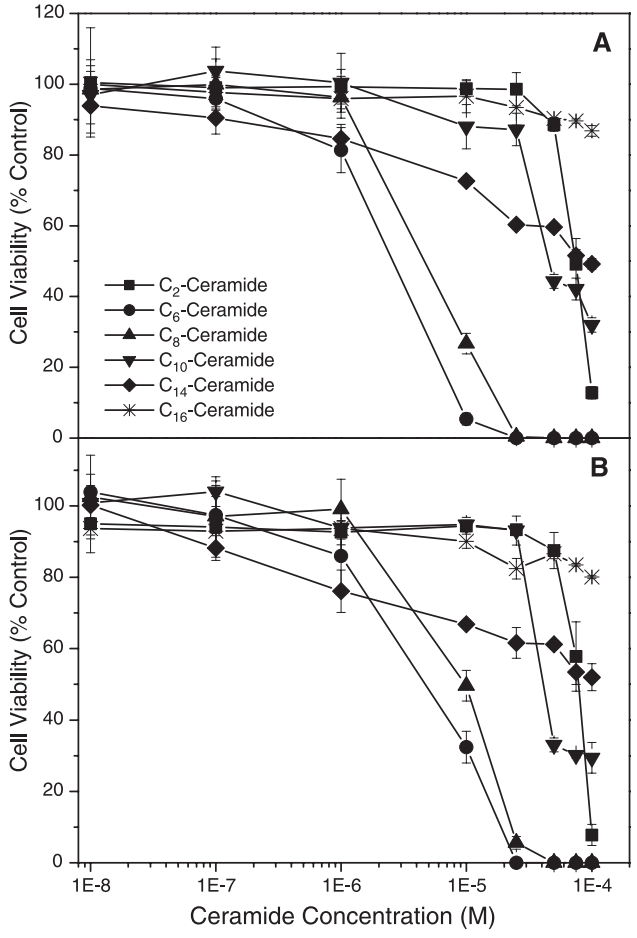

Fig. 1. Cytotoxicity of various acyl chain length free ceramide lipids on wild-type (A) and MDR-1 gene transfected (B) MDA435/LCC6 human breast cancer cells. Cells were incubated with the indicated ceramide concentrations for 72 h and cell viability was measured using the MTT assay. Data are averaged means from three triplicate experiments. Each value represents the mean from at least three independent experiments; bars, S.D.

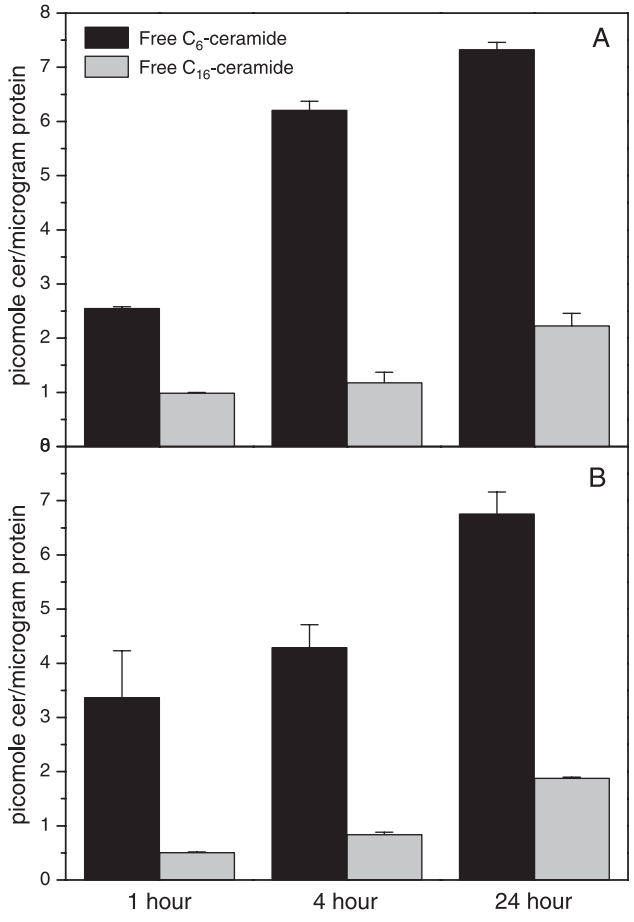

Fig. 2. Cellular uptake of C_6- and C_{16}-ceramide by wild-type (A) and MDR-1 gene transfected (B) MDA435/LCC6 cells. Cells were incubated with 1.0 μM C_6-ceramide or 50 μM C_{16}-ceramide for the times indicated. [^{14}C]C_6- or [^{14}C]C_{16}-ceramide was added at 0.1 μCi/nmol ceramide for quantitation by scintillation counting. Cellular protein content was measured spectrophotometrically (Abs 562 nm) using the micro BCA protein assay kit. Data are averaged means from two triplicate experiments; bars, S.D.

3.2. Correlation of MTT cytotoxicity results with ceramide uptake levels

In order to examine whether acyl chain length determines ceramide cytotoxicity because of differences in cell-permeability, we conducted cellular uptake studies using radioactive C_6- and $C1_6$-ceramides. Fig. 2 shows that for both the wild-type and resistant cell lines, [^{14}C]C_6-ceramide levels steadily increased to 7 pmol ceramide/μg protein over the 24-h incubation period while [^{14}C]C_{16}-ceramide levels remained under 2 pmol ceramide/μg protein. These relative differences are consistent with the lack of C_{16}-ceramide-induced cytotoxicity.

Table 1
IC_{50} values for various chain length free ceramide lipids incubated with wild-type and MDR-1 gene-transfected MDA435/LCC6 human breast cancer cells

Cell line	IC_{50}[a] ± S.E. (μM)					
	C_2-Cer	C_6-Cer	C_8-Cer	C_{10}-Cer	C_{14}-Cer	C_{16}-Cer
MDA435/LCC6	73.60 ± 7.92	2.71 ± 0.69	7.00 ± 3.25	44.10 ± 2.69	>100	>100
MDA435/LCC6^{MDR1}	79.20 ± 6.04	5.67 ± 0.70	13.95 ± 2.19	46.00 ± 1.39	>100	>100

[a] Cells were incubated with increasing concentrations of free ceramide lipid over 72 hours and cell viability was measured using the MTT assay. The IC_{50} value was taken as the ceramide concentration that inhibits cell growth by 50% relative to untreated control cells. Data are average IC_{50} values obtained from three independent experiments conducted in triplicate. The S.E.M. represents the standard error associated with the resulting three mean values.

3.3. Formulation and activity of C_6-ceramide liposomes in MDA435/LCC6 cells

Since C_6-ceramide was identified as the most potent exogenous ceramide lipid, we attempted to formulate it into liposomes to further enhance its intracellular delivery. Liposomes containing up to 15 mol% C_6-ceramide were successfully formulated, with an overall composition of C_6-ceramide/DSPC/Chol (15:40:45) and a mean diameter of 98–117 nm. Fig. 3 demonstrates that C_6-ceramide liposomes are cytotoxic to cells in vitro, with IC_{50} values of 15.9 and 18.2 μM for the wild-type and MDR-1 gene-transfected MDA435/LCC6 cells, respectively. The cytotoxicity was ceramide-specific, as control liposomes composed of DSPC/Chol (55:45) that do not contain ceramide in the bilayer displayed no activity. These results were then correlated with cellular uptake. For these studies lipid

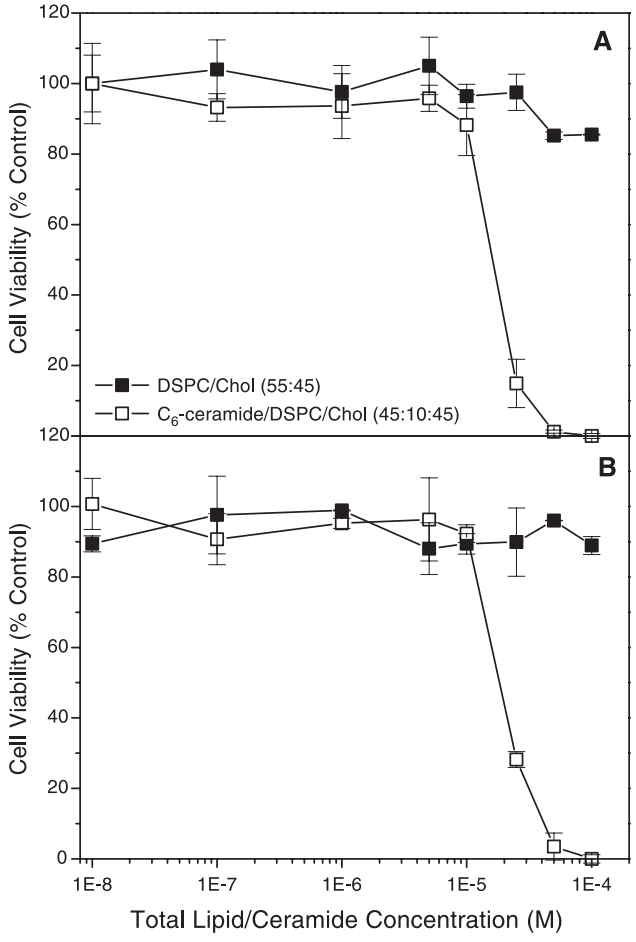

Fig. 3. Cytotoxicity of control (DSPC/Chol, 55:45) and C_6-ceramide (C_6-ceramide/DSPC/Chol, 15:40:45) liposomes on wild-type (A) and MDR-1 gene-transfected (B) MDA435/LCC6 human breast cancer cells. Cells were incubated with the indicated concentrations of liposomes for 72 h and cell viability was measured using the MTT assay. The indicated liposome concentration represents total lipid for control liposomes and was corrected for ceramide content for ceramide-containing liposomes. Data are averaged means from three triplicate experiments; bars, S.D.

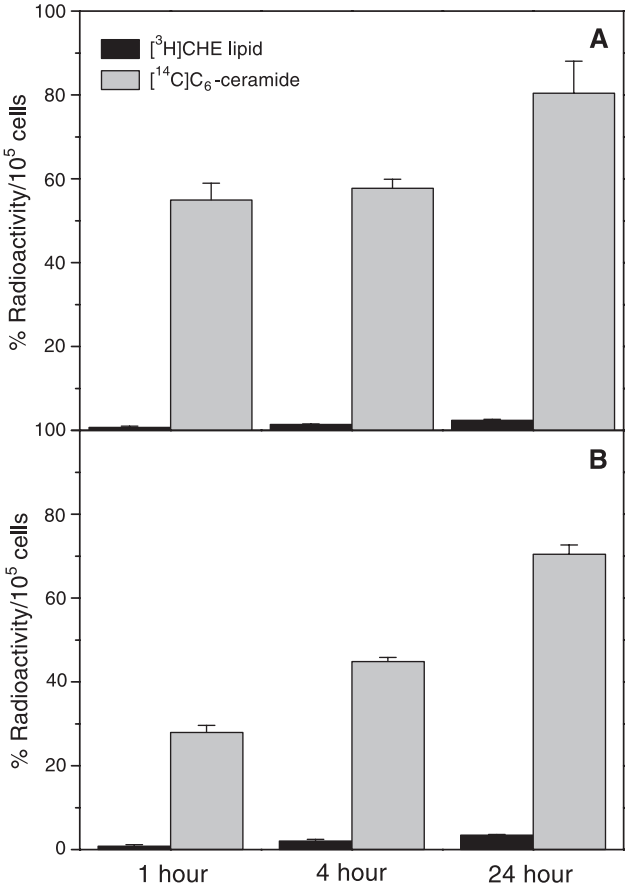

Fig. 4. Cellular uptake of C_6-ceramide/DSPC/Chol (15:40:45) liposomes by wild-type (A) and MDR-1 gene-transfected (B) MDA435/LCC6 cells. Uptake of [^{3}H]CHE bulk liposomal lipid and [^{14}C]C_6-ceramide is expressed as percent of the total radioactivity added, normalized to 10^5 cells. Data are averaged means from two triplicate experiments; bars, S.D.

uptake was expressed as a percent of the total radioactivity added in order to allow for direct comparisons to be made between the ceramide lipid and bulk liposomal lipid. The results in Fig. 4 indicate that the [^{14}C]C_6-ceramide component of the liposomes was taken up by the tumor cells, approaching 80% internalization after 24 h, whereas less than 10% of the [^{3}H]CHE liposome label was cell-associated after 24 h. These results suggest that the C_6-ceramide is being delivered not via the liposomes, but rather by exchange from the liposome bilayer into cellular membranes in a manner similar to that following addition of non-liposomal C_6-ceramide.

3.4. Formulation and activity of C_{16}-ceramide liposomes in MDA435/LCC6 cells

Since formulation of C_6-ceramide into liposomes did not appear to afford any delivery benefit, we decided to investigate whether the more physiologically relevant long-chain C_{16}-ceramide, which was not cytotoxic in its free form, could be made active by delivering it via liposomes. We

determined that up to 15 mol% C_{16}-ceramide could be successfully formulated into liposomes with an overall composition of C_{16}-ceramide/DSPC/Chol (15:40:45) and a mean diameter in the range of 91–124 nm. However, when incubated with cells in the MTT assay, we found that the cytotoxicity of C_{16}-ceramide-containing liposomes was comparable to control DSPC/Chol (55:45) liposomes. This was correlated with cellular uptake studies indicating that less than 10% of the [^{3}H]CHE and [^{14}C]C_{16}-ceramide labels were internalized after 24 h (data not shown).

The inability of liposomes to enhance the cytotoxicity of C_{16}-ceramide appeared to be attributed to a lack of internalization of the liposomes by the MDA435/LCC6 cells. In order to enhance internalization of ceramide-containing liposomes, we formulated C_{16}-ceramide into negatively charged liposomes in an attempt to increase the interaction of the liposomes with the target cells. To accomplish this goal, we utilized cholesteryl hemisuccinate (CHEMS), an acidic cholesterol ester that can be incorporated into liposomes to impart pH sensitivity and fusogenic properties [19,20]. Our objectives in incorporating this lipid into our ceramide-containing liposomes were first, to use its negative charge to increase the interaction between the liposomes and cells, and second, to exploit the pH sensitivity of the lipid to trigger liposome destabilization upon intracellular delivery via endocytosis. We observed that C_{16}-ceramide could be incorporated into CHEMS containing liposomes at up to 50 mol%, for a final liposome composition of C_{16}-ceramide/CHEMS (50:50). These liposomes were stable and displayed a mean diameter range of 97–132 nm. Fig. 5 shows that C_{16}-ceramide/CHEMS liposomes provided a modest increase in the cytototoxicity of free C_{16}-ceramide; however, the IC_{50} remained greater than 100 μM. This improvement in activity was not attributable to the negative charge of the CHEMS liposomes, as control liposomes composed of DPPC/CHEMS (50:50) showed no activity. The non-ceramide lipid of the control liposomes in this case was changed from DSPC to DPPC to more closely match the 16-carbon acyl chain length of the ceramide.

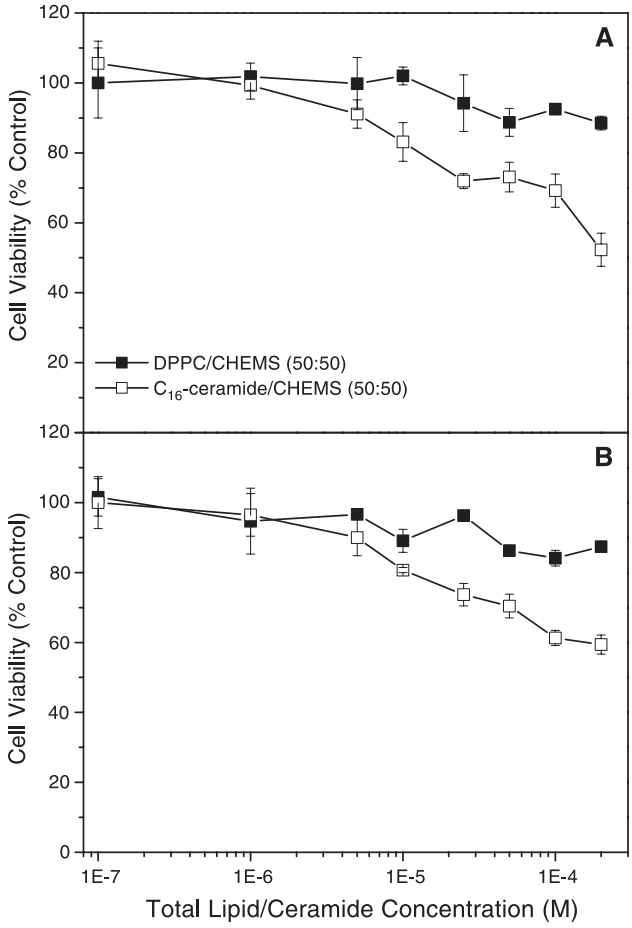

Fig. 5. Cytotoxicity of control (DPPC/CHEMS, 50:50) and C_{16}-ceramide (C_{16}-ceramide/CHEMS, 50:50) liposomes on wild-type (A) and MDR-1 gene-transfected (B) MDA435/LCC6 human breast cancer cells. Cells were incubated with the indicated concentrations of liposomes for 72 h and cell viability was measured using the MTT assay. The indicated liposome concentration represents total lipid for control liposomes and was corrected for ceramide content for ceramide-containing liposomes. Data are averaged means from three triplicate experiments; bars, S.D.

3.5. Cytotoxicity of ceramide lipids in the J774 murine macrophage cell line

The modest improvement in activity afforded by the CHEMS liposomes gave us a preliminary indication that liposomal delivery of C_{16}-ceramide may be effective if efficient intracellular delivery could be achieved. In order to demonstrate this from a proof-of-principle perspective, we utilized the J774 murine macrophage cell line, which provides enhanced liposome endocytosis and therefore intracellular delivery of the liposomes to their intended target (endosomes/lysosomes) where pro-apoptotic ceramide is known to be endogenously generated by aSMases [21–23]. Identification of the endosomal acidic aspartate protease cathepsin D as a ceramide binding target has been implicated as a mediator of this response [24,25].

Before investigating the effect of ceramide-containing liposomes on J774 cells, we first examined whether free ceramide lipids had the same effect on J774 cells as we observed in the MDA435/LCC6 cells. As shown in Fig. 6, the same chain length dependence of free ceramide cytotoxicity was observed, with the short-chain C_6- and C_8-ceramides being the most active (IC_{50} values of 14.4 μM for both) and C_{16}-ceramide showing no activity.

The MTT cytotoxicity results shown in Fig. 7 indicate that liposomes composed of C_{16}-ceramide/CHEMS (50:50) dramatically improved the cytotoxicity of C_{16}-ceramide in the J774 macrophage cell line. Specifically, while the IC_{50} value of C_{16}-ceramide, when exogenously applied to these cells in non-liposomal form, was well in excess of 100 μM, its formulation and delivery via CHEMS liposomes decreased the IC_{50} to 36.1 μM, bringing it into the range of cytotoxicity observed with free C_6-ceramide (14.4 μM).

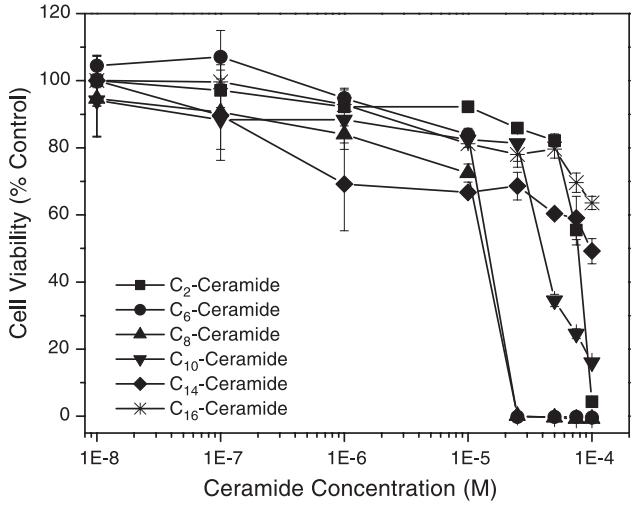

Fig. 6. Cytotoxicity of various acyl chain length free ceramide lipids on J774 murine macrophage cells. Cells were incubated with the indicated ceramide concentrations for 72 h and cell viability was measured using the MTT assay. Data are averaged means from three triplicate experiments. Each value represents the mean from at least three independent experiments; bars, S.D.

This cytotoxic effect was ceramide-specific and was not attributed to the presence of CHEMS lipid alone, as control DPPC/CHEMS (50:50) liposomes were non-cytotoxic. Cellular uptake studies showed that both the liposome and the ceramide components were internalized, as evidenced by uptake of the [^{3}H]CHE and [^{14}C]C_{16}-ceramide labels, which both approached 50% after 24 h (Fig. 8). This indicates that the C_{16}-ceramide lipid is being delivered via liposomes (rather than by passive lipid exchange), and demonstrates

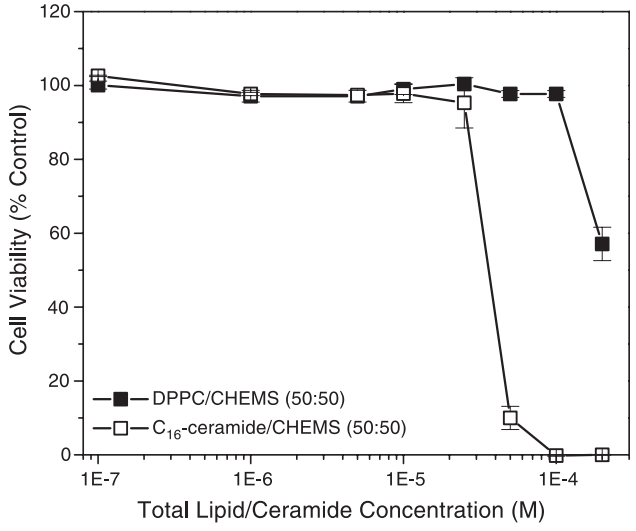

Fig. 7. Cytotoxicity of control (DPPC/CHEMS, 50:50) and C_{16}-ceramide (C_{16}-ceramide/CHEMS, 50:50) liposomes on J774 murine macrophage cells. Cells were incubated with the indicated concentrations of liposomes for 72 h and cell viability was measured using the MTT assay. The indicated liposome concentration represents total lipid for control liposomes and was corrected for ceramide content for ceramide-containing liposomes. Data are averaged means from three triplicate experiments; bars, S.D.

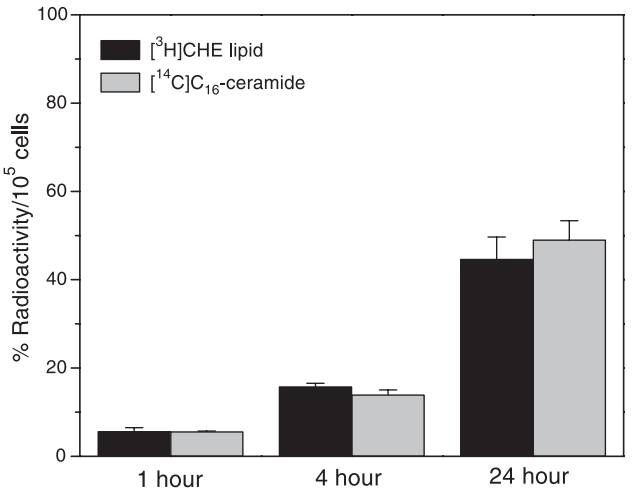

Fig. 8. Cellular uptake of C_{16}-ceramide/CHEMS (50:50) liposomes by J774 murine macrophage cells. Uptake of [^{3}H]CHE bulk liposomal lipid and [^{14}C]C_{16}-ceramide are expressed as percent of the total radioactivity added, normalized to 10^5 cells. Data are averaged means from two triplicate experiments; bars, S.D.

that endosomal delivery of these liposomes can enhance ceramide-induced apoptosis.

4. Discussion

The field of sphingolipid biology is rapidly developing, with great emphasis on ceramide lipids as mediators of diverse biological effects. Of these effects, research in the area of ceramide lipids and apoptosis has received considerable interest, both from a mechanistic [6,7,26–28] and a therapeutic [29,30] perspective. Modulation of intracellular ceramide levels is not a novel concept. Ceramide has been shown to increase dramatically following exposure to a number of cytotoxic insults, including anticancer drugs and radiation. Addition of exogenous, cell-permeable ceramides has been shown to induce apoptosis in many cell lines. In this approach research has largely focused on the C_6-ceramide form, which has received some criticism in the literature because it is perhaps not a physiologically relevant ceramide form. Nevertheless, it does induce apoptosis and therefore may be useful from a therapeutic, if not a mechanistic, perspective. Other approaches aimed at promoting endogenous ceramide formation and/or inhibiting endogenous ceramide metabolism have also shown promise in both in vitro and in vivo models [11]; reviewed in Refs. [29,31].

In the present investigation, our first objective was to examine the effect of different acyl chain length synthetic ceramides on in vitro cytotoxicity using the MDA435/LCC6 wild-type and MDR-1 gene-transfected MDA435/LCC6 cell lines. We observed an inverse relationship between acyl chain length and cytotoxicity for the C_6 to C_{16}-ceramide forms, with C_6- showing the most activity (IC_{50} values in the 3–14 μM) whereas C_{16}-ceramide showed no cytotoxic

activity (IC_{50} values well in excess of 100 μM). We were able to correlate these differences with uptake of radioactive [^{14}C]C_6- and [^{14}C]C_{16}-ceramide and demonstrated that short-chain ceramide uptake was more than threefold greater than that of long-chain ceramide (7 versus 2 pmol cer/μg protein). It is important to highlight that no differences were observed between the wild-type and MDR-1 gene-transfected cells with respect to ceramide-induced cytotoxicity or uptake. This is significant because it suggests that ceramide-based therapies may be equally effective in the treatment of sensitive and MDR tumors that overexpress Pgp.

As noted by Hannun and Luberto in a recent review, it is important to consider that the relevant concentration of cell-associated ceramide represents the total membrane ceramide concentration. Since this is highly dependent on factors such as cell density, it is important that experimental conditions be uniform and consistent in order for valid comparisons to be made between different treatments and/or experiments [30].

The observed relationship between acyl chain length, cytotoxicity and uptake led us to speculate about the possibility of incorporating ceramide lipids into liposome bilayers to enhance intracellular delivery. Liposomes containing 15 mol% C_6-ceramide were successfully formulated into DSPC/Chol liposomes and were demonstrated to be cytotoxic to MDA435/LCC6 cells in vitro. However, uptake studies demonstrated that the activity was attributed to ceramide lipid exchange from the liposome bilayer to the cell membranes, rather than by delivery via liposomes (80% cell-associated C_6-ceramide versus less than 10% cell-associated liposomal lipid). This supports our contention that C_6-ceramide is cytotoxic due to its ability to readily exchange between intracellular membrane pools. The amphipathic nature of C_6-ceramide affords it sufficient solubility to disperse in aqueous cell culture media, but also provides the hydrophobic character required for the permeation of cell membranes to reach intracellular target(s). This exchangeability is supported by observations of C_6-ceramide transfer between lipid vesicles [32]. Thus, the liposomes neither enhanced ceramide delivery nor improved ceramide cytotoxicity beyond that observed following delivery of short-chain ceramide in its free form, and therefore the liposomes provided no therapeutic advantage to the already active short-chain ceramide. This prompted us to turn our attention to natural ceramide in an attempt to design liposomes that incorporate C_{16}-ceramide in the bilayer. This was done on the basis that C_{16}-ceramide is more likely to remain associated with the liposome during delivery and does not passively exchange from the liposome bilayer in which it is stably incorporated. This is supported by the work of Simon et al., who observed that exchange of [^{14}C]C_{16}-ceramide between phospholipid vesicles occurred on the order of days ($t_{1/2}$ of 49–105 h) [33], as well as similar observations made by our lab [32]. Furthermore, C_{16}-ceramide is the more physiologically relevant form, and both short- and long-term increases in C_{16}-ceramide accumulation have been observed during apoptosis [34]. We found that up to 15 mol% C_{16}-ceramide could be successfully formulated into DPPC/Chol liposomes. However, these liposomes did not appear to be active in vitro, with IC_{50} values well in excess of 100 μM in the MDA435/LCC6 cell line. Uptake studies revealed that this was attributed to lack of internalization of the liposomes themselves. This was perhaps not surprising, as cells do not readily endocytose liposomes in the absence of targeting ligands or surface charge.

In order to address this issue, we changed the liposome formulation to incorporate the pH-sensitive CHEMS lipid, which would increase liposome interaction with the target cells due to the negative surface charge and also become fusogenic once exposed to the acidic pH of the endosomes and lysosomes. We also changed the target cells to the murine macrophage J774 cell line, which is known to readily endocytose liposomes. This helped to ensure that the ceramide would be specifically delivered to the endosomal target site. We observed that up to 50 mol% C_{16}-ceramide could be successfully formulated into CHEMS-containing liposomes, and we demonstrated that these liposomes were indeed cytotoxic with an IC_{50} value of 36.1 μM, which approached that of free C_6-ceramide (14.4 μM). We further demonstrated that both the ceramide and lipid components of the C_{16}-ceramide formulation are internalized, which indicates that the liposome is acting as a biologically active ceramide delivery vehicle.

These results clearly demonstrate the importance intracellular delivery for the application of exogenous ceramide lipids as therapeutically active inducers of apoptosis. Although short-chain, cell-permeable ceramides show activity on their own, their rapid exchange between lipid membranes limits their ability to be used for controlled ceramide delivery purposes. Furthermore, one must be cautious when extrapolating observations obtained using short-chain, exogenous ceramides to the physiological setting, as their biophysical properties differ from those of natural ceramides. The results presented here address both of these issues. First, by using a liposome-based system the ceramide lipids can be delivered in a much more controlled and specific manner. Second, this work avoids potential complications associated with the use of synthetic ceramide analogues by delivering physiologically relevant endogenous C_{16}-ceramide to specific organelles (endosomes/lysosomes) associated with natural ceramide production in response to apoptotic stimuli. Taken together, these results provide the basis for the development of therapeutically active ceramide-based liposomes, which have the potential to be pursued as novel inducers of apoptosis.

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