Short-Chain Ceramides for Enhancing Cytotoxicity of Liposome-Encapsulated Doxorubicin Toward Human Breast Cancer (MDA-MB-231) and Prostate Cancer (PC-3) Cells

By

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Abstract

Co-delivery of short chain ceramide, C_6-Ceramide (C_6-Cer) and C_8-Ceramide (C_8-Cer) and doxorubicin (DOX) using a liposomal system in MDA-MB-231 breast cancer and PC3 prostate cancer cell lines for synergistic cytotoxic effects was investigated. Liposomes, containing disparate ceramides, were prepared in a molar ratio of 44:40:4:12 mol% of DOTAP/ cholesterol/PEG2000-DSPE/Ceramide, respectively using lipid film hydration method and loaded with doxorubicin (ratio of 0.2:1). Liposomes were characterized by measuring size, polydispersity index, release profile and doxorubicin content. In addition, *in vitro* cytotoxicity and cellular uptake were evaluated.

Doxorubicin liposomes enriched with either C_6 or C_8 ceramide exhibited high drug encapsulation efficiency (>90%) and small size (~ 94 nm). Enhanced cytotoxic effect was noticed between doxorubicin and both C_6 and C_8 ceramide in both cell lines. Doxorubicin enriched with C_6 and C_8-ceramide exhibited the highest cytotoxicity against MDA-MB-231 and PC3 cells compared to liposome formulation that does not contain ceramide and free doxorubicin. Furthermore, cellular uptake of liposomal doxorubicin enriched with C_6 and C_8-ceramide was higher than both free doxorubicin and liposome formulation without ceramide.

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Akt	Protein Kinase B (serine/threonine kinase)
C6-Ceramide	N-hexanoyl-D-erythro-sphingosine
C8-Ceramide	N-Octanoyl-D-erythro-sphingosine
DMSO	Dimethylsulfoxide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOX	Doxorubicin
Fas-L	Fas ligand
1L	Interleukin
MLV	Multilamellar
mPEG-DSPE(2000)	MethoxypolyEthyleneGly-col-DistearoylPhosphatidyl Ethanolamine
PBS	Phosphate-Buffered Saline
RES	Reticuloendothelial system
PEG	PolyEthylene Glycol
PI3K	Phosphoinositide 3-kinase
UV	Unilamellar vesicle

1. Review

1.1 Structure and Properties:

Ceramides compose of a long-chain, called sphingoid, base attached to a fatty acid via an amide bond. Sphingoid base (a 2-aminoalk[ane or ene]1,3-diol with 2S,3R stereochemistry) (1) can be differentiated by the chain length of the fatty acid (2). For instance, in case of C-$_6$ ceramide, fatty acid chain contains six carbons. Ceramides have been identified as minor cell components, except in stratum corneum where they compose up to 50% of total lipid, which are engaged in biological functions in different aspects.

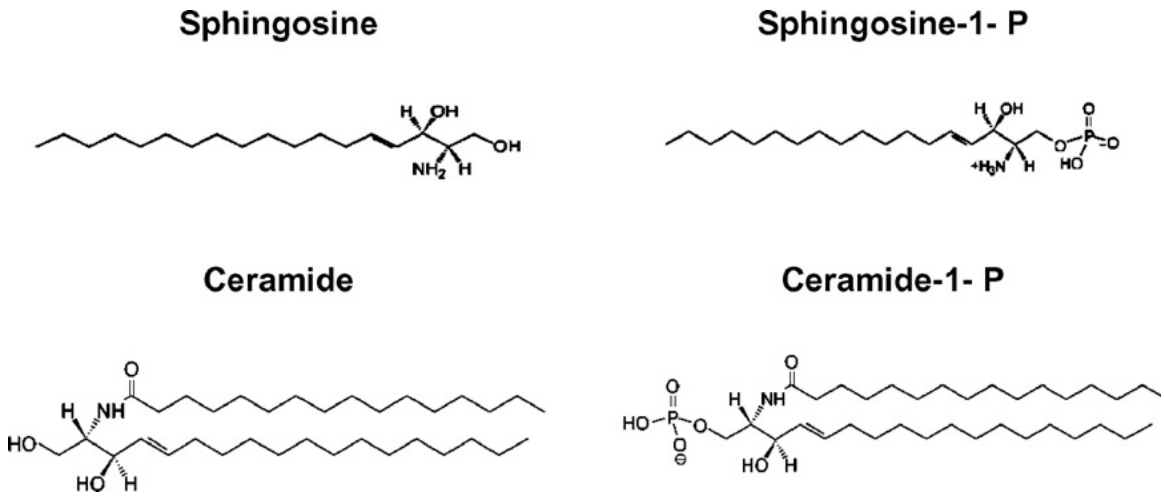

Fig.1. Simple sphingolipids structure (adapted from Goni *et al.* (3))

Fatty acid chain contains 2 to 28 carbon atoms, which can be saturated, or unsaturated (3-5). The length of fatty acid chain can have considerable influence on the physical properties of different ceramides; nevertheless, the biological activities of different ceramides, in general, are considered to be similar (6-9). Sphingoid bases

display a positive charge at pH 7 (10). In addition, they have an adequate aqueous solubility that permits them to move easily across cell membrane (11-12).

The term Ceramide is used particularly to describe N-acylsphingosines (Figure 1). When sphingoid bases, which is partially aqueous soluble, link to fatty acids with variable length to form ceramides, the physical properties of the new compounds differ to a great extent. The aqueous solubility, especially of long chain ceramides, is low (cmc $<10^{-10}$ M) (10). In addition, the transition temperature of creamides is ordinarily high >37 ^{O}C .This is attributed to the fact that both alkyl chains of ceramides are completely saturated and they possess highly hydrophobic properties (13-14). It must be noted that despite the poor aqueous solubility of ceramides, they are generally considered amphiphilic due to presence of the hydroxyl and the amide bond, which are hydrophilic in nature. Organic solvent such as chloroform and ethanol are used in preparing formulations that contain ceramides. When dissolving ceramides in an organic solvent, it is recommended to purge the solution with inert gas like nitrogen to prevent the oxidation and the solution should be stored at -20 ^{O}C for long-term stability.

1.2 Synthesis:

Ceramides compose the basic building unit of complex sphingolipids such as sphingomyelin, which regulate cholesterol distribution and homostasis within cell membrane. Ceramides can be synthesis by two pathways (i) De novo synthesis where four enzymes form ceramides from non- sphingolipids precursor. It is believed that the cooperation action of 3- ketodihydrosphingosine reductase, serine palmitoyltransferase, and (dihydro) ceramide synthases will result in transforming cytosolic and palmitoyl CoA into a single membrane of lipid. Dihydroceramide desaturase will introduce a double

bound to the bound lipid. (ii) Breakdown of sphingomyelin to its original building block by sphingomyelinase enzyme is another approach for ceramide formation (Figure 2) (15-20).

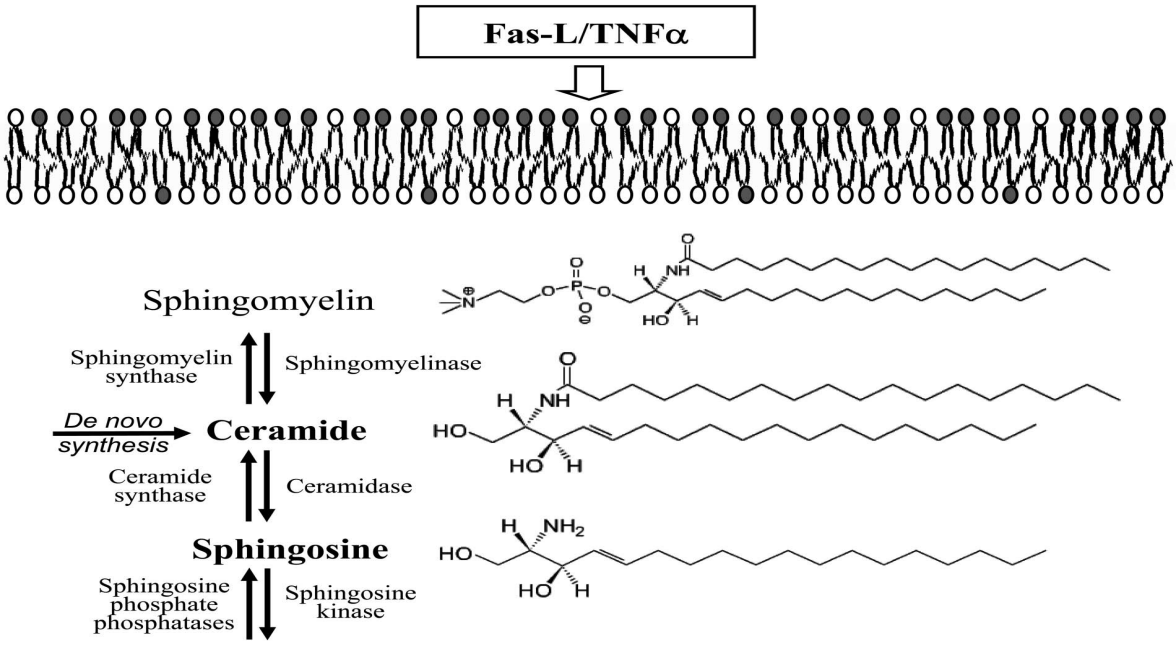

Fig.2. Ceramides biosynthesis (adopted from Woodcock (15))

1.3 Biological Functions:

When normal cells are exposed to certain stress conditions, sphingomyelin breakdown occurs, which lead to increase in the ceramides level (21-22). There is significant elevation in ceramides level in the cells treated with agents such as tumor necrosis factor alpha and interleukin (1L) 1β (16,23). Moreover, ceramides were detected in inflammatory diseases like irritable bowel syndrome. Research studies show that ceramide promotes cell apoptosis, a programmatic cell death in response to the stress.

Mechanisms in which ceramides induce cell apoptosis are still unclear. However, ceramides might stimulate cell apoptosis by modifying intracellular signaling. Mitochondria are the main site for apoptosis induced by ceramides (21). Ceramides change the biophysical properties of the mitochondrial membrane possibly by creating channels (prompt permeabilization) in the membrane. Consequently, several apoptotic mediators such as Fas ligand (Fas-L) are released (24-25). Another theory indicates that creamides potentiate the apoptotic signaling due to accumulation of receptors and signaling molecules. The mechanism is not understood fully, but it is hypothesized that when ceramides released in abundant amounts, as a result of sphingomyelin turnover, they tend to form lipid rafts. The lipid rafts have the capability to coalesce together, so more receptors are available for signaling (26-27).

Ceramides can exert some biological functions via modifying the composition of cell membrane. They can promote membrane leakiness to water-soluble solute (28-29). Such property makes ceramides an ideal candidate for enhancing drug delivery across cell membrane. Although the process involved in ceramides-induce membrane deformation is still ambiguous, several observations have been proposed that ceramides affect organizational structure of cell membrane. This will be discussed in detail in the following section.

1.4 Ceramides Effect on Cell-Membrane Structure:

Ceramides biological activities triggering apoptosis are probably due to the fact that they can re-structure rafts and caveolae of the cell membrane. They can induce membrane curvature and permeabilization through transbilayer lipid movement, a phenomenon called flip-flop (30-31). In order to understand flip-flop principle, we must

know normal lipids distribution in cell membrane. Phosphatidylcholine, glycolipids and sphingomyelin are predominantly localized in the outer leaflet of membrane, whereas phosphatidylserine and phosphatidylethanolamine are mainly localized in the inner leaflet (32) (Figure 3) Obviously, Flip-Flop will change the lipids distribution pattern.

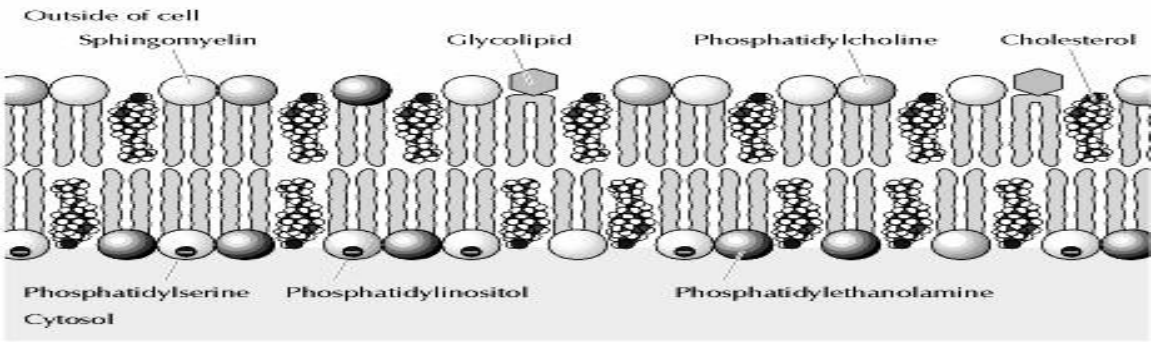

Fig.3. Lipids distribution in cell membrane

It is important to mention that the transbilayer lipid motion, or flip-flop movement, induced by ceramides, is an active process that requires certain enzymes that are ATP-dependent, referred to as flippase and floppase, beside ATP-independent lipid translocator, referred to as scramblases (33-34) (Figure 4).

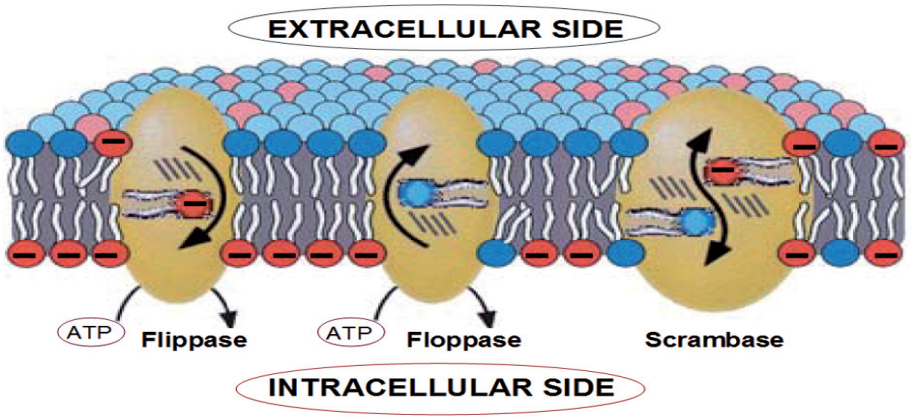

Fig.4. Membrane asymmetry created as a result of phospholipids exchange between extracellular and intracellular leaflets (adapted from A.P. Demchenko (35))

Flippase catalyzes lipid movement into the inward lipid monolayer. On the other hand, floppsae catalyzes lipid movement toward the outward of lipid monolayer. Unlike flippase and floppsase, scramblases are bidirectional lipid translocators that can transfer lipid either inward or outward across monolayers (30,36). Indeed, such lipid motion, promoted by ceramides as they alter the dynamic state of the membrane through destabilizing the lipid bilayer, will modify the membrane physical structure, particularly in mitochondria, which eventually leads to ceramide-induced apoptosis (15).

Furthermore, when ceramides introduced to cell membrane either by hydrolysis of sphingomyelin by sphingomyelinase or by externally inserted, liposomes enriched with ceramides for instance, they accumulate in the outer leaflet of membrane (37).
Consequently, ceramides form unique lipid domains in the outer leaflet of the membrane; hence, the tension between the inner and the outer layer are disturbed. As a result, ceramides diffuse quickly to the inner layer and cause disparate signaling event that stimulate cell apoptosis by one of the mechanisms described above (38-40).

1.5 Ceramides for Enhancing Transdermal Delivery:

Transdermal delivery route is an attracting substitute to conventional routes, such as oral and intravenous, as it offers tremendous advantages include bypass hepatic first-pass metabolism, easy application and removal of dose, providing drug release for a long period of time up to 7 days and avoiding of pain because it is a non-invasive route (41). Poor penetration through the skin is considered the main obstacle in transdermal delivery route. Stratum corneum is the major barrier that limits drugs penetration across the skin and it is represent the rate-limiting barrier for drugs absorption throughout skin (42). It is

composed of lipid matrix (5-15%), which includes ceramides, free fatty acids and cholesterol, and protein (75-85%), which is keratin in majority (43, 44).

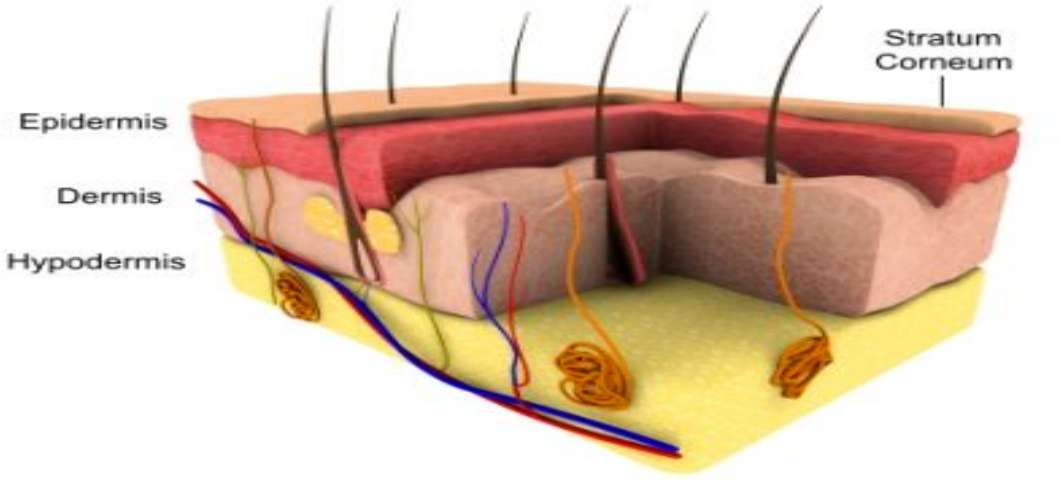

Fig.5. Stratum corneum is the major barrier that limits drugs penetration across the skin

In order to overcome this barrier, chemical penetration enhancers such as ceramides are employed. They are defined as inert compounds that can reduce stratum corneum resistance to drug transport by interaction and partition within the lipid components of the stratum corneum (45-46). The mechanism by which ceramides enhance drugs permeation across skin will be discussed in detail in the following section.

Ceramides compose the major type of lipid, about 40%, in lipid matrix of the stratum corneum. The penetration of hydrophilic drugs can be enhanced by the incorporating of ceramides and their analogs in the formulation (47). Although the mechanism of action of ceramides and their analogs still to be elucidated, enriching transdermal formulation with lipids such as ceramides similar to those in the skin will induce a higher interaction between skin and formulation; as a result, drugs will retain

and accumulate to great extent in the tissue (47). Ceramides could diminish the lipid bilayer continuity of the skin because they have the ability to place their hydrophobic tail between the creamides constituents, hence increase the drugs transportation (48). Also, ceramides increase the penetration of polar solutes through the skin by disrupting normal organization of intercellular lamellae. It is important to note that the optimal length of fatty acid chain, particularly in ceramides analogs, to enhance drugs penetration is between 10 and 20 (49-50).

Schroeter *et al.* (51) investigated the effect of different ceramides on the lamellae architecture of a phytoshingosine based nanoparticle formulation CER (NP). The effect of the nanoparticle formulation, CER (NP) on the stratum corneum lipid was studied using neutron diffraction and H2 NMR spectroscopy. The CER (NP) modified the composition of the stratum corneum into two lamellae with different hydration features, which coexisted. Furthermore, their study revels that CER (NP) has influence the lipid morphology as it forms a firm and stable bilayer when applied on a nanostructure of stratum corneum.

A drug delivery system composed of ceramide-3 liposomes in a hydrogel formulation for enhanced delivery of quercetin was reported (52). Liposomal hydrogel formulation increased the transdermal permeation of quercetin when applied to mice skin. It demonstrated higher skin permeation compared to either ceramides liposomes (67.42 vs. 48.35 %) or hydrogel alone (67.42 vs. 31.77 %). The liposomal hydrogel complex enhanced the transdermal diffusion rate of quercetin because incorporation of ceramide helped to sequester the drug within the stratum corneum.

Ceramides do not always have a great impact when included in transdermal formulation. Ceramides might perform two divergent effects: (i) they may induce intracellular lamellae lipid disorder as discussed earlier or (ii) they could make drugs penetration even more difficult as they enhance the lamellae phase (53). Moreover, the action of ceramides might be declined in presence of others compounds used in the formulation. A formulation mixture containing ceramide 2, 5-aminolevulinic acid (a hydrophilic drug for non-melanoma skin cancer) and its hexyl-ester or octyl-ester and propylene glycol was developed (54). The permeation study across pig ear skin showed that ceramide 2 did increase both penetration and retention for the drug under this study.

To understand the relationship between the structure and skin permeability relationships in ceramides, various analogs of N-lignoceroylsphingosine were studied for their behavior in skin and in model lipid membranes (55). Ceramide analogs with C_{15}-sphingosine chains were found to be more barrier-perturbing than C_{12}- and C_{18}-sphingosine ceramides. Ceramide analogs with C_4 to C_6 showed 15 times higher skin permeability as compared to an untreated control and up to 79 times higher permeability compared to native very long-chain ceramides. The authors presume that the skin permeation properties of some short ceramides can be explored for the rational design of permeation enhancers for transdermal drug delivery.

A formulation containing ceramides, fatty acids, cholesterol and hydrocortisone (HC) was developed for xerosis and atopic dermatitis for achieving combined antiinflammatory/barrier repair properties. The ceramide based emulsion (Cer-E) skin permeation performance was compared to a blank emulsion (No Cer-E). The Cer-E showed smaller droplet sizes and higher viscosity values compared to NoCer-E. The

Cer-E showed a similar anti-inflammatory activity in vivo when compared with a commercially available 1% HC emulsion. In this work, the authors developed a new non-ionic Cer-E for a topical therapy for the improvement of skin barrier abnormalities in atopic dermatitis (56).

The human skin penetration effect of phytosphingosine and 9 derived phytoceramides on several transdermal model drugs (i.e. caffeine, testosterone, ibuprofen) was investigated by Veryser *et al.* (57) The phytoceramides 1-6 exhibited a penetration-enhancing ratio of more than 2 fold for caffeine and testosterone but none of them had an influence on ibuprofen. The authors did not attribute any reason for the lack of skin permeability of ceramides for ibuprofen in their study.

One of the intercellular lipid components of the stratum corneum, ceramides, is known to play an essential role in maintaining and structuring the lipid barrier of the skin. Internal wool lipids (IWL), which are also rich in ceramides, have a composition similar to that of the stratum corneum lipids. The topical application of IWL-formulated liposomes on intact and compromised skin has been demonstrated to improve barrier skin properties. The IWL liposomes improved skin barrier integrity and increased skin hydration when applied onto intact skin (58).

Finally, many ceramides analogs have been synthesized in order to increase the penetration across the cell membrane and stabilized metabolic compounds that have a crucial role in lipids structural organization. Ceramide analogues with different polar head and hydrophobic chain were synthesized as skin permeation enhancers. The polar head is responsible for the permeation of the ceramide into the stratum corneum lipids

and the length of the hydrophobic chain is important for disordering the lipid packing (59).

1.6 Ceramides for Oral Delivery:

Lipids have been exploited to increase bioavailability and solubility of Biopharmaceutical Classification System (BCS) class II (high permeability-low solubility) and class IV (low permeability-low solubility) drugs (60-61). Phosphosphingolipids such as ceramides might modulate the physical structure of cell membrane, which facilitate drugs pharmacological activities (62). Incorporation of ceramides into nanocarriers, like liposomes, could assist to circumvent the pharmacokinetic problems associated with drugs that pose low aqueous solubility profile (63). Such drugs can be dispersed inside the bilayers of the liposome. It should be mentioned that the major drawback when applying liposomes for oral delivery is that they dissociate due to the harsh acidic environment of gastrointestinal system (64).

Lahiani *et al.* (65) reported that liposomes enriched with various ceramides enhanced the bioavailability of oral amphotericin B. Authors selected those ceramides specifically because they exhibit a rigid structure; therefore, they are resistant to bile salts and digestive enzymes degrading activities. Amphotericin B was entrapped in various liposomes contain glucosylceramides. To examine the bioavailability and stability, all preparations were applied in an artificial-stomac-duodenum model and the results were compared with phosphotidylcholine liposomes. Liposomal formulations with ceramides display better stability in digestive system. Authors suggested that ceramides minimized the degradation impact of bile salt and other gastrointestinal enzymes on liposomes membrane.

It is important to point out that ceramides may enhance the therapeutic efficacy of certain drugs by altering cells signaling. When superoxide dismutase, an anti-inflammatory drug, was encapsulated into liposomes with ceramides (62), the activity of the drug was increased. It was postulated that ceramides alter the membrane signal transduction via effecting both protein kinase and phospholipase. In addition, ceramides modify the membrane receptors and also release of different cellular oxidants.

1.7 Ceramides for Ocular Delivery:

Several anatomical and physiological barriers exist for ocular delivery such as permeation through cornea and conjunctiva, drug reflex and rapid drainage, which limit achieving the effective drug level in target ocular tissue (66-70). Ceramides regulate disparate signaling responsible for cells apoptosis and inflammation response. In general, because ceramides provoke photoreceptor apoptosis in ocular tissue, their application in ocular delivery is limited.

Photoreceptor death is the most common finding in retinal degenerative diseases. Although there is no definitive evidence that support the involvement of sphingolipids in retinal degenerative diseases and inflammatory eye disorders, some findings claim that ceramides play a crucial role in regulating several ocular inflammatory disorders. Ceramides have been identified as a key secondary messenger in inducing photoreceptor death through oxidative-stress mechanism (71-73). According to the earlier studies, accumulation of ceramides was associated with photoreceptor apoptosis as the photoreceptors mutate with increase in ceramides levels. The increase in ceramides levels was due to mutation in ceramide kinase gene, phospholipase C and arrestin 2 (74). The exact mechanism in which ceramides induce cells apoptosis is not understood fully;

however, many research studies have hypothesized that disturbance in the sphingolipids metabolism and lack of sufficient degradation of ceramide could contribute to the pathophysiology of retinal degenerative diseases.

In human, defects in Ceramide Kinase Like genes, which is responsible for ceramides breakdown, is often associated with retinitis pigmentosa (RP 26), a sign that indicates that ceramides are involved in retinal degenerative disorders. In case of retinal pigment epithelium, ceramides might induce apoptosis by different mechanisms including initiating permeability of mitochondria membrane, increasing reactive oxygen species (ROS) and caspase-3 activation (75). Furthermore, accumulation of ceramides, as a result of ceramidase gen mutation, was detected in the retina of patients suffer from Farber disease (76).

Since it is thought that ceramides play various roles in photoreceptor death and inflammatory eye disorder, their use in ocular formulation has been restricted. However, Sun and his coworkers (77) conducted a study that showed that short chain ceramide (Cer $_6$) decreased corneal inflammation. In their study, a liposomal formulation was applied on human corneal epithelia cells *in vitro* as well as *in vivo* in a murine model of corneal inflammation induced by S. aureus in order to stimulate the release of neutrophil chemokines by HCE cells. The results indicated that the liposomal formulation did not induce any apoptosis of corneal epithelia cells in both in vitro and in vivo. Furthermore, the data gathered showed that liposomal formulation of c6-ceramide decreased inflammation of corneal epithelia cells by decreasing production of chemotactic chemokines, such as CXCL1, CXCL5 and CXCL8, and bloking CXC chemokine.

In conclusion, the role of ceramides in inflammation regulation and cells apoptosis is not well understood. Their accumulation might induce cells apoptosis; however, they might reduce inflammation without significant cellular apoptosis. The use of ceramides to enhance ocular delivery is limited as they have some adverse effects despite of their anti-inflammatory effects.

1.8 Ceramides for Enhancing Cytotoxicity and Cellular Uptake toward Cancer Cells:

Ceramides play an important role as a mediator in the cell-signaling in the cell differentiation, cell cycle arrest and apoptosis. These processes involve P13K/Akt signaling pathway (78). However, due to hydrophobicity of ceramides, the bioavailability is low and limits their use in the systemic delivery in the cancer treatment. Liposomes can blend ceramides in the lipid component and provide better delivery across the biological membrane.

Two major obstacles encountered in the cancer treatment are insufficient delivery of drugs to their intended target and inadequate uptake of drugs by cancer cells (79). Encapsulation of chemotherapeutic drugs in Polyethylene glycol (PEG) – coated liposomes have significantly reduced non-selective cytotoxicity, cardiotoxicity and nephrotoxicity (80). The targeted delivery of chemotherapeutic agents to the tumor site is dependent on their physico-chemical properties, which in turn affect the stability, distribution and circulation time (81) leading to enhanced therapeutic effect. Indeed, increasing drugs influx across the cell membrane of targeted cancer cells will definitely elevate their intercellular concentration.

As discussed earlier, the distribution of lipid across the plasma membrane is asymmetrical among the inner and the outer layers. The outer layer is predominantly composed of sphingolipids, e.g., sphingomyelin and glycosphingolipids. It has been

found that using of lipids analogue to those located in outer membrane, like C_6-Ceramide and C_8-Ceramide, enhanced the membrane permeability and increase the cellular uptake of chemotherapeutic medications by cancer cells (82). In addition, short chain ceramides are easily metabolized because they are similar to the components of the plasma membrane (83). Incorporation of short chain ceramides into liposomes with lipid bilayers along with anticancer drugs are known to produce promising results such as enhanced cell permeation, retention and anti-tumor effects (82).

To investigate the effect of sphingolipids on cytotoxicity and cellular delivery of liposomal DOX toward A4321 epidermoid carcinoma cells, Veldman *et al.* (84) formulated Polyethylene glycol (PEG) – coated liposomes with N -octanoyl – glucosylceramide (C_8-GlcCer). Insertion of short chain sphingolipids into liposome bilayer improved the delivery of DOX with (C_8-GlcCer) incorporated in different Mol% (3,6,9,13,17 Mol%). The cellular uptake of DOX was increased by about 4-folds when A4321 epidermoid carcinoma cells incubated for 24 hours with liposomes enriched with 17 Mol% C8-GlcCer. In the same study, C_8-GlcCer, 17 Mol %, was post -inserted into Calyx, DOX liposomes that are available commercially. Incorporation of (C_8-GlcCer) into Caelyx enhanced the cytotoxicity of DOX as cells viability dropped for 76.2 %, cells viability for Caelyx alone, to about 18.2 %.

In another study, the enhancement of cellular uptake of DOX by the short-chain sphingolipid was demonstrated (85). N-hexanoyl-sphingomyelin (C_6-SM) was used instead of C_6-Ceramide. Unlike C_6-Ceramide, 6-SM was non-toxic and did not induce apoptosis by itself as it is an inert metabolite of C_6-Ceramide. Primary bovine aortic endothelial cells (BAEC) exposed to DOX in presence of 10 mM of C_6-SM and in vitro

cellular uptake of the drug was studied. The cellular uptake of DOX was 300% higher compared to cells treated without adding C_6-SM. Enhanced permeability of plasma membrane and creation voids in lipid bilayers facilitated the penetration of DOX. C_6-SM did not elevate the cellular level of transferrin, a marker for clathrin, and the CTB, a marker for raft-mediated endocytosis, in BAEC cells. Furthermore, the enhanced DOX cellular by C_6-SM was not related to inhibition of drug efflux.

In a previous study, we proved that incorporation of short-chain ceramide into liposomal DOX would enhance its cytotoxicity (86). A liposomal formulation consists of DOTAP/cholesterol,PEG2000-DSPE,C_8-ceramide and DOX at molar ratio of 10:10:1:2:2 was tested in vitro against B16BL6, melanoma cell line. Liposomal formulation with C_8-Ceramide exhibited highest cytotoxicity when it was compared to both free DOX and liposomal formulation without C_8-Ceramide. Cells viability, at 0.5 μg/ml DOX, of liposomal formulation with C_8-Ceramide was five times higher than the formulation without short-chain ceramide and nine times higher than DOX solution. The author assumed that increased cytotoxicity was due to ceramide-induce apoptosis through PI3K/Akt pathway. Furthermore, packing ceramide into liposome structure enhanced DOX permeability by providing better cell penetration.

Apparently, co-formulation of exogenous short-chain ceramides with liposomes enhanced the cellular uptake of amphiphilic drugs as they rapidly exchange and accumulate in the plasma membrane of different cancer cells (87-88).

1.9 References

1- Zheng, W., Kollmeyer, J., Symolon, H., Momin, A., Munter, E., Wang, E., . . . Merrill, A. H. (2006). Ceramides and other bioactive sphingolipid backbones in health and disease: Lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy. *Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1758*(12), 1864-1884

2- Pruett, S. T., Bushnev, A., Hagedorn, K., Adiga, M., Haynes, C. A., Sullards, M. C., . . . Merrill, A. H. (2008). Thematic Review Series: Sphingolipids. Biodiversity of sphingoid bases ("sphingosines") and related amino alcohols. *The Journal of Lipid Research, 49*(8), 1621-1639

3- Goñi, F. M., & Alonso, A. (2006). Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. *Biochimica Et Biophysica Acta (BBA) - Biomembranes,1758*(12), 1902-1921

4- Hakomori, S. (1983). Chemistry of Glycosphingolipids. *Sphingolipid Biochemistry,* 1-165. doi:10.1007/978-1-4757-0396-2_1

5 Kolter, T., Wilkening, G., Döring, T., Werth, N., & Sandhoff, K. (1999). Recent Advances in the Biochemistry of Glycosphingolipid Metabolism. *Biochm. Soc. Trans. Biochemical Society Transactions, 27*(3). doi:10.1042/bst027a079a

6- Sot, J., Goñi, F. M., & Alonso, A. (2005). Molecular associations and surface-active properties of short- and long-N-acyl chain ceramides. *Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1711*(1), 12-19

7- Sot, J., Aranda, F. J., Collado, M., Goñi, F. M., & Alonso, A. (2005). Different Effects of Long- and Short-Chain Ceramides on the Gel-Fluid and Lamellar-Hexagonal

Transitions of Phospholipids: A Calorimetric, NMR, and X-Ray Diffraction Study. *Biophysical Journal, 88*(5), 3368-3380

8- Ghidoni, R., Sala, G., & Giuliani, A. (1999). Use of sphingolipid analogs: Benefits and risks1The ganglioside nomenclature is that of Svennerholm, L. (1969) J. Lipid Res. 5, 145–155.1. *Biochimica Biophysica Acta, 1439*(1), 17-39

9- Kolesnick, R. N., Goñi, F. M., & Alonso, A. (2000). Compartmentalization of ceramide signaling: Physical foundations and biological effects. J. Cell. Physiol. *Journal of Cellular Physiology, 184*(3), 285-300

10- Bieberich, E., Hu, B., Silva, J., Mackinnon, S., Yu, R. K., Fillmore, H., . . . Ottenbrite, R. M. (2002). Synthesis and characterization of novel ceramide analogs for induction of apoptosis in human cancer cells. *Cancer Letters, 181*(1), 55-64. doi:10.1016/s0304-3835(02)00049-6

11- Brockman, H. L., Momsen, M. M., Brown, R. E., He, L., Chun, J., Byun, H., & Bittman, R. (2004). The 4,5-Double Bond of Ceramide Regulates Its Dipole Potential, Elastic Properties, and Packing Behavior.*Biophysical Journal, 87*(3), 1722-1731.

12- Park, J., & Schuchman, E. H. (2006). Acid ceramidase and human disease. *Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1758*(12), 2133-2138

13- Sullards, M. C., Allegood, J. C., Kelly, S., Wang, E., Haynes, C. A., Park, H., . . . Merrill, A. H. (2007). Structure-Specific, Quantitative Methods for Analysis of Sphingolipids by Liquid Chromatography–Tandem Mass Spectrometry: "Inside-Out" Sphingolipidomics. *Methods in Enzymology Lipidomics and Bioactive Lipids: Mass-Spectrometry–Based Lipid Analysis*, 83-115. doi:10.1016/s0076-6879(07)32004-1

14- Maggio, B., Fanani, M., Rosetti, C., & Wilke, N. (2006). Biophysics of sphingolipids II. Glycosphingolipids: An assortment of multiple structural information transducers at the membrane surface. *Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1758*(12), 1922-1944

15- Woodcock, J. (2006). Sphingosine and ceramide signalling in apoptosis. IUBMB Life (International Union of Biochemistry and Molecular Biology: Life), 58(8), 462-466.

16- Gault, C. R., Obeid, L. M., & Hannun, Y. A. (2010). An Overview of Sphingolipid Metabolism: From Synthesis to Breakdown. *Advances in Experimental Medicine and Biology Sphingolipids as Signaling and Regulatory Molecules,* 1-23. doi:10.1007/978-1-4419-6741-1_1

17- Hanada, K. (2003). Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1632*(1-3), 16-30

18- Mizutani, Y., Kihara, A., & Igarashi, Y. (2005). Mammalian Lass6 and its related family members regulate synthesis of specific ceramides. *Biochemical Journal, 390*(1), 263-271

19- Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., & Kolesnick, R. (1995). Ceramide synthase mediates daunorubicin-induced apoptosis: An alternative mechanism for generating death signals. *Cell, 82*(3), 405-414

20- Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Sullards, M. C., . . . Futerman, A. H. (2002). Upstream of Growth and Differentiation Factor 1 (uog1), a Mammalian Homolog of the Yeast Longevity Assurance Gene 1 (LAG1), RegulatesN-Stearoyl-sphinganine (C18-(Dihydro)ceramide) Synthesis in a

Fumonisin B1-independent Manner in Mammalian Cells. *Journal of Biological Chemistry, 277*(38), 35642-35649

21- Siskind, L. J. (2005). Mitochondrial Ceramide and the Induction of Apoptosis. *Journal of Bioenergetics and Biomembranes, 37*(3), 143-153

22- Pettus, B. J., Chalfant, C. E., & Hannun, Y. A. (2002). Ceramide in apoptosis: An overview and current perspectives. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1585*(2-3), 114-125

23- Krut, O., Wiegmann, K., Kashkar, H., Yazdanpanah, B., & Kronke, M. (2006). Novel Tumor Necrosis Factor-responsive Mammalian Neutral Sphingomyelinase-3 Is a C-tail-anchored Protein. *Journal of Biological Chemistry, 281*(19), 13784-13793.

24- Ruvolo, P. (2003). Intracellular signal transduction pathways activated by ceramide and its metabolites.*Pharmacological Research, 47*(5), 383-392

25- Reynolds, C., Maurer, B. J., & Kolesnick, R. N. (2004). Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Letters, 206*(2), 169-180

26- Senchenkov, A., Litvak, D. A., & Cabot, M. C. (2001). Targeting Ceramide Metabolism--a Strategy for Overcoming Drug Resistance. *Journal of the National Cancer Institute, 93*(5), 347-357

27- Liu, B., Jayadev, S., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., . . . Hannun, Y. A. (1995). Role for Ceramide in Cell Cycle Arrest. *Journal of Biological Chemistry, 270*(5), 2047-2052

28- Montes, L., López, D. J., Sot, J., Bagatolli, L. A., Stonehouse, M. J., Vasil, M. L., . . . Alonso, A. (2008). Ceramide-Enriched Membrane Domains in Red Blood Cells and the

Mechanism of Sphingomyelinase-Induced Hot−Cold Hemolysis. *Biochemistry, 47*(43), 11222-11230

29- Hinkovska-Galcheva, V. (2005). Ceramide 1-Phosphate, a Mediator of Phagocytosis. *Journal of Biological Chemistry, 280*(28), 26612-26621.

30- Contreras, F., Sánchez-Magraner, L., Alonso, A., & Goñi, F. M. (2009). Transbilayer (flip-flop) lipid motion and lipid scrambling in membranes. *FEBS Letters, 584*(9), 1779-1786

31- Devaux, P. F., Herrmann, A., Ohlwein, N., & Kozlov, M. M. (2008). How lipid flippases can modulate membrane structure. *Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1778*(7-8), 1591-1600

32- Contreras, F., Villar, A., Alonso, A., & Goñi, F. M. (2008). Ceramide-Induced Transbilayer (Flip-Flop) Lipid Movement in Membranes. *Methods in Molecular Biology Lipid Signaling Protocols,* 1-11. doi:10.1007/978-1-60327-115-8_10

33- Daleke, D. L. (2002). Regulation of transbilayer plasma membrane phospholipid asymmetry. *The Journal of Lipid Research, 44*(2), 233-242

34- Sanyal, S., & Menon, A. K. (2009). Flipping Lipids: Why an' What's the Reason for? *ACS Chemical Biology, 4*(11), 895-909

35- Demchenko, A. P. (2012). The Change of Cellular Membranes on Apoptosis: *Fluorescence Detection.Experimental Oncology, 34*(3), 263-268

36- Poulsen, L. R., López-Marqués, R. L., & Palmgren, M. G. (2008). Flippases: Still more questions than answers. *Cell. Mol. Life Sci. Cellular and Molecular Life Sciences, 65*(20), 3119-3125

37- Ogushi, F., Ishitsuka, R., Kobayashi, T., & Sugita, Y. (2012). Rapid flip-flop motions of diacylglycerol and ceramide in phospholipid bilayers. *Chemical Physics Letters*, 522, 96-102

38- López-Montero, I., Rodriguez, N., Cribier, S., Pohl, A., Vélez, M., & Devaux, P. F. (2005). Rapid Transbilayer Movement of Ceramides in Phospholipid Vesicles and in Human Erythrocytes. *Journal of Biological Chemistry, 280*(27), 25811-25819

39- Kahya, N., Scherfeld, D., Bacia, K., Poolman, B., & Schwille, P. (2003). Probing Lipid Mobility of Raft-exhibiting Model Membranes by Fluorescence Correlation Spectroscopy. *Journal of Biological Chemistry,278*(30), 28109-28115

40- Kok, J. W., Babia, T., Klappe, K., Egea, G., & Hoekstra, D. (1998). Ceramide transport from endoplasmic reticulum to Golgi apparatus is not vesicle-mediated. *Biochemical Journal, 333*(3), 779-786

41- Prausnitz, M. R., & Langer, R. (2008). Transdermal drug delivery. *Nature Biotechnology,26*(11), 1261-1268

42- Chantasart, D., & Li, S. K. (2012). Structure Enhancement Relationship of Chemical Penetration Enhancers in Drug Transport across the Stratum Corneum. *Pharmaceutics, 4*(4), 71-92. doi:10.3390/pharmaceutics4010071

43- Scheuplein, R. J. (2011, January 1). Permeability of the Skin. *Comprehensive Physiology,* 299-322. doi:10.1002/cphy.cp090119

44- Bouwstra, J. A., & Ponec, M. (2006). The skin barrier in healthy and diseased state. Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1758(12), 2080-2095.

45- Williams, A. C., & Barry, B. W. (2004). Penetration enhancers. *Advanced Drug Delivery Reviews, 56*(5), 603-618

46- Sinha, V. R., & Kaur, M. P. (2000). Permeation Enhancers for Transdermal Drug Delivery. Drug Development and Industrial Pharmacy, 26(11), 1131-1140.

47- Chen, Y., Quan, P., Liu, X., Wang, M., & Fang, L. (2014). Novel chemical permeation enhancers for transdermal drug delivery. *Asian Journal of Pharmaceutical Sciences, 9*(2), 51-64. doi:10.1016/j.ajps.2014.01.001

48- Vávrová, K., Hrabálek, A., Doležal, P., Holas, T., & Zbytovská, J. (2003). L-Serine and glycine based ceramide analogues as transdermal permeation enhancers: Polar head size and hydrogen bonding.*Bioorganic & Medicinal Chemistry Letters, 13*(14), 2351-2353

49- Takács, M., Bubenyák, M., Váradi, A., Blazics, B., Horváth, P., & Kökösi, J. (2011). Synthesis of novel ceramide-like penetration enhancers. *Tetrahedron Letters, 52*(16), 1863-1865

50- Vávrová, K., Hrabálek, A., Doležal, P., Šámalová, L., Palát, K., Zbytovská, J., . . . Klimentová, J. (2003). Synthetic ceramide analogues as skin permeation enhancers: Structure–Activity relationships. Bioorganic & Medicinal Chemistry, 11(24), 5381-5390.

51- Schroeter, A., Engelbrecht, T., & Neubert, R. H. (2013). Influence of short chain ceramides and lipophilic penetration enhancers on the nano-structure of stratum corneum model membranes studied using neutron diffraction. Frontiers of Chemical Science and Engineering, *7*(1), 29-36

52- Park, S. N., Lee, M. H., Kim, S. J., & Yu, E. R. (2013). Preparation of quercetin and rutin-loaded ceramide liposomes and drug-releasing effect in liposome-in-hydrogel complex system. *Biochemical and Biophysical Research Communications, 435*(3), 361-366

53- Coderch, L., Lopez, O., Maza, A. D., & Parra, J. L. (2003). Ceramides and Skin Function. *American Journal of Clinical Dermatology, 4*(2), 107-129

54- Pierre, M. B., Lopez, R. F., & Bentley, M. V. (2009). Influence of ceramide 2 on in vitro skin permeation and retention of 5-ALA and its ester derivatives, for Photodynamic Therapy. *Brazilian Journal of Pharmaceutical Sciences, 45*(1), 109-116

55- Školová, B., Janůšová, B., & Vávrová, K. (2016). Ceramides with a pentadecasphingosine chain and short acyls have strong permeabilization effects on skin and model lipid membranes. *Biochimica Et Biophysica Acta (BBA) - Biomembranes, 1858*(2), 220-232. doi:10.1016/j.bbamem.2015.11.019

56- Carneiro, R., Salgado, A., Raposo, S., Marto, J., Simões, S., Urbano, M., & Ribeiro, H. M. (2011). Topical emulsions containing ceramides: Effects on the skin barrier function and anti-inflammatory properties. *European Journal of Lipid Science and Technology, 113*(8), 961-966. doi:10.1002/ejlt.201000495

57- Veryser, L., Boonen, J., Taevernier, L., Guillaume, J., Risseeuw, M., Shah, S. N., . . . Spiegeleer, B. D. (2014). The Influence of the Acyl Chain on the Transdermal Penetration-Enhancing Effect of Synthetic Phytoceramides. *Skin Pharmacology and Physiology, 28*(3), 124-136. doi:10.1159/000365730

58- Ramírez, R., Martí, M., Barba, C., Méndez, S., Parra, J. L., & Coderch, L. (2010). Skin efficacy of liposomes composed of internal wool lipids rich in ceramides. *International Journal of Cosmetic Science, 32*(6), 472-472. doi:10.1111/j.1468-2494.2010.00619_3.x

59-Vávrová, K., Hrabálek, A., Doležal, P., Šámalová, L., Palát, K., Zbytovská, J., . . . Klimentová, J. (2003). Synthetic ceramide analogues as skin permeation enhancers:

Structure–Activity relationships. *Bioorganic & Medicinal Chemistry, 11*(24), 5381-5390. doi:10.1016/j.bmc.2003.09.034

60- Amidon, G. L., Lennernas, H., Shah, V. P., & Crison, J. R. (1995). A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm Res, 12*(3), 413-420

61- Stuchlik, M., & Zak, S. (2001). Lipid-based vehicle for oral drug delivery. *Biomedical Papers, 145*(2), 17-26. doi:10.5507/bp.2001.008

62- Regnault, C., Roch-Arveiller, M., Tissot, M., Sarfati, G., Giroud, J. P., Postaire, E., & Hazebroucq, G. (1995). Effect of encapsulation on the anti-inflammatory properties of superoxide dismutase after oral administration. *Clinica Chimica Acta, 240*(2), 117-127

63- Battogtokh, G., & Ko, Y. T. (2014). Self-assembled Chitosan-Ceramide Nanoparticle for Enhanced Oral Delivery of Paclitaxel. *Pharmaceutical Research, 31*(11), 3019-3030

64- Chen, M., Mi, F., Liao, Z., Hsiao, C., Sonaje, K., Chung, M., . . . Sung, H. (2013). Recent advances in chitosan-based nanoparticles for oral delivery of macromolecules. *Advanced Drug Delivery Reviews, 65*(6), 865-879

65- Skiba-Lahiani, M., Hallouard, F., Mehenni, L., Fessi, H., & Skiba, M. (2015). Development and characterization of oral liposomes of vegetal ceramide based amphotericin B having enhanced dry solubility and solubility. *Materials Science and Engineering*: C, 48, 145-149

66- Cholkar, K., Patel, S. P., Vadlapudi, A. D., & Mitra, A. K. (2013). Novel Strategies for Anterior Segment Ocular Drug Delivery. *Journal of Ocular Pharmacology and Therapeutics, 29*(2), 106-123

67- Dartt, D. A. (2002). Regulation of mucin and fluid secretion by conjunctival epithelial cells. *Progress in Retinal and Eye Research, 21*(6), 555-576

68- Shi, X., & Candia, O. A. (1995). Active sodium and chloride transport across the isolated rabbit conjunctiva.*Current Eye Research, 14*(10), 927-935

69- Karla, P. K., Earla, R., Boddu, S. H., Johnston, T. P., Pal, D., & Mitra, A. (2009). Molecular Expression and Functional Evidence of a Drug Efflux Pump (BCRP) in Human Corneal Epithelial Cells. *Current Eye Research, 34*(1), 1-9

70- Daemen, M., Thijssen, H., & Struyker-Boudier, H. (1991). Pharmacokinetic considerations in local drug delivery. *Advanced Drug Delivery Reviews, 6*(1), 1-18

71- Chen, H., Tran, J. A., Brush, R. S., Saadi, A., Rahman, A. K., Yu, M., . . . Mandal, M. N. (2011). Ceramide Signaling in Retinal Degeneration. *Retinal Degenerative Diseases Advances in Experimental Medicine and Biology,* 553-558. doi:10.1007/978-1-4614-0631-0_70

72- German, O. L., Miranda, G. E., Abrahan, C. E., & Rotstein, N. P. (2006). Ceramide is a Mediator of Apoptosis in Retina Photoreceptors. *Investigative Opthalmology & Visual Science Invest. Ophthalmol. Vis. Sci., 47*(4), 1658. doi:10.1167/iovs.05-1310

73- Zhu, D., Sreekumar, P. G., Hinton, D. R., & Kannan, R. (2010). Expression and regulation of enzymes in the ceramide metabolic pathway in human retinal pigment epithelial cells and their relevance to retinal degeneration. *Vision Research, 50*(7), 643-651

74- Chen, H., Chan, A. Y., Stone, D. U., & Mandal, N. A. (2014). Beyond the cherry-red spot: Ocular manifestations of sphingolipid-mediated neurodegenerative and inflammatory disorders. *Survey of Ophthalmology, 59*(1), 64-76

75- Kannan, R., Jin, M., Gamulescu, M., & Hinton, D. (2004). Ceramide-induced apoptosis: Role of catalase and hepatocyte growth factor. *Free Radical Biology and Medicine, 37*(2), 166-175

76- Zarbin, M. A., Green, W. R., Moser, A. B., & Tiffany, C. (1988). Increased Levels of Ceramide in the Retina of a Patient With Farber's Disease. *Archives of Ophthalmology, 106*(9), 1163-1163

77- Sun, Y., Fox, T., Adhikary, G., Kester, M., & Pearlman, E. (2008). Inhibition of corneal inflammation by liposomal delivery of short-chain, C-6 ceramide. *Journal of Leukocyte Biology, 83*(6), 1512-1521

78- Oskouian, B., & Saba, J. D. (2010). Cancer treatment strategies targeting sphingolipid metabolism. Advances in Experimental Medicine and Biology, 688, 185-205. doi:10.1007/978-1-4419-6741-1_13

79- Shen, F., Chu, S., Bence, A. K., Bailey, B., Xue, X., Erickson, P. A., . . . Erickson, L. C. (2007). Quantitation of Doxorubicin Uptake, Efflux, and Modulation of Multidrug Resistance (MDR) in MDR Human Cancer Cells. *Journal of Pharmacology and Experimental Therapeutics, 324*(1), 95-102

80- Sanson, C., Schatz, C., Meins, J. L., Soum, A., Thévenot, J., Garanger, E., & Lecommandoux, S. (2010). A simple method to achieve high doxorubicin loading in biodegradable polymersomes. *Journal of Controlled Release, 147*(3), 428-435. doi:10.1016/j.jconrel.2010.07.123

81- Valetti, S., Mura, S., Stella, B., & Couvreur, P. (n.d.). Rational design for multifunctional non-liposomal lipid-based nanocarriers for cancer management: Theory to practice. *Journal of Nanobiotechnology, 11*(Suppl 1). doi:10.1186/1477-3155-11-s1-s6

82 Lummel, M. V., Blitterswijk, W. J., Vink, S. R., Veldman, R. J., Valk, M. A., Schipper, D., . . . Koning, G. A. (2010). Enriching lipid nanovesicles with short-chain glucosylceramide improves doxorubicin delivery and efficacy in solid tumors. *The FASEB Journal, 25*(1), 280-289. doi:10.1096/fj.10-163709

83- Fenske, D. B., Chonn, A., & Cullis, P. R. (2008). Liposomal Nanomedicines: An Emerging Field. *Toxicologic Pathology, 36*(1), 21-29

84- Veldman, R. J. (2005). Coformulated N-Octanoyl-glucosylceramide Improves Cellular Delivery and Cytotoxicity of Liposomal Doxorubicin. *Journal of Pharmacology and Experimental Therapeutics,315*(2), 704-710

85- Veldman, R., Zerp, S., Blitterswijk, W., & Verheij, M. (2004). N-hexanoyl-sphingomyelin potentiates in vitro doxorubicin cytotoxicity by enhancing its cellular influx. *British Journal of Cancer*, 917-925

86- Chen, L. (2015). Codelivery of Doxorubicin and Ceramide as a Nanoparticle. Retrieved April 28, 2016, from https://etd.auburn.edu/handle/10415/4490

87- Zolnik, B. S., Stern, S. T., Kaiser, J. M., Heakal, Y., Clogston, J. D., Kester, M., & Mcneil, S. E. (2008). Rapid Distribution of Liposomal Short-Chain Ceramide in Vitro and in Vivo. *Drug Metabolism and Disposition,36*(8), 1709-1715

88- Stover, T. (2003). Liposomal Delivery Enhances Short-Chain Ceramide-Induced Apoptosis of Breast Cancer Cells. *Journal of Pharmacology and Experimental Therapeutics, 307*(2), 468-475

2. Short -Chain Ceramides for Enhancing Cytotoxicity of Liposome-Encapsulated Doxorubicin toward Human Breast Cancer (MDA-MB231) and Prostate Cancer (PC-3) Cells

2.1 Introduction:

In the United States, cancer is the most common cause for death after heart diseases. By far, prostate cancer is the leading cancer among men in the United States (1). On the other hand, breast cancer is the leading cancer among women (2-3). In 2015, it is estimated that 231,840 women were diagnosed with breast cancer and 40,290 will die as a result of it. Likewise, 220,800 men were diagnosed with prostate cancer and 27,540 will die from it (4).

Table 1. Number of new cancer cases and deaths by sex, US, 2015 (adapted from American Cancer Society (4))

Site	New cases			Estimated deaths		
	Both sexes	Male	Female	Both sexes	Male	Female
Prostate	220,800	220,800	-	27,540	27,540	-
Breast	234,190	2,350	231,840	40,730	440	40,290

Both breast and prostate cancer are being treated by standard therapies such as chemotherapy, radiotherapy, immunotherapy, targeted therapy and combinations of these therapies (5-7).

Introduced in 1970s, DOX, anthracycline antibiotic, has become widely used for treatment different types of cancers including both solid tumors, such as prostate and breast, and hematological cancer, such as leukemia and lymphoma (8-10).

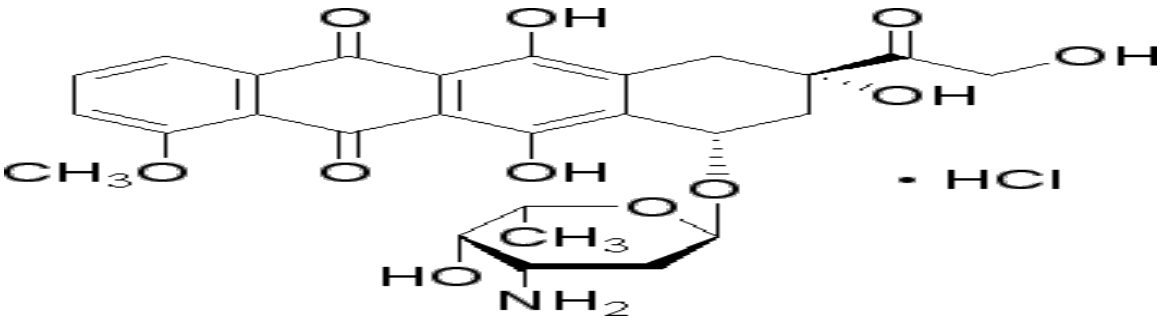

Fig.6. Chemical structure of Doxorubicin

Although the mechanism of action of DOX is not fully understood, it is known that it intercalates between DNA base pairs; as a result, it inhibits the activity of topoisomerase II enzyme (11-12). In addition, DOX triggers Reactive Oxygen Species (ROS) generation in different cancer cells through exhausting endogenous antioxidants, such as glutathione (15). Furthermore, DOX is reduced endogenously to semiquinone, a free radical that produces $O_2^{\bullet -}$ in presence of oxygen. $O_2^{\bullet -}$ will be eventually converted to hydrogen peroxide H_2O_2 (13-15).

Despite the fact of being widely used as antineoplastic drug, DOX still exhibits serious adverse effects, like cardio and bone marrow toxicity, which result from non-specific toxicity toward normal cells (16-17). Furthermore, it is not taken up sufficiently by tumor cells as it diffuses slowly through plasma membrane (18).

The main aim for developing of an optimum delivery system for the anticancer drugs is to enhance their selectivity and minimize their harm effects (19). Nanotechnology is considered one approach to encapsulate poor-soluble drugs, modify circulation time and tissue distribution of therapeutic agents, enhance cellular uptake by cells and decrease drugs toxicity (20-23).

Table 2. Some benefits of using nanocarrier for drug delivery (adapted from Valetti *et al* (24))

Advantages of nanocarriers
• Prevention of undesired drug interaction with the biological environment (i.e., drug inactivation by metabolization) • Control on pharmacokinetic/pharmacodynamic parameters • Enhanced drug accumulation at the tumor target site and improved intracellular uptake • Safety (i.e.; decrease of drug toxicity and side-effects).

Liposomes are spherical lipid vesicles that are formed upon hydration of different lipid (25). They consist of lipid bilayer with aqueous phase inside. Liposomes might be classified to unilamellar vesicle (UV) or multilamellar vesicles (MLV) based on diameter size (26) (Table 3).

Table 3. Types of liposomes vesicles

Different types of liposomes	
Type	Size range
Multilamellar vesicles	500 to 5,000 nm
Small unilamellar vesicles	<200 nm
Large unilamellar vesicles	200 to 800 nm

Liposomes circulation time was increased by incorporation of PEGylated lipid (27) (Figure 7). PEGylated liposomes, also called sterically stabilized liposomes, possess a prolong circulation time because PEG moiety located on the surface of the liposome eliminates the faster uptake of liposome by the Reticuloendothelial system (RES) by

forming a protective layer “steric effect” that minimizes protein binding to liposomes and subsequent uptake by macrophage (28-29).

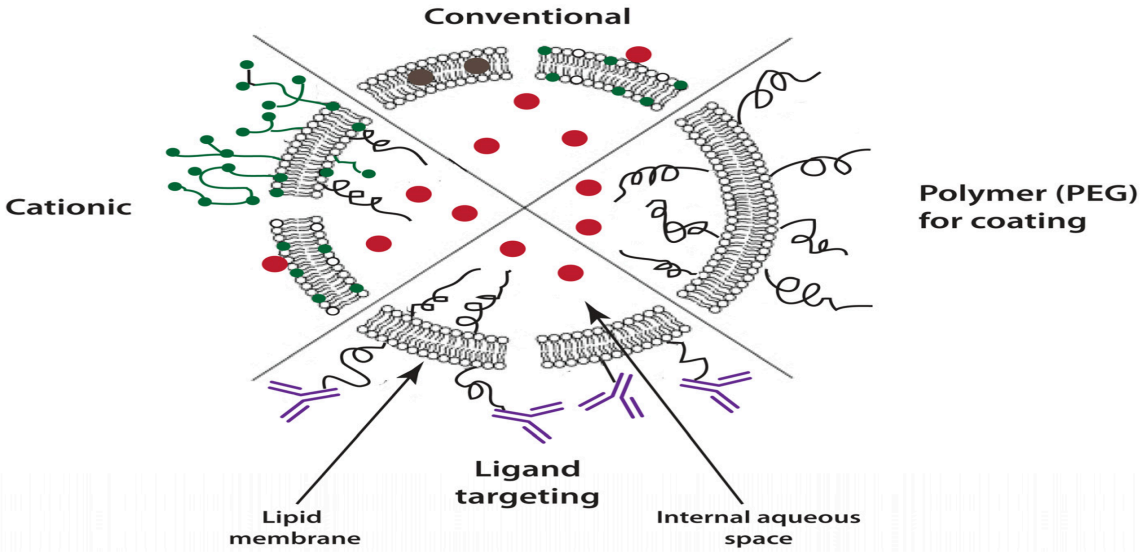

Fig.7. Diagram represents the difference between sterically stabilized liposomes (SSL) and conventional liposomes (adapted from Ait-Oudhia *et al.* (30))

Liposomes have been often studied as a drug delivery carrier because their ability to encapsulate a therapeutically suitable amount of the drugs (Table 4). Hydrophilic drugs can be entrapped in the aqueous compartment of liposome, while the lipid bilayer can be utilized to encapsulate lipophilic drugs.

Table 4. Some liposomal drugs that have been approved for clinical use (adapted from Torchilin *et al* (31))

Liposomal drugs approved for clinical application		
Active drug	Product name	Indications
Daunorubicin	DaunoXome	Kaposi's sarcoma
Doxurubicin	Mycet	Combinational therapy of recurrent breast cancer
Doxorubicin in PEG-liposomes	Doxil/Caelyx	Refractory Kaposi's sarcoma; ovarian cancer; recurrent breast cancer
Amphotericin B	AmBisome	Fungal infections
Cytarabine	DepoCyt	Lymphomatous meningitis
Vincristine	Onco TCS	Non-Hodgkin's lymphoma
Lurtotecan	NX211	Ovarian cancer
Nystatin	Nyotran	Topical antifungal agent
All-trans retinoic acid	Altragen	Acute promyelocytic leukaemia; non-Hodgkin's lymphoma; renal-cell carcinoma; Kaposi's sarcoma
Platinum compounds	Platar	Solid tumours

Ceramides, a type of sphingolipid metabolites, are well known for their biological activities such as regulating cell proliferation and/or apoptosis by modifying intracellular signaling (32-33). Furthermore, ceramides are known to induce transbilayer movement of lipids in cell membrane and to alter the bilayer asymmetry across cell membrane (34-35). Increasing intracellular level of ceramides has been linked cell apoptosis (36). Although the exact mechanisms responsible for ceramide-induced cell death remain unclear, it has been proposed that ceramides induce cell apoptosis by decreasing the activity of serine/threonine kinase (Akt) (37).

One major problem with liposomal drug delivery is that the liposomes are somewhat difficult to cross the cell membranes, particularly when the drugs are required

to enter the cell through passive diffusion. In this study, we hypothesized that co-delivery of DOX with ceramides, using liposomal delivery system, will facilitate the trans-membrane diffusion of DOX, leading to increase both cytotoxicity and cellular uptake of DOX. We have used two cell lines, (MDA-MB-231) breast cancer and (PC-3) prostate cancer, to study various liposomal formulations with and without ceramide for their cell uptake behavior and cytotoxic effects.

2.2 Thesis Objectives:

The objective of this thesis is to determine the cytotoxicity and cellular uptake of DOX liposomal formulations enriched with ceramides (C_8-Cer and C_6-Cer). Ceramides have anti-tumor activity *in vitro* and *in vivo* (33). Ceramides target the PI3K/Akt pathway through dephosphorylation of Akt, leading to increased cytotoxicity and cell apoptosis and they act synergistically when in combination with other chemotherapeutics (37). Furthermore, ceramide could facilitate the transmembrane diffusion of DOX, leading to increased cytotoxicity and apoptosis of human breast cancer (MDA-MB-231) and prostate cancer (PC-3) cells.

Incorporation of cationic lipid such as DOTAP into liposomes could enhance the chemotherapeutic activity of cancer drugs (43). The higher cytotoxicity of DOTAP liposomes might be partially explained by the interaction between the positively charged liposome and the negatively charged cell membrane

The thesis has the following research objectives:

1. To develop, characterize and evaluate pegylated cationic liposome formulation loaded with both DOX and ceramides. Characterization of different formulations for particle size (dynamic light scattering), *in vitro* release behavior (by dialysis method) and drug-to-

lipid ratio effects on recovery and loading efficiency. Different types of ceramides (ceramide-6 and ceramide-8) will be used in the formulation of liposomes.

2. To evaluate the *in vitro* efficacy of optimized formulations and their molecular mechanisms of action. Determination of the cell cytotoxicity against human breast cancer (MDA-MB-231) and prostate cancer (PC-3) cells by MTT assay, DOX uptake in the above cell lines, measurement of oxidative stress generated by DOX inside the cells and confirmation of DOX uptake by fluorescence microscopy.

3. Materials and Methods:

3.1 Materials

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-mPEG (2000)), C_6-Ceramide (C_6-Cer) and C_8-Ceramide (C_6-Cer)) were purchased from Avanti Polar Lipids Inc (Alabaster, AL). Cholesterol and Ammonium sulfate were purchased from JT Baker (Phillipsburg, NJ). Fetal bovine serum, Ham's F-12 medium, 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12, and other reagents for cell culture were purchased from Mediatech (Manassa, VA). Doxorubicin hydrochloride (DOX) was purchased from AvaChem Scientific (San Antonio, TX). 2′,7′-Dichlorofluorescin diacetate and PBS were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid protein kit was purchased form Thermo Scientific (IL, USA). Tetrazolium bromide (MTT) was purchased from Calbiochem (Darmstadt, Germany). Polycarbonate membrane (0.08 μm) was purchased from Whatman Maidstone, UK). Prostate cancer (PC3) and breast cancer (MDA-MB-231) were obtained from American Type Culture Collection (Manassa, VA).

3.2 Methods

3.2.1 Liposomes Preparation

Liposomes were prepared by lipid film hydration technique using rotary evaporator. Briefly, DOTAP was dissolved at 10 mg/ml in chloroform. Similarly, 10 mg/ml solution of cholesterol, 10 mg/ml solution of one of the ceramides (C_6-ceramide and C_8-ceramide) and 10 mg/ml solution of DSPE-mPEG (2000) were prepared in chloroform. The solutions, mixed at a molar ratio of 44:40:4:12 for lipid/ cholesterol/DSPE-mPEG(2000)/ ceramide, was flash evaporated on a rotavapor (Rotavapor, Büchi, Germany) by applying vacuum of about 25mmHg at 65 ^{o}C, until it forms a thin film on the inner wall of the flask. The lipid film was further dried under a stream of nitrogen for 1h, followed by vacuum desiccation for 2 h. The dry lipid film was then hydrated in 250 mM ammonium sulfate solution (pH 5.5). This mixture was then placed in a water-bath incubator (65 ^{o}C) for 1 h to form coarse liposomes. The liposome mixture was then extruded through 80 nm (10 passes) polycarbonate filter using Lipex 100 ml (Transferra Nanosciences Inc, Burnaby, BC. Canada). The free ammonium sulfate outside the liposomes was removed by dialysis (using 12, 000 to 14,000 Daltons molecular weight cut off dialysis tubing) against sucrose solution (10% w/v, 250 ml) at 4 ^{o}C. Sucrose medium was discarded and replaced with fresh medium after 1,4,8 h and then left overnight.

3.2.2 Drug Encapsulation in Liposomes (Active Loading)

DOX solution was prepared by adding the required quantities of drug in the PBS and this drug solution, after adjusting the pH to 8, was added to the lipid solution at appropriate drug-to-lipid ratios (0.3:1 & 0.2:1). Excess DOX was removed by dialysis against sucrose solution (10%) at 4 ^{o}C. Based on initial results of drug loading efficiency,

0.2:1 drug-to-lipid ratio was found to be optimum and this ratio was used for all formulations.

3.2.3 Recovery and Loading Efficiency Measurement

The amount of DOX entrapped into liposomes was determined fluorometrically at 480 nm (excitation) and 590 nm (emission) using a microplate reader 142 (Fluostar, BMG labtechnologies, Germany). Briefly, Triton X-100 (1%) was added to different liposomal DOX to break the liposome bilayer and release the entrapped DOX. Liposomal drug was compared to a DOX standard curve. All the experiments were run in triplicate and mean data were presented. The recovery % was calculated as follows:

$$\text{Recovery (\%)} = \frac{\text{amount of liposomal drug}}{\text{total amount of drug}} \times 100$$

The loading efficiency (LE %) was calculated as follows:

(amount of liposomal drug / (amount of drug added + amount of excipients)) $\times 100$

3.2.4 Particle Size Determination of Liposomal Formulations

Particle size distribution of the liposomal formulations was carried out by dynamic light scattering method using Nicomp 380 ZLS particle size analyzer (Particle Sizing Systems, Santa Barbara, CA). Mean particle size and polydispersity index of the formulations after appropriate dilutions were calculated.

3.2.5 *In Vitro* Release

The release profile of DOX from liposome formulations was determined by dialysis method. Phosphate buffered saline (PBS) pH 7.4, 250 ml, in a conical flask was used as a receptor phase. Dialysis tubing (12,000 to 14,000 Daltons molecular weight cut off), 30 mm × 25 mm release area, pre-soaked in buffer solution for one hour, was used. 1 ml of the formulation or DOX solution was placed in the dialysis tubing. All flasks

were incubated at 37 ^{o}C in a rotary shaker set at 150 rpm. Samples (1 ml) were collected at different time intervals (1h, 3h, 6h, 12h, 24h) and the sample volumes were replenished with fresh buffer immediately. The concentration of DOX in the receptor buffer (dialysate) was analyzed fluorometrically at 480 nm (excitation) and 590 nm (emission) using a microplate reader 142 (Fluostar, BMG labtechnologies, Germany). The cumulative amount of DOX released versus time was plotted. All the experiments were run in triplicate and mean data was presented.

3.2.6 Stability Studies

A short-term stability was conducted to monitor physical stabilities of the liposomes. All liposomal formulations were stored at 4 ^{o}C for up to one month. The stability parameter, such as particle size and polydispersity were determined after the storage.

3.2.7 Cell Culture

PC3 cells were cultured in Ham's F-12 medium, whereas MAD- MB-231 cells were cultured in 1:1 mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12. Both media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were performed at a confluence of 90 to 95%.

3.2.8 Measurement of cytotoxicity by MTT Assay

For viability assessment, PC3 and MAD- MB-231 cells were cultured in flat-bottom 96-well plates for 24 hours. The cell density in the wells was 8×10^3 cells/well. The cells received treatments of various liposomal formulations for 48 h prior to MTT assay. After experimental treatments, 10 µl of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) was added to each well and the cells were incubated at 37 ^{o}C

for an additional 2 hours. Finally, the medium was aspirated and 100 µl dimethylsulfoxide (DMSO) was added to each well to solubilize the dye remaining in the plates. The absorbance was measured using a microplate reader (spectramax M5, molecular devices, Sunnyvale, CA, USA) at 544 nm. All the experiments were run in triplicate and mean data were presented.

3.2.9 Cellular Doxorubicin Uptake

PC3 and MAD- MB-231 cells were cultured in flat bottom 96-well plates. At confluence, cells were exposed to 20 µM liposomal DOX or free DOX for 24 hours. After extensive washing with PBS, cells were lysed in 100 µl of 1% Triton X-100. DOX fluorescence was then measured by a microplate reader (spectramax M5, molecular devices, Sunnyvale, CA, USA) using 480- and 590-nm filters for excitation and emission, respectively. After calculating cellular DOX contents with the aid of standard amounts, all contents were corrected for any differences in protein content, as determined with the bicinchoninic acid assay (38). In addition, all values were corrected for background fluorescence. All the experiments were run in triplicate and mean data were presented.

3.2.10 Measurement of Oxidative Stress

The determination of intracellular reactive oxidant species generated by DOX was based on the oxidation of 2',7'-dichlorodihydrofluorescein diacetate to the fluorescent product, 2',7'-dichlorofluorescein. PC3 and MAD- MB-231 cells were cultured in flat bottom 24-well plates for 24 hours. At confluence, cells were exposed to 20 µM of different liposomal formulations or free DOX for 24 hours. Following treatment with various formulations, medium was aspirated and the cells were washed three times with PBS before being placed into 1 ml of cell culture medium without FBS. 2',7'-dichlorodihydrofluorescein diacetate was added to a final concentration of 10 µM, and

cells were incubated for 20 min. The cells were again washed twice with PBS and maintained in 1 ml of culture medium. Intracellular fluorescence was measured at wavelengths of 480nm (excitation) and 535 nm (emission) using a microplate reader (spectramax M5, molecular devices, Sunnyvale, CA USA). Each study was repeated three times and the mean fluorescence was presented.

3.2.11 Fluorescence Microscopy

PC3 and MAD- MB-231 cells were seeded in a flat bottom 24-well plate for 24 hours. After exposure to liposomal DOX or free DOX for 24 hours, cells were washed and fixed [15 min in 4% (w/v) paraformaldehyde in phosphate-buffered saline]. All samples were examined with fluorescence microscope (EVOS fl, ZP-PKGA-0494 REV A, USA) and photographed through a 20 X objective.

3.2.12 Statistical Analysis

The DOX % released from liposomes was plotted as a function of time (h). All the data were presented as mean ± standard deviation. Graph Pad Prism software was used to determine the standard deviation and statistical levels of significance. All data were subjected to one-way analysis of variance (ANOVA) to determine the statistical levels of significance. P value less than 0.05 was considered to be statistically significant.

4. Results and Discussion

4.1 Formulation Optimization

The liposomes prepared with and without ceramides were evaluated for particle size and recovery %. The particle size of all formulations was in the range of 92-98 nm with a narrow particle size distribution as known from the polydispersity values. As shown in Table 5.1, there is no significant difference in particle size between liposomes formulations with or without ceramides indicating that the addition of short-chain

ceramides (C_6-Cer, C_8-Cer) has no effect on particle size ($p > 0.05$).

To obtain liposomes with desirable recovery %, DOX was mixed with lipid (Dox:lipid) at two different ratios 0.3:1 and 0.2:1. DOX was loaded into the aqueous phase of liposome by active loading, using ammonium sulfate 250 mM. As shown in Table 5.2, 0.2:1 drug-to-lipid ratio demonstrated higher recovery % compared to 0.3:1 drug-to-lipid ratio. The recovery was above 90% for both 0.2:1 and 0.3:1 drug-to-lipid ratio; however, since 0.2:1 drug-to-lipid ratio provided higher recovery %, especially for liposomes enriched with C_8-Cer (95% vs 90% in case of 0.3:1 drug-to-lipid ratio), it was used for all liposomal formulations.

The mechanism by which the drug-to-lipid ratio influences the recovery is of particular interest. Inverse relationship was noticed between recovery and DOX concentration. The recovery % decreases with increased DOX concentration. Existence of drug precipitate in the liposome interior may explain the inverse relationship between recovery and DOX concentration. Increasing drug-to-lipid ratio will cause the drug to precipitate inside the liposomes leading to significant deformation and disruption of the liposomal membrane, which cause leakage of encapsulated drug from liposomes (39).

High recovery of amphipathic weak bases might be achieved by a transmembrane ammonium sulfate gradient in and out of liposomes (active loading) (40). Similar to most drugs, DOX was not entrapped into the aqueous phase of the liposome without a pH gradient. DOX active loading process is dependent on the base exchange with ammonium ions in the liposomes.

Table 5.1. Particle diameter, PI, Recovery (%) and drug loading of different liposomes. Values are expressed as mean ± standard deviation (n = 3).

Dox Liposomal Formulation (0.2:1 ratio)	Particle Diameter (nm)	Polydispersity Index (PI)	Drug Loading (%)	Recovery (%)
DOTAP-C6-Cer	95.0 ± 2.6	0.19 ± 0.02	15.0 ± 0.6	94.0 ± 2.5
DOTAP-C8-Cer	98.0 ± 2.0	0.24 ± 0.02	16.0 ± 0.6	95.0 ± 2.0
DOTAP	92.0 ± 1.1	0.17 ± 0.01	16.0 ± 0.6	97.0 ± 0.6

Table 5.2. Recovery % of different liposomes at different drug to lipid ratio (0.3:1 & 0.2:1). Values are expressed as mean ± standard deviation (n = 3).

Recovery %	Dox to lipid ratio 0.3:1	Dox to lipid ratio 0.2:1
DOTAP-C6-Cer	92.0 ± 2.1	94.0 ± 2.5
DOTAP-C8-Cer	90.0 ± 1.7	95.0 ± 2.0
DOTAP	91.0 ± 1.4	97.0 ± 0.6

4.2 Release Profiles

In order to evaluate whether C_6 and C_8 could affect the release of DOX from the liposomes, we performed an *in vitro* drug release analysis at 37 ^{o}C. The release profiles of DOX from different liposomal formulations are shown in Figure 8. The control formulation DOX solution reached 100% release within 6 hours, which confirmed that the dialysis membrane did not restrict diffusion of the released DOX into the medium. The release of DOX from the liposomes with different ceramides was also investigated. The amounts released after 24 hours were 35%, 43% and 31 % for DOTAP-C_6-Cer, DOTAP-C_8-Cer and DOX liposomal formulation without ceramide, respectively.

Liposome formulations exhibited significantly slower release profile compared to

free DOX, because of additional time required for the release of drug from the liposome. In addition, cholesterol decreased the flexibility of the lipid bilayer, leading to slower release of DOX from liposomes. Incorporation of short chain ceramide into the liposomal bilayer did not lead to enhanced DOX leakage ($p > 0.05$). Apparently, incorporation of sphingolipid (C_6-Cer or C_6-Cer) did not affect DOX diffusion through the liposomal bilayer; as a result, they do not enhance its release rate.

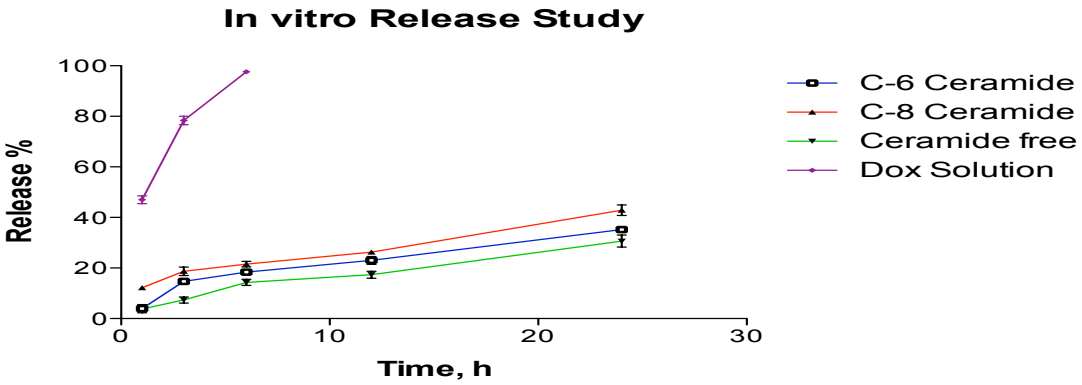

Fig. 8. In vitro release profiles of DOX encapsulated liposome with various ceramides, DOX encapsulated liposome without ceramide and free DOX solution. Mean±S.D., n = 3 separate experiments.

4.3 Stability Study

Physical stability of different PEGylated liposomes during storage (at 4°C for one month) was followed by time-dependent changes in liposome size and Polydispersity Index (Table 6). No significant changes in size and Polydispersity Index (PI) during the course of stability study when compared the data to that of the initial analysis ($p > 0.05$).

The introduction of cholesterol, less than 50%, decreased the fluidity of the lipid bilayer, leading to higher physical stability (41). In addition, ceramide stabilizes lipid rafts; as a result, long-term storage instability of PEGylated liposomes in the presence of ceramide was not affected (42).

Table 6. Particle size and polydispersity index of DOX-loaded PEGylated liposomes, with/without ceramides, before/after storage at 4°C for one month. Values are expressed as mean ± standard deviation (n = 3).

	Freshly prepared		4°C for one month	
Liposomal formulation	Particle Diameter (nm)	Polydispersity Index (PI)	Particle Diameter (nm)	Polydispersity Index (PI)
DOTAP-C_6-Cer	95.0 ± 2.6	0.19 ± 0.015	96.0 ± 2.0	0.2± 0. 10
DOTAP-C_8-Cer	98.0 ± 2.0	0.24 ± 0.015	101.0 ± 1.0	0.25± 0.01
DOTAP	92.0 ± 1.1	0.17 ± 0.01	95.0 ± 3.2	0.17 ± 0.01

4.4 Cell Cytotoxicity

Liposomes carrying various ceramides (C_6-Cer and C_8-Cer) in the lipid bilayer and DOX in the aqueous phase were formulated and their cytotoxicities were tested in prostate cancer (PC3) and breast cancer (MDA-MB-231) cell lines. In the case of PC3, liposome with DOTAP/C_8-Cer exhibited the highest cytotoxicity, followed by DOTAP/C_6-Cer, DOTAP (liposome without ceramide) and finally DOX solution ($p < 0.001$) (Fig 9A). As shown in Figure 9B, the cell toxicity (5 uM, 48 hours) due to C_8-Cer, C_6-Cer, DOTAP and DOX was 26, 36, 40 and 51 %, respectively. The IC_{50} of DOTAP/ C_8-Cer was about 10-fold lower than DOX solution (0.5 uM compared to 5 uM). Furthermore, both C_6-Cer and DOTAP exhibited higher cytotoxicity compared to DOX solution ($p < 0.001$); however, their cytotoxicities were less than DOTAP/ C_8-Cer. IC_{50} of both C_6-Cer and DOTAP was 5-fold lower than DOX solution (1 uM compared to 5 uM).

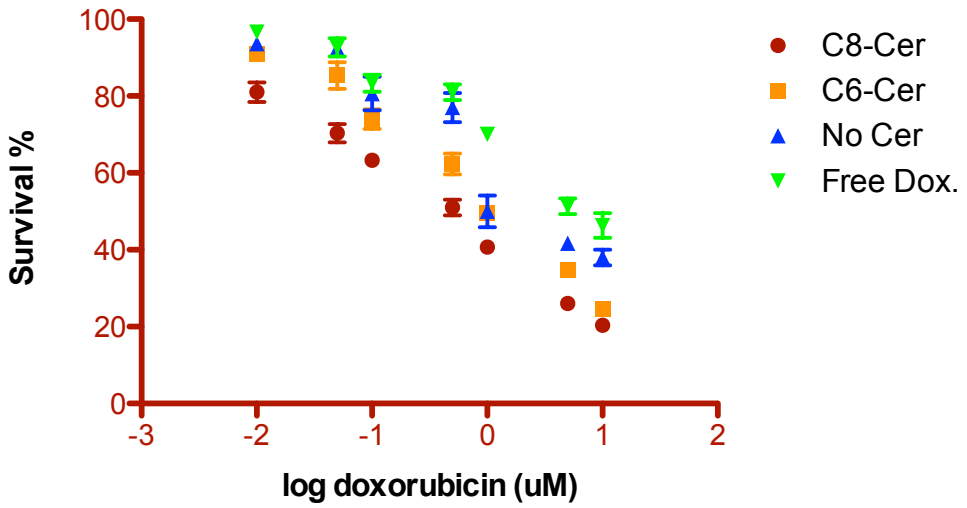

Fig. 9 A. C_6-Cer and C_8-Cer potentiates the cytotoxic effect of DOX against PC3 cell line. Cells were incubated with various concentrations of different Liposomal formulations (0.01 µM, 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM) for 48 hours. All data are expressed as mean percentages (n=3) to untreated control cells.

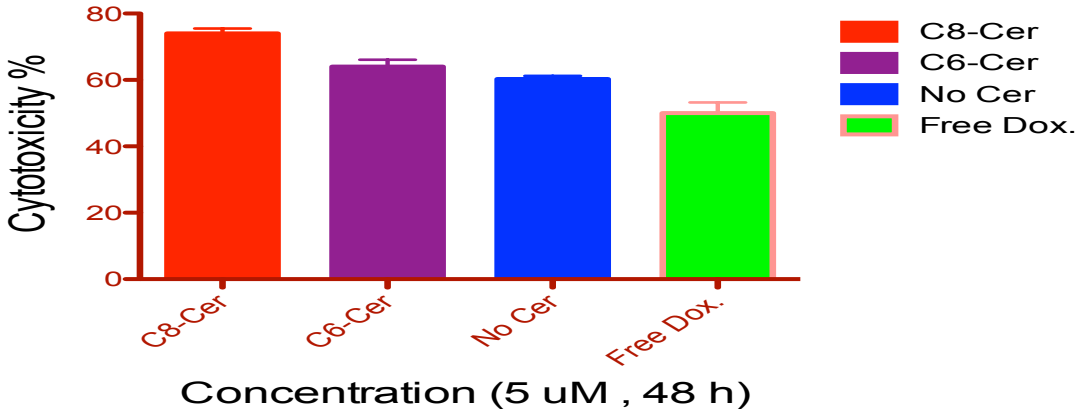

Fig. 9 B. In vitro cytotoxicity of formulations containing different ceramides in PC3 prostate cell line. All data are expressed as mean ± S.E. of n = 3 separate experiments. $p < 0.001$ when C_8-Cer and C_6-Cer compared to free DOX. $p < 0.01$ when DOTAP (liposome without ceramide) compared to free DOX. $p < 0.05$ when C_8-Cer compared to C_6-Cer.

In the case of MDA-MB-231, both C_8-Cer and C_6-Cer exhibited the highest cytotoxicity, followed by DOTAP and finally DOX solution (Fig 10A). As shown in Figure 10B, the cell cytotoxicity (5 uM, 48 hours) due to C_8-Cer, C_6-Cer, DOTAP and

DOX was 25,22, 42 and 55, respectively. There is no statistically significant difference in the cytotoxicity of C_8-Cer and C_6-Cer; however, both have a much higher cytotoxicity than DOTAP and free drug. IC_{50} of DOTAP/ C_8-Cer and DOTAP/C_6-Cer is about 12-fold lower than DOX solution (0.4 uM compared to 5 uM).

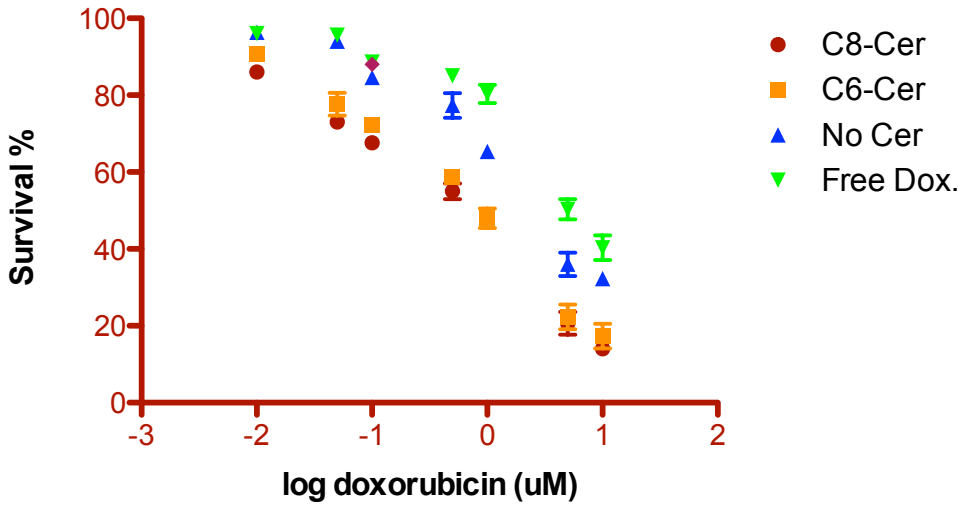

Fig. 10 A. C_6-Cer and C_8-Cer potentiates the cytotoxic effect of DOX toward MDA-MB-231 cell line. Cells were incubated with various concentrations of different Liposomal formulations (0.01 µM, 0.05 µM, 0.1 µM, 0.5 µM, 1 µM, 5 µM, 10 µM) for 48 hours. All data are expressed as mean percentages (n=3) to untreated control cells.

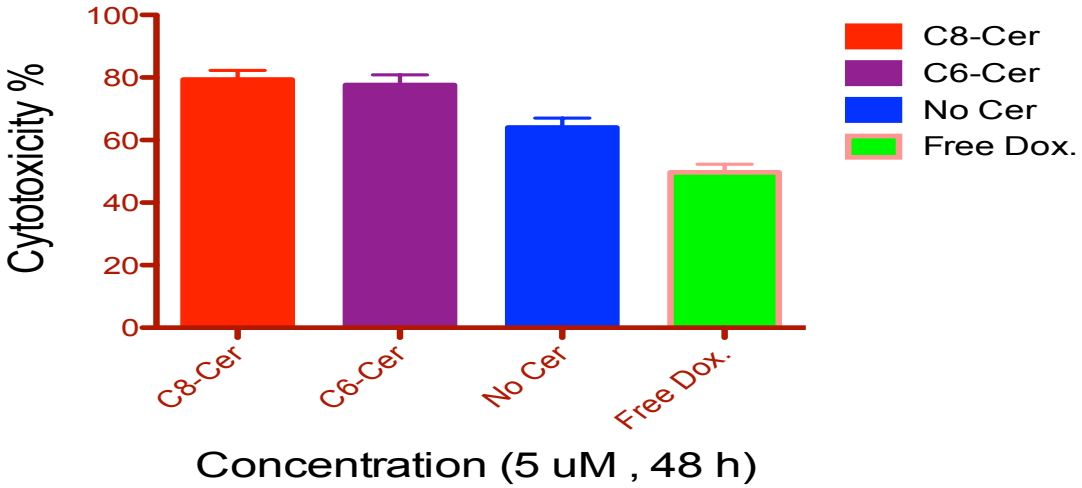

Fig. 10 B. In vitro cytotoxicity of formulations containing different ceramides in MDA-MB-231breast cell line. Mean ± S.E. of n = 3 separate experiments. $p < 0.001$ when C_8-Cer and C_6-Cer compared to free DOX. $p < 0.05$ when DOTAP (liposome without ceramide) compared to free DOX. $p > 0.05$ when C_8-Cer compared to C_6-Cer.

The results demonstrated that the liposome delivery system enhanced the delivery of DOX to both prostate cancer (PC3) and breast cancer (MDA-MB-231) cell lines significantly. The higher cytotoxicity of DOTAP liposomes might be partially explained by the interaction between the positively charged liposome (DOTAP is a cationic lipid) and the negatively charged cell membrane (43).

Ceramide can enhance the chemotherapeutic activity of numerous antineoplastic agents such as Adriamycin (44). Incorporation of short chain ceramides such as C_8-Cer and C_6-Cer into cationic lipid formulation (cationic liposomes) enhanced DOX cytotoxicity against prostate cancer (PC3) and breast cancer (MDA-MB-231). Regarding PC3 cell line, when C_8-Cer and C_6-Cer compared to DOTAP (liposome without ceramide), p value was <0.001 and <0.05, respectively. In the case of MDA-MB-231, p value was <0.05 when C_8-Cer and C_6-Cer compared to DOTAP (liposome without ceramide). The enhanced cytotoxic effect of DOTAP liposomes by ceramides might be explained by two possible mechanisms. First, ceramide enhances the permeation of DOX as it has a strong impact on the integrity of cell membrane lipid rafts (45). Second, ceramide induces cell cycle arrest, and/or apoptosis through the PI3K/Akt pathway (46).

4.5 Correlation of Cytotoxicity Results with Doxorubicin Uptake Levels

In order to examine whether short chain ceramides enhance the cytotoxicity of DOX because of differences in cell-permeability, we conducted cellular uptake studies. Both prostate and breast cancer cells were exposed to 20 μM liposomal DOX for 24 hours. This concentration was selected because it closely related to the plasma

concentrations that are achieved upon intravenous bolus administrations of liposomal DOX (20–40 mg/m^2 is the standard dosages of liposomal DOX) into average-sized persons.

All liposomal formulations exhibited higher cellular uptake of DOX when they were compared to free DOX solution. In the case of PC3, when using liposomes that were enriched with C_8-Cer and C_6-Cer, DOX cellular uptake increased up to 5-fold and 3-fold, respectively, when compared to DOTAP (liposome without ceramide) (Fig. 11A).

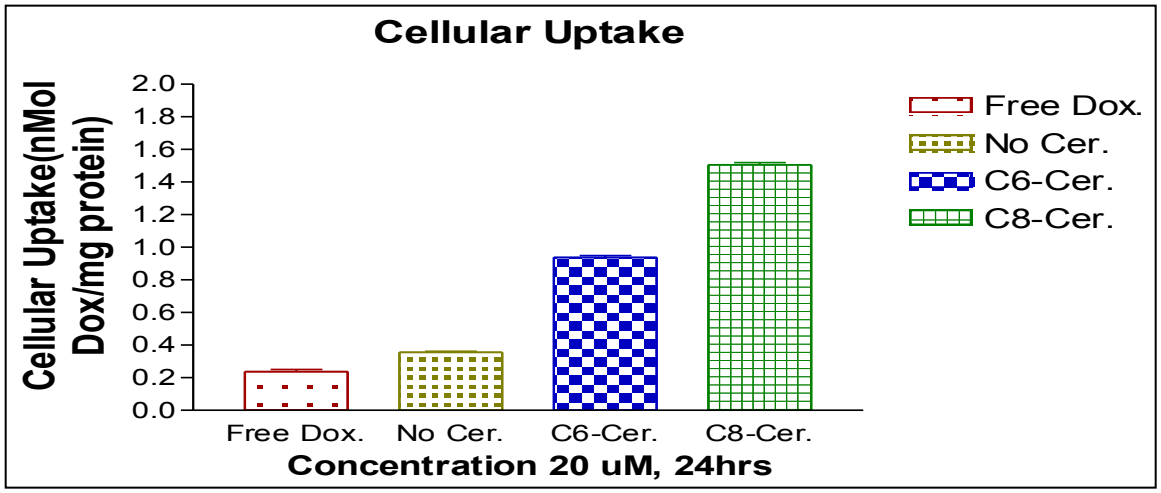

Fig. 11A. In PC3 cancer cells, C_8-Cer and C_6-Cer enriched DOX liposomes enhanced cellular uptake of doxorubicin from liposomes. p value <0.001 when C_8-Cer and C_6-Cer enriched liposomes is compared to DOTAP (liposome without ceramide). DOX fluorescence was quantified, corrected for cellular protein content, and expressed as mean ± S.D. (n = 3 independent experiments).

Similar results were observed in MDA-MB-231breast cell line. Liposomes that were enriched with C_8-Cer and C_6-Cer enhanced DOX cellular uptake up to 4-fold and 3-fold, respectively, when compared to DOTAP (liposome without ceramide) (Fig. 11B).

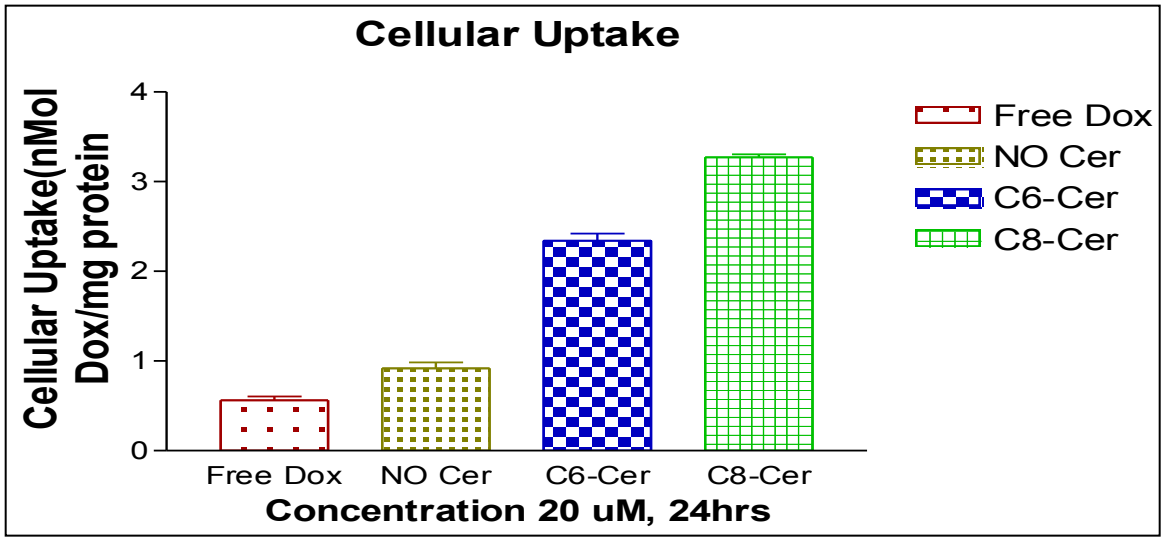

Fig. 11B. In MDA-MB-231 cancer cells, C_8-Cer and C_6-Cer enriched doxorubicin liposomes enhanced cellular uptake of DOX from liposomes. p value <0.001 when C_8-Cer and C_6-Cer enriched liposomes is compared to DOTAP (liposome without ceramide). DOX fluorescence was quantified, corrected for cellular protein content, and expressed as mean ± S.D. (n = 3 independent experiments).

PEG-liposomes do not permeate the plasma membrane and they are not taken adequately by cells as a result of their hydrophilic property (47). Cellular uptake of DOX from C_8-Cer and C_6-Cer enriched DOX liposomes is superior to DOTAP (liposome without ceramide). For both PC3 and MDA-MB-231 cell lines, when the cellular uptake of C_8-Cer and C_6-Cer enriched liposomes is compared to DOTAP (liposome without ceramide), p value <0.001. The mechanism by which short chain ceramides enhance the cellular uptake of DOX is not fully understood; however, it was proposed that ceramides, when incorporated into the liposomal bilayer, facilitate DOX trans-membrane diffusion by making the plasma membrane more permeable as they damage the bilayer asymmetry across cell membrane (48).

4.6 Fluorescence Microscopy

The cellular uptake enhanced by ceramide-based formulation was further confirmed by fluorescence microscopy experiments followed by imaging. Both cell lines

were exposed to 20 μM liposomal DOX for 24 hours. In PC3 cell line, C_8-Cer fluorescence intensity of DOX in the nucleus was much higher than both C_6-Cer and DOTAP. On the other hand, C_6-Cer and DOTAP (liposome without ceramide) the fluorescence intensity due to DOX accumulation in cell nuclei was almost similar (Fig. 12A). In MDA-MB-231 cell line, DOX accumulation in cell nuclei with C_8-Cer and C_6-Cer enriched liposomes was much higher compared to DOTAP (liposome without ceramide) (Fig. 12B).

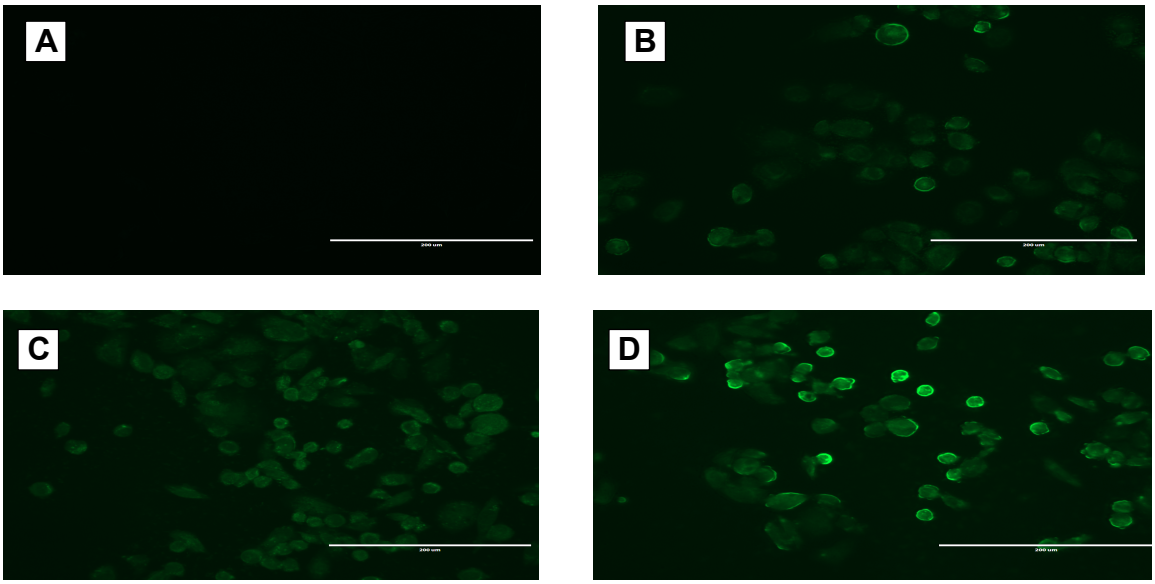

Fig. 12A. Fluorescence microscopy showing short chain ceramides C_8-Cer and C_6-Cer enhanced DOX uptake from liposomes. PC3 cells were cultured for 24 h (A) and then were treated with DOTAP (liposome without ceramide) (B), or C_6-Cer PEG-liposomes (C), or C_8-Cer PEG-liposomes (D). Final liposomal DOX concentrations were 20 uM. Dox fluorescence was taken with a X20 lens.

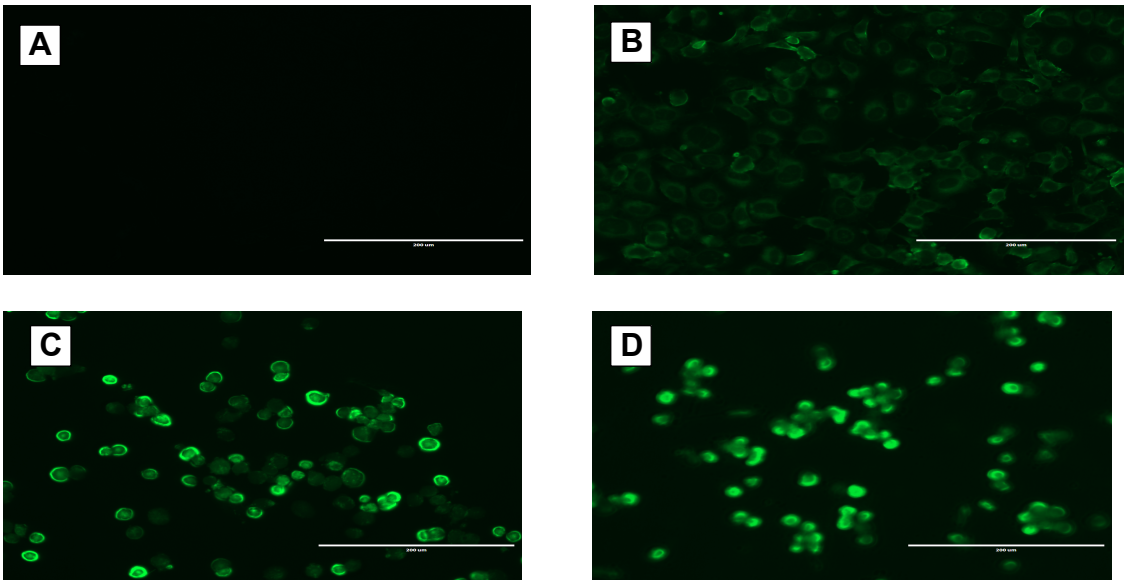

Fig. 12B. Fluorescence microscopy showing short chain ceramides C_8-Cer and C_6-Cer enhanced doxorubicin uptake from liposomes. MDA-MB-231 cells were cultured for 24 h (A) and then were treated with DOTAP (liposome without ceramide) (B), or C_6-Cer PEG-liposomes (C), or C_8-Cer PEG-liposomes (D). Final liposomal DOX concentrations were 20 uM. Dox fluorescence was taken with a X20 lens.

Our fluorescence microscopy results demonstrated that short chain ceramides enhanced delivery of DOX into tumor cells. The results correlate with both cytotoxicity and cellular uptake results. As mentioned above, C_8-Cer greatly improves the cellular uptake and cytotoxicity of DOX toward PC3 cell line. As showing in Fig.12A, fluorescence microscopy experiment demonstrated that C_8-Cer enriched liposomes enhanced nuclear accumulation of DOX into tumor cells. Similar observation was noticed with C_8-Cer rand C_6-Cer enriched liposomes, which greatly improves the cellular uptake and cytotoxicity compared to liposome without ceramide, in the case of MDA-MB-231 cell line. Both C_8-Cer and C_6-Cer enriched liposomes increased nuclear accumulation of DOX to a greater extent. Short chain ceramides could change the physical properties of plasma membrane such as thickness and permeability through creating voids (49). The voids within the lipid bilayer could facilitate DOX accumulation in the cells.

4.7 Reactive Oxygen Species (ROS)

Anthracycline drugs such as DOX might elevate the intracellular production of reactive oxygen species like hydrogen peroxide (13). There is a direct relationship between DOX concentration inside cells and ROS generation. In both cell lines, ceramide enriched liposomes produced higher ROS compared to free DOX solution. In the case of PC3 cell line, C_8-Cer enriched liposomes exhibited the highest ROS production compared to C_6-Cer and DOTAP liposomes, p value <0.001 (Fig. 13A). On the other hand, there is no statistical significance between C_6-Cer and DOTAP liposomes ($p > 0.05$). In MDA-MB-231 cell line, C_8-Cer and C_6-Cer enriched liposomes produced much higher ROS than DOTAP liposomes, p value <0.001 and <0.05, respectively. There is statistical significance between C_8-Cer and C_6-Cer enriched liposomes, p value <0.001(Fig. 13B).

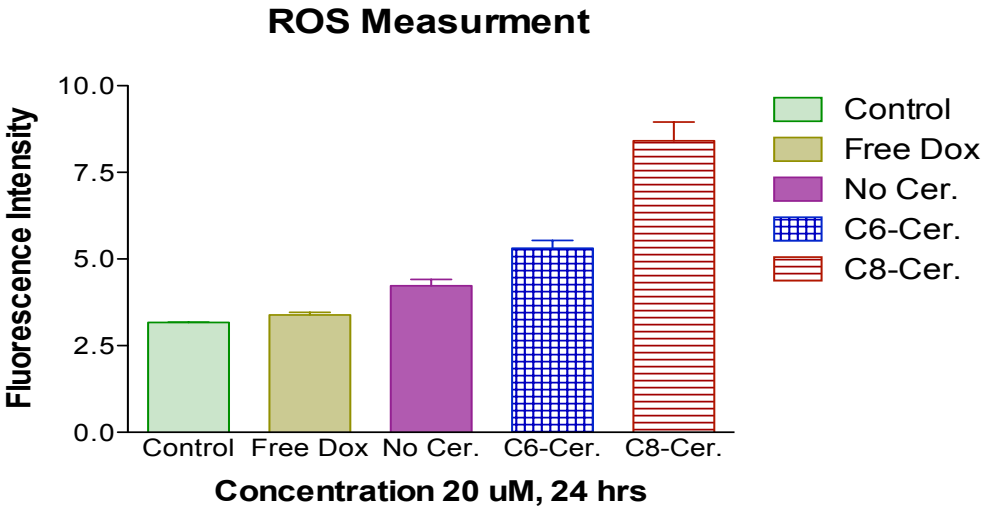

Fig. 13A. Ceramide increased DOX-induced ROS generation in PC3 cell line. Cells were treated for 24 h with free and liposomal DOX (20 uM). Data are represented as means ± S.E. of three independent experiments.

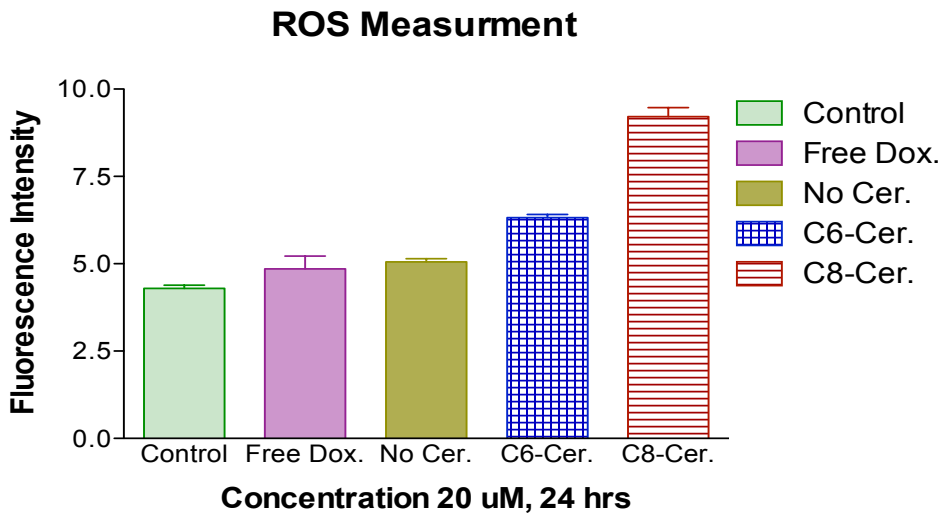

Fig. 13B. Ceramide increased DOX-induced ROS generation in MDA-MB-231 cell line. Cells were treated for 24 h with free and liposomal DOX (20 uM). Data are represented as means ± S.E. of three independent experiments.

Elevated level of reactive oxygen species (ROS) has been noticed in most cancer cells (50). DOX is reduced by cancer cells to semiquinone, a free radical that produces $O_2^{\bullet -}$ in presence of Oxygen. $O_2^{\bullet -}$ will be ultimately converted to hydrogen peroxide H_2O_2 (51). ROS results in both cell lines are strongly correlated to cellular uptake results, which indicate that combining short chain ceramides into liposomes enhance DOX uptake by the tumor cells. It is important to mention that elevation in ROS level is not merely due to DOX. Ceramide stimulates ROS production and influences certain cellular oxidative stress through manganese-dependent superoxide dismutase pathway (52).

5. Conclusion

We prepared a formulation for simultaneous delivery of DOX and a ceramide into tumor cells. The liposomes were prepared at a 0.2:1molar ratio of drug-to-lipid, with a narrow particle size distribution and high recovery %. The final optimum liposome had 44:40:4:12 molar ratio for DOTAP/ cholesterol/DSPE-mPEG (2000)/ ceramide. C_8-Cer enriched liposomes exhibited a higher cytotoxic and cellular uptake effect on PC3 cell

line than free DOX or liposomes with no ceramide. In MDA-MB-231 cell line, both C_8-Cer and C_6-Cer enriched liposomes exhibited a higher cytotoxic and cellular uptake effect than free DOX or liposomes with no ceramide.

6. Future Direction

In the present study, we demonstrated that DOX-loaded PEGylated liposomes with C_6 or C_8 ceramide are useful for delivering amphiphilic anticancer drugs, such as DOX. Cytotoxicity and cellular uptake of DOX liposomes enriched with ceramide were greater than that of free DOX and liposomal DOX without ceramide.

Due to the enhanced in vitro efficacy of C_8-Cer and C_6-Cer enriched liposomes, our next step is to investigate the effectiveness of the formulations *in vivo* in a tumor bearing mouse model. In order to assess the feasibility of clinical application of DOX-loaded PEGylated liposomes with C_6 or C_8 ceramide, extensive toxicity and pharmacokinetics studies will be conducted in the future. Furthermore, the exact synergistic mechanism between ceramide and DOX will be further explored in detail.

7. References

1- Bell, K. J., Mar, C. D., Wright, G., Dickinson, J., & Glasziou, P. (2015). Prevalence of incidental prostate cancer: A systematic review of autopsy studies. *International Journal of Cancer Int. J. Cancer, 137(7)*, 1749-1757.

2- Hunt, B. R., Whitman, S., & Hurlbert, M. S. (2014). Increasing Black:White disparities in breast cancer mortality in the 50 largest cities in the United States. *Cancer Epidemiology, 38(2)*, 118-123.

3- Baquet, C. R., Mishra, S. I., Commiskey, P., Ellison, G. L., & Deshields, M. (2008). Breast Cancer Epidemiology in Blacks and Whites: Disparities in Incidence, Mortality, Survival Rates and Histology. *Journal of the National Medical Association, 100(5)*, 480-489.

4- American Cancer Society. Cancer Facts & Figures 2015. Atlanta: American Cancer Society; 2015.

5- American Cancer Society. Breast Cancer Facts & Figures 2011-2012. Atlanta: American Cancer Society, Inc.

6- Akaza, H., Hinotsu, S., Usami, M., Arai, Y., Kanetake, H., Naito, S., & Hirao, Y. (2009). Combined androgen blockade with bicalutamide for advanced prostate cancer. *Cancer, 115(15)*, 3437-3445.

7- Bostrom, P. J., & Soloway, M. S. (2007). Secondary Cancer After Radiotherapy for Prostate Cancer: Should We Be More Aware of the Risk? *European Urology, 52(4),* 973-982.

8- Tsakalozou, E., Eckman, A. M., & Bae, Y. (2012). Combination Effects of Docetaxel and Doxorubicin in Hormone-Refractory Prostate Cancer Cells. *Biochemistry Research International, 2012*, 1-10.

9- Smith, L. (2006). The analysis of doxorubicin resistance in human breast cancer cells using antibody microarrays. *Molecular Cancer Therapeutics, 5(8),* 2115-2120.

10- Szwed, M., Laroche-Clary, A., Robert, J., & Jozwiak, Z. (2014). Induction of apoptosis by doxorubicin–transferrin conjugate compared to free doxorubicin in the human leukemia cell lines. *Chemico-Biological Interactions, 220*, 140-148.

11- Mohan, P., & Rapoport, N. (2010). Doxorubicin as a Molecular Nanotheranostic Agent: Effect of Doxorubicin Encapsulation in Micelles or Nanoemulsions on the Ultrasound-Mediated Intracellular Delivery and Nuclear Trafficking. *Molecular Pharmaceutics, 7(6)*, 1959-1973.

12- Orzechowska, E., Girstun, A., Staron, K., & Trzcinska-Danielewicz, J. (2015). Synergy of BID with doxorubicin in the killing of cancer cells. *Oncology Reports*, 33(5), 2143-2150. doi:10.3892/or.2015.3841

13- Wang, S., Konorev, E. A., Kotamraju, S., Joseph, J., Kalivendi, S., & Kalyanaraman, B. (2004). Doxorubicin Induces Apoptosis in Normal and Tumor Cells via Distinctly Different Mechanisms: intermediacy of h2o2- and p53-dependent pathways. *Journal of Biological Chemistry, 279*(24), 25535-25543. doi:10.1074/jbc.m400944200

14- Kalivendi, S., Konorev, E., Cunningham, S., Vanamala, S., Kaji, E., Joseph, J., & Kalyanaraman, B. (2005). Doxorubicin activates nuclear factor of activated T-lymphocytes and Fas ligand transcription: Role of mitochondrial reactive oxygen species and calcium. *Biochemical Journal, 389*(2), 527-539. doi:10.1042/bj20050285

15- Shi, R., Huang, C., Aronstam, R. S., Ercal, N., Martin, A., & Huang, Y. (2009). N-acetylcysteine amide decreases oxidative stress but not cell death induced by doxorubicin in H9c2 cardiomyocytes. *BMC Pharmacology,9*(1), 7. doi:10.1186/1471-2210-9-7

16- Minotti, G., Licata, S., Saponiero, A., Menna, P., Calafiore, A. M., Giammarco, G. D., . . . Maggi, C. A. (2000). Anthracycline Metabolism and Toxicity in Human Myocardium: Comparisons between Doxorubicin, Epirubicin, and a Novel Disaccharide Analogue with a Reduced Level of Formation and [4Fe-4S] Reactivity of Its Secondary Alcohol Metabolite. *Chemical Research in Toxicology, 13*(12),1336-1341. doi:10.1021/tx000143z

17- Maejima, Y., Adachi, S., Ito, H., Hirao, K., & Isobe, M. (2008). Induction of premature senescence in cardiomyocytes by doxorubicin as a novel mechanism of myocardial damage. *Aging Cell, 7*(2), 125-136. doi:10.1111/j.1474-9726.2007.00358.x

18- Speelmans, G., Staffhorst, R. W., Kruijff, B. D., & Wolf, F. A. (1994). Transport Studies of Doxorubicin in Model Membranes Indicate a Difference in Passive Diffusion across and Binding at the Outer and Inner Leaflet of the Plasma Membrane. *Biochemistry, 33*(46), 13761-13768. doi:10.1021/bi00250a029

19- Kamba, S., Ismail, M., Hussein-Al-Ali, S., Ibrahim, T., & Zakaria, Z. (2013). In Vitro Delivery and Controlled Release of Doxorubicin for Targeting Osteosarcoma Bone Cancer. *Molecules, 18*(9), 10580-10598. doi:10.3390/molecules180910580

20- Bertrand, N., Wu, J., Xu, X., Kamaly, N., & Farokhzad, O. C. (2014). Cancer nanotechnology: The impact of passive and active targeting in the era of modern cancer biology. *Advanced Drug Delivery Reviews, 66*, 2-25. doi:10.1016/j.addr.2013.11.009

21- Zheng, L., Gou, M., Zhou, S., Yi, T., Zhong, Q., Li, Z., . . . Zhao, X. (2011). Antitumor activity of monomethoxy poly(ethylene glycol)-poly (epsilon-caprolactone) micelle-encapsulated doxorubicin against mouse melanoma. *Oncol Rep, 25*(6), 1557-1564. doi:10.3892/or.2011.1243

22- Kipp, J. (2004). The role of solid nanoparticle technology in the parenteral delivery of poorly water-soluble drugs. *International Journal of Pharmaceutics, 284*(1-2), 109-122. doi:10.1016/j.ijpharm.2004.07.019

23- Alexis, F., Pridgen, E., Molnar, L. K., & Farokhzad, O. C. (2008). Factors Affecting the Clearance and Biodistribution of Polymeric Nanoparticles. *Molecular Pharmaceutics, 5*(4), 505-515. doi:10.1021/mp800051m

24- Valetti, S., Mura, S., Stella, B., & Couvreur, P. (2013). Rational design for multifunctional non-liposomal lipid-based nanocarriers for cancer management: Theory to practice. *Journal of* Nanobiotechnology 1 Suppl 1, S6. doi:10.1186/1477-3155-11-s1-s6

25- Samad, A., Sultana, Y., & Aqil, M. (2007). Liposomal Drug Delivery Systems: An Update Review. *Current Drug Delivery, 4*(4), 297-305. doi:10.2174/156720107782151269

26- Fenske, D. B., Chonn, A., & Cullis, P. R. (2008). Liposomal Nanomedicines: An Emerging Field. *Toxicologic Pathology, 36*(1), 21-29. doi:10.1177/0192623307310960

27- Nie, Y., Ji, L., Ding, H., Xie, L., Li, L., He, B., . . . Gu, Z. (2012). Cholesterol Derivatives Based Charged Liposomes for Doxorubicin Delivery: Preparation, In Vitro and In Vivo Characterization. *Theranostics, 2*(11), 1092-1103. doi:10.7150/thno.4949

28- Yang, T., Cui, F., Choi, M., Cho, J., Chung, S., Shim, C., & Kim, D. (2007). Enhanced solubility and stability of PEGylated liposomal paclitaxel: In vitro and in vivo evaluation. *International Journal of Pharmaceutics, 338*(1-2), 317-326. doi:10.1016/j.ijpharm.2007.02.011

29- Klibanov, A. L., Maruyama, K., Torchilin, V. P., & Huang, L. (1990). Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Letters, 268*(1), 235-237. doi:10.1016/0014-5793(90)81016-h

30- Ait-Oudhia, S., Mager, D., & Straubinger, R. (2014). Application of Pharmacokinetic and Pharmacodynamic Analysis to the Development of Liposomal Formulations for Oncology. *Pharmaceutics, 6*(1), 137-174. doi:10.3390/pharmaceutics6010137

31- Torchilin, V. P. (2005). Recent advances with liposomes as pharmaceutical carriers. *Nature Reviews Drug Discovery, 4*(2), 145-160. doi:10.1038/nrd1632

32- Burenjargal, M., Lee, Y., Yoo, J., Kim, Y., Lee, Y., Oh, S., . . . Yoo, H. (2007). Endogenous sphingolipid metabolites related to the growth in Sphingomonas chungbukensis. *Archives of Pharmacal Research, 30*(3), 317-322. doi:10.1007/bf02977612

33- Kolesnick, R. N., & Kronke, M. (1998). Regulation of ceramide production and apoptosis. Annu Rev Physiol, 60, 643-665. doi:10.1146/annurev.physiol. 60.1.643

34- Zhou, Q., Zhao, J., Stout, J. G., Luhm, R. A., Wiedmer, T., & Sims, P. J. (1997). Molecular Cloning of Human Plasma Membrane Phospholipid Scramblase: a protein mediating transbilayer movement of plasma membrane phospholipids. *Journal of Biological Chemistry, 272*(29), 18240-18244. doi:10.1074/jbc.272.29.18240

35- Paulusma, C. C., & Elferink, R. P. (2006). Diseases of intramembranous lipid transport. *FEBS Letters, 580*(23), 5500-5509. doi:10.1016/j.febslet.2006.06.067

36- Barth, B. M., Cabot, M. C., & Kester, M. (2011). Ceramide-Based Therapeutics for the Treatment of Cancer. *Anti-Cancer Agents in Medicinal Chemistry, 11*(9), 911-919. doi:10.2174/187152011797655177

37- Zhou, H., Summers, S. A., Birnbaum, M. J., & Pittman, R. N. (1998). Inhibition of Akt Kinase by Cell-permeable Ceramide and Its Implications for Ceramide-induced Apoptosis. *Journal of Biological Chemistry, 273*(26), 16568-16575. doi:10.1074/jbc.273.26.16568

38- Smith, P., Krohn, R., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., . . . Klenk, D. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry, 150*(1), 76-85. doi:10.1016/0003-2697(85)90442-7

39- Johnston, M. J., Edwards, K., Karlsson, G., & Cullis, P. R. (2008). Influence of Drug-to-Lipid Ratio on Drug Release Properties and Liposome Integrity in Liposomal Doxorubicin Formulations. *Journal of Liposome Research, 18*(2), 145-157. doi:10.1080/08982100802129372

40- Haran, G., Cohen, R., Bar, L. K., & Barenholz, Y. (1993). Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases Biochim Biophys Acta, 1151(2), 201-215*, 1151*(2), 201-215. doi:10.1016/0005-2736(93)90105-9

41- Love, W. G., Amos, N., Kellaway, I. W., & Williams, B. D. (1990). Specific accumulation of cholesterol-rich liposomes in the inflammatory tissue of rats with adjuvant arthritis. *Annals of the Rheumatic Diseases, 49*(8), 611-614. doi:10.1136/ard.49.8.611

42- Zolnik, B. S., Stern, S. T., Kaiser, J. M., Heakal, Y., Clogston, J. D., Kester, M., & Mcneil, S. E. (2008). Rapid Distribution of Liposomal Short-Chain Ceramide in Vitro and in Vivo. *Drug Metabolism and Disposition, 36*(8), 1709-1715. doi:10.1124/dmd.107.019679

43- Ruttala, H. B., & Ko, Y. T. (2014). Liposome Encapsulated Albumin-Paclitaxel Nanoparticle for Enhanced Antitumor Efficacy. *Pharmaceutical Research, 32*(3), 1002-1016. doi:10.1007/s11095-014-1512-2

44- Lucci, A., Han, T. Y., Liu, Y. Y., Giuliano, A. E., & Cabot, M. C. (1999). Modification of ceramide metabolism increases cancer cell sensitivity to cytotoxics. *International Journal of* Oncology,15(3), 541-546. doi:10.3892/ijo.15.3.541

45- Lummel, M. V., Blitterswijk, W. J., Vink, S. R., Veldman, R. J., Valk, M. A., Schipper, D., . . . Koning, G. A. (2010). Enriching lipid nanovesicles with short-chain glucosylceramide improves doxorubicin delivery and efficacy in solid tumors. *The FASEB Journal, 25*(1), 280-289. doi:10.1096/fj.10-163709

46- Stover, T. (2003). Liposomal Delivery Enhances Short-Chain Ceramide-Induced Apoptosis of Breast Cancer Cells. *Journal of Pharmacology and Experimental Therapeutics, 307*(2), 468-475. doi:10.1124/jpet.103.054056

47- Koning, G. A., Gorter, A., Scherphof, G. L., & Kamps, J. A. (1999). Antiproliferative effect of immunoliposomes containing 5-fluorodeoxyuridine-dipalmitate on colon cancer cells. *British Journal of Cancer, 80*(11), 1718-1725. doi:10.1038/sj.bjc.6690588

48- Veldman, R. J. (2005). Coformulated N-Octanoyl-glucosylceramide Improves Cellular Delivery and Cytotoxicity of Liposomal Doxorubicin. *Journal of Pharmacology and Experimental Therapeutics, 315*(2), 704-710. doi:10.1124/jpet.105.087486

49- Veldman, R. J., Zerp, S., Blitterswijk, W. J., & Verheij, M. (2004). N-hexanoyl-sphingomyelin potentiates in vitro doxorubicin cytotoxicity by enhancing its cellular influx. *British Journal of Cancer, 90*(4), 917-925. doi:10.1038/sj.bjc.6601581

50- Prasad, S., Gupta, S. C., & Tyagi, A. K. (2016). Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. *Cancer Lett.* doi:10.1016/ j.canlet.2016.03.042

51- Wagner, B. A., Evig, C. B., Reszka, K. J., Buettner, G. R., & Burns, C. P. (2005). Doxorubicin increases intracellular hydrogen peroxide in PC3 prostate cancer cells. *Archives of Biochemistry and Biophysics, 440*(2), 181-190. doi:10.1016/j.abb.2005.06.015

52- Pahan, K., Dobashi, K., Ghosh, B., & Singh, I. (2002). Induction of the Manganese Superoxide Dismutase Gene by Sphingomyelinase and Ceramide. *Journal of Neurochemistry, 73*(2), 513-520. doi:10.1046/j.1471-4159.1999.0730513.x