THE ROLE OF CERAMIDE IN METASTATIC PROCESSES IN BREAST CANCER

A Dissertation in

Cell and Molecular Biology

by

Jeremy Kenneth Haakenson

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The dissertation of Jeremy Kenneth Haakenson was reviewed and approved* by the following:

Mark Kester
G. Thomas Passananti Professor of Pharmacology
Dissertation Co-advisor
Co-chair of Committee

Andrea Manni
Professor of Medicine
Dissertation Co-advisor
Co-chair of Committee

Gary A. Clawson
Professor of Pathology, and Biochemistry and Molecular Biology

Cheng Dong
Department Head and Distinguished Professor, Bioengineering

Rosalyn Irby
Associate Professor of Medicine

Sarah K. Bronson
Program Chair, Cell and Molecular Biology

*Signatures are on file in the Graduate School.
ABSTRACT

Ceramide is a bioactive sphingolipid that is capable of inducing apoptosis in mammalian cells. However, the role of ceramide in cancer metastasis remains largely unexplored. In this dissertation, I have demonstrated that ceramide induces anoikis, inhibits extravasation, and blocks the epithelial-mesenchymal transition (EMT) in metastasis-competent human breast cancer cell lines. Mechanistically, the effects of ceramide on anoikis and extravasation are mediated by lysosomal degradation of CD44, independent of palmitoylation or proteasome targeting. SiRNA down-regulation of CD44 mimics ceramide-induced anoikis and diminished extravasation of cancer cells. On the other hand, the ability of ceramide to prevent IL-6-induced EMT is dependent on its ability to inhibit STAT3 activation. Taken together, the data in this dissertation indicate that ceramide limits CD44-dependent breast cancer cell migration and IL-6/STAT3-dependent EMT, suggesting that ceramide analogs could be used to prevent and treat solid tumor metastasis.
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<td>2BP</td>
<td>2-bromopalmitate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C1P</td>
<td>ceramide-1-phosphate</td>
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<tr>
<td>CerS6</td>
<td>ceramide synthase 6</td>
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<tr>
<td>CNL</td>
<td>ceramide nanoliposome</td>
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<tr>
<td>CSC</td>
<td>cancer stem cell</td>
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<tr>
<td>CTC</td>
<td>circulating tumor cell</td>
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<tr>
<td>CXC</td>
<td>cysteine-X-cysteine</td>
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<tr>
<td>DISC</td>
<td>death-inducing signalling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DSPC</td>
<td>1,2-distearyl-sn-glycero-3-phosphocholine</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<td>EpCAM</td>
<td>epithelial cell adhesion molecule</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor 1 alpha</td>
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<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IL-6</td>
<td>interleukin 6</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>IL-8</td>
<td>interleukin 8</td>
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<tr>
<td>KSR</td>
<td>kinase suppressor of Ras</td>
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<tr>
<td>MDCK</td>
<td>Madin Darby canine kidney</td>
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<td>MET</td>
<td>mesenchymal-epithelial transition</td>
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<tr>
<td>NK-LGL</td>
<td>natural killer-large granular lymphocytic</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
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<tr>
<td>Q</td>
<td>volumetric flow rate</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>Scr</td>
<td>scrambled</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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siCD44  CD44 siRNA

SIK1  salt-inducible kinase 1

siRNA  small interfering RNA

TGFβ  transforming growth factor beta
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DEDICATION

In memory of Michelle Haakenson (1956-2013)
Chapter 1

INTRODUCTION

1.A. Carcinomatous Metastasis

In 1889, Paget articulated Fuchs’ predisposition hypothesis in English, providing his own analysis of 735 breast cancer patient necropsies, which led him to conclude that the site of metastasis was not random, but that certain cancers had a predisposition for certain secondary sites, a phenomenon which he likened to a seed requiring a congenial soil in which to grow (Paget 1889). Today, we know that metastasis of a carcinoma occurs as a cascade of events that results in the spread of a tumor to secondary sites in the body. The first step of this metastatic cascade takes place when cells detach from the tumor. Metastatic cells then secrete proteases to degrade the extracellular matrix (ECM) and migrate towards the circulatory or lymphatic systems, which tend to be leaky near tumors. Metastatic cells then migrate between the endothelial cells that make up blood vessels in order to enter the bloodstream. In order to survive in the bloodstream, metastatic carcinoma cells must become resistant to anoikis, which is defined as programmed cell death caused by the detachment of epithelial cells from the epithelial sheet. Even if tumor cells are able to overcome anoikis and survive in the bloodstream, they still have to exit the bloodstream, or extravasate, before they can form a secondary tumor. The process of extravasation takes place under shear forces and begins when cancer cells come to a stop in the bloodstream, either by binding to endothelial cells or
reaching a capillary that is too narrow to allow further travel. At this point, metastatic cells may then secrete proteases to degrade cell-cell junctions between the endothelial cells lining the blood vessels, and then migrate out of the bloodstream. Finally, tumor cells must survive in a foreign microenvironment and proliferate in order to form a secondary tumor.

1.A.i. Resistance to Anoikis

Anoikis is a term used to describe cell death that occurs when cells detach from the ECM (Frisch and Francis 1994). When integrins detach from the ECM, three cell death pathways are triggered: the intrinsic apoptotic pathway; the extrinsic apoptotic pathway; and a third, caspase-independent cell death pathway.

The intrinsic apoptotic pathway is triggered by mitochondrial damage, causing cytochrome c to be released from the outer mitochondrial membrane (Simpson, Anyiwe et al. 2008), which is a process mediated by Bax and Bak (Tan, Goldstein et al. 2013). This activates caspase 9, which in turn activates caspase 3 and caspase 7, leading to intracellular protein degradation and cell death (Simpson, Anyiwe et al. 2008). XIAP inhibits the intrinsic apoptotic pathway by binding and inhibiting caspases 3, 7, and 9 (Simpson, Anyiwe et al. 2008). Similarly, survivin prevents apoptosis by inhibiting caspases (Berezovskaya, Schimmer et al. 2005). Mcl-1 and Bcl-XL, on the other hand, inhibit the intrinsic pathway by inhibiting Bax and Bak (Tan, Goldstein et al. 2013).
The extrinsic apoptotic pathway is initiated when FAS or TRAIL bind to their death receptors on the cell surface (Simpson, Anyiwe et al. 2008), causing the formation of the death-inducing signaling complex (DISC) (Tan, Goldstein et al. 2013). The DISC then activates caspase 8 (Tan, Goldstein et al. 2013), which activates caspases 3 and 7, leading to cell death (Simpson, Anyiwe et al. 2008). In addition, death receptors activate Bmf, which inhibits the anti-apoptotic Bcl2 protein, which normally blocks cytochrome c release from the mitochondria. In this way, death receptors activate the intrinsic pathway as well. The extrinsic pathway can be inhibited by FLIP (which prevents caspase 8 activation) and XIAP (as in the intrinsic pathway) (Simpson, Anyiwe et al. 2008).

In addition to the intrinsic and extrinsic apoptotic pathways, there is another cell death mechanism that is activated in anoikis. In this pathway, integrin detachment from the ECM causes Bit1 to be released from the mitochondria, leading to caspase-independent cell death (Jenning, Pham et al. 2013).

In addition to the intrinsic, extrinsic, and caspase-independent pathways of anoikis, a number of other signaling pathways have been implicated. For instance, salt-inducible kinase 1 (SIK1) and the tumor suppressor p53 act together to cause anoikis (Cheng, Liu et al. 2009). In addition, upregulation of PTEN inhibits the activation of focal adhesion kinase (FAK), causing anoikis (Kim, Koo et al. 2012). Furthermore, the androgen receptor (AR) has been shown to induce anoikis in hepatocellular carcinoma (HCC) cells (Ma, Hsu et al. 2012). Finally, the production of reactive oxygen species
(ROS) can induce anoikis (Kamarajugadda, Cai et al. 2013). How SIK1, p53, PTEN, AR, and ROS affect the intrinsic, extrinsic, and caspase-independent pathways of anoikis remains to be elucidated.

Of course, the problem in oncology is not anoikis, but resistance to anoikis. Unfortunately, a number of circulating growth factors and chemokines can cause anoikis resistance in carcinoma cells. For example, when brain-derived neurotrophic factor (BDNF) binds its receptor, Trk-B, anti-apoptotic Akt and Zeb1 are activated, leading to anoikis resistance (Rennebeck, Martelli et al. 2005, Smit and Peeper 2011). Similarly, when insulin-like growth factor 1 (IGF-1) binds its receptor, IGF-1R, Akt and anti-apoptotic survivin are activated, inhibiting anoikis (Rennebeck, Martelli et al. 2005). Anoikis can also be blocked when the chemokines CXCR4 and CCR7 bind to their receptors (CXCL12 and CCL21, respectively), which upregulate anti-apoptotic Bcl-XL (Kochetkova, Kumar et al. 2009). Furthermore, inducers of the epithelial-mesenchymal transition (EMT), such as transforming growth factor beta (TGFβ) and fibroblast growth factor (FGF), and other growth factors, such as hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) cause resistance to anoikis (Kim, Koo et al. 2012). HGF promotes anoikis resistance in breast cancer cells by binding to its receptor, met, which then activates pro-metastatic Src and hypoxia inducible factor 1 alpha (HIF-1α) (Maroni, Bendinelli et al. 2014).

Besides growth factors and chemokines, multiple cell surface molecules are responsible for anoikis resistance. One of these is HER2, which increases levels of α5
integrin and activates Src, leading to the inhibition of apoptotic Bim and anoikis resistance (Kim, Koo et al. 2012). In HCC, cells that are resistant to anoikis express high levels of CD147, which activates Akt (Ke, Li et al. 2012).

In addition to growth factors, chemokines, and cell surface molecules, several transcription factors have been implicated in anoikis resistance. Interestingly, transcription factors that cause EMT also induce anoikis resistance. These include Twist (Rennebeck, Martelli et al. 2005), Zeb1 (Smit and Peeper 2011), and Snail (Kim, Koo et al. 2012). Furthermore, NFκB has been shown to contribute to anoikis resistance (Kochetkova, Kumar et al. 2009).

Besides the genes mentioned above, a number of cytosolic proteins are implicated in anoikis resistance. For instance, FAK has been shown to mediate anoikis resistance by inhibiting caspase activity in pancreatic cancer cells (Duxbury, Ito et al. 2004). In addition, CTTN, an actin-associated scaffolding protein, causes anoikis resistance in esophageal squamous cell carcinoma by activating Akt (Luo, Shen et al. 2006). Furthermore, Talin1, a focal adhesion protein, causes resistance to anoikis by activating FAK, Akt, and Src (Sakamoto, McCann et al. 2010). In addition, 14-3-3ζ inhibits apoptotic Bad and Bim, thus inhibiting the intrinsic apoptotic pathway (Tan, Goldstein et al. 2013). Moreover, there are several cytosolic proteins whose mechanism of causing anoikis resistance is unknown. These include PKCζ (Liu, Wang et al. 2011), eEF2 kinase (Zhang, Zhang et al. 2011), and FER kinase (Ivanova, Vermeulen et al. 2013).
1.A.ii. **Cell Migration**

This thesis focuses on three aspects of the metastatic cascade: cell migration, resistance to anoikis, and EMT. Cell migration through the ECM is driven by actin polymerization at the leading edge of the cell, causing a protrusion (i.e. filopodia, lamellipodia, or pseudopodia), and actin depolymerization behind the leading edge. This is followed by attachment of the leading edge to the ECM by integrins to form focal adhesions, which are regulated by FAK. Once focal adhesions have been established, myosin II in the rear of the cell contracts, pulling the rear of the cell forward. Then the cycle repeats, with a new protrusion being extended forward and new focal adhesions being made (Alberts, Johnson et al. 2002).

Intracellularly, the Rho GTPases (Rho, Rac, and Cdc42) mediate cell migration by regulating the actin cytoskeleton and cell polarity (Raftopoulou and Hall 2004). On the other hand, cell migration can be inhibited by RKIP1, which binds and inhibits Raf kinase (Millarte and Farhan 2012). Extracellularly, there are a number of factors that can induce cell migration, including laminins (Pouliot and Kusuma 2013), growth factors (e.g. PDGF, EGF) (Sieg, Hauck et al. 2000), chemokine gradients (Balkwill 2004), and lipids (i.e. sphingosine-1-phosphate) (Sadahira, Ruan et al. 1992).
1.8. **Ceramide**

Ceramide is a membrane lipid that is enriched in lipid rafts and acts as a second messenger. Its structure consists of an acyl chain of variable length attached to a sphingosine backbone (Fig. 1.1).

Ceramide can be produced via the *de novo* pathway when serine and palmitoyl CoA are combined by serine palmitoyl transferase to form 3-keto-dihydrosphingosine, which is converted to dihydrosphingosine by 3-keto-dihydrosphingosine reductase (Fig. 1.2). Dihydrosphingosine can then be acylated by ceramide synthase to form dihydroceramide, which is desaturated by dihydroceramide desaturase to form ceramide. In addition to the *de novo* pathway, there are several ways to generate ceramide: sphingomyelin can be converted to ceramide by sphingomyelinase; glucosylceramide can be converted to ceramide by glucosylceramidase; galactosylceramide can be converted to ceramide by galactosylceramidase; ceramide-1-phosphate (C1P) can be converted to ceramide by C1P phosphatase; and sphingosine can be converted to ceramide by ceramide synthase (Bartke and Hannun 2009).

Upon formation, ceramide can be metabolized into a number of other lipids. The addition of a phosphocholine group by sphingomyelin synthase yields sphingomyelin; the addition of a glucose by glucosylceramide synthase yields glucosylceramide, which can have more sugar groups added to produce more complex
Fig. 1.1  Structure of C6 Ceramide

Ceramide is composed of a sphingosine backbone (blue) with an amide-linked acyl chain (red), which varies in length from 14 to 26 carbons \textit{in vivo}. C6 ceramide, with an acyl chain length of 6 carbons, is shown here, as this is the short chain ceramide that was used in the ceramide nanoliposome (CNL). Absence of the double bond between carbons 4 and 5 yields an inactive form known as dihydroceramide.
Fig. 1.1  Structure of C6 Ceramide
Fig. 1.2  Sphingolipid Metabolism

SPT, serine palmitoyl transferase; KDS, 3-keto-dihydrosphingosine reductase; DES, dihydroceramide desaturase; SPPase, sphingosine phosphate phosphatase; CK, ceramide kinase; C1PP, C1P phosphatase; SMS, sphingomyelin synthase; PC, phosphatidylcholine; DAG, diacylglycerol; GCS glycosylceramide synthase; GCase, glucosyl ceramidase. (Bartke and Hannun 2009)
Fig. 1.2  Sphingolipid Metabolism
glycosphingolipids; the addition of galactose by galactosylceramide synthase yields galactosylceramide; the addition of a phosphate by ceramide kinase yields C1P; and the removal of the amide-linked acyl chain by ceramidase yields sphingosine, which can be phosphorylated to produce sphingosine-1-phosphate (S1P) (Bartke and Hannun 2009).

If ceramide is not metabolized into other lipids, it can directly bind and activate several enzymes, including cathepsin D, protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), kinase suppressor of Ras (KSR), cRAF, and protein kinase c zeta (PKCζ), all of which lead to apoptosis downstream (Pettus, Chalfant et al. 2002).

1.B.i. **Ceramide Nanoliposomes**

Given the ability of ceramide to induce apoptosis, it would appear to be a promising cancer therapeutic. However, long chain ceramides, such as those found endogenously, are not cell-permeable. In addition, ceramide has low solubility in aqueous solutions and is subject to degradation in the bloodstream (Stover and Kester 2003). To circumvent these issues, I used short-chain ceramide liposomes in my dissertation work. These liposomes have been shown to enhance the delivery of C6 ceramide to breast cancer cells (Stover and Kester 2003). The ceramide in these liposomes has an amide-linked acyl chain of six carbons (C6 ceramide). Thus, the ceramide nanoliposome (CNL) that I used consisted of a spherical lipid bilayer composed of phosphatidylcholine, phosphatidylethanolamine, and C6 ceramide, with polyethylene
glycol (PEG) coating the outer surface to prevent degradation (Stover, Sharma et al. 2005).

The metabolism of CNL indicates that it could be a useful drug in the clinic. It has a half-life ($t_{1/2}$) of 11 hours in balb/c mice (Stover, 2005) and 14 hours in rats (Zolnik, Stern et al. 2008). From two minutes to two hours after dosing, the majority of CNL is found in plasma. 48 hours after dosing, it accumulates in the liver and spleen. Interestingly, the C6 ceramide component of CNL is taken up by cells via a flippase mechanism, in which ceramide is transferred from the liposome to the plasma membrane of the cell while the liposome remains intact (Zolnik, Stern et al. 2008). Once C6 ceramide is taken up by a cell, it is typically converted to C6 sphingomyelin by sphingomyelin synthase, C6 glucosylceramide by glucosylceramide synthase, or long chain ceramide via de-acylation by ceramidase, followed by re-acylation by ceramide synthase (Chapman, Gouaze-Andersson et al. 2010).

A number of studies examining the ability of CNL to inhibit various types of cancer have been completed. CNL has been shown to induce apoptosis in breast cancer cells (Stover and Kester 2003, Stover, Sharma et al. 2005), melanoma cells (Tran, Smith et al. 2008), natural killer-large granular lymphocytic (NK-LGL) leukemia cells (Liu, Ryland et al. 2010), HCC cells (Tagaram, Divittore et al. 2011), pancreatic cancer cells (Jiang, DiVittore et al. 2011), and colorectal cancer cells (Adiseshaiah, Clogston et al. 2013). CNL does not induce apoptosis in normal mammary epithelial cells (Stover, Sharma et al. 2005) or peripheral blood mononuclear cells (PBMC’s) (Liu, Ryland et al. 2010).
addition, CNL has been shown to inhibit tumor growth in in vivo models of cancer when given as either a monotherapy or duotherapy. As a monotherapy, CNL inhibited tumor growth in animal models of breast cancer (Stover, Sharma et al. 2005) and HCC (Tagaram, Divittore et al. 2011) and increased survival in an in vivo model of NK-LGL leukemia (Liu, Ryland et al. 2010). As a duotherapy, it decreased tumor growth in an animal model of melanoma when given in combination with sorafenib (Tran, Smith et al. 2008), and it inhibited tumor growth in an in vivo model of pancreatic cancer when given in combination with gemcitabine (Jiang, DiVittore et al. 2011). Several molecular mechanisms help to explain the ability of CNL to target cancer cells and inhibit tumor growth, including inhibition of IL-8, p38 (Sun, Fox et al. 2008), Akt (Tran, Smith et al. 2008, Tagaram, Divittore et al. 2011), ERK, survivin (Liu, Ryland et al. 2010), and the neurotensin receptor, which mediates breast cancer cell migration (Heakal and Kester 2009).

1.C. CD44

CD44 is a transmembrane adhesion molecule that is a major player in metastasis. It consists of three domains: extracellular, transmembrane, and intracellular. CD44 has 10 standard exons and 10 variant exons, the latter of which can be inserted into the standard form of CD44 (CD44s) to create variant isoforms of CD44 (CD44v's). All isoforms of CD44 can undergo extensive post-translational modification, including
glycosylation (Zoller 1995), palmitoylation (Lesley, Hyman et al. 1997), and ubiquitination (Bartee, Eyster et al. 2010). In this dissertation, I deal solely with CD44s.

TGFβ has been shown to upregulate CD44 (Jothy 2003), which is found at high levels in breast cancer (Zoller 1995), melanoma, and pancreatic cancer (Lesley, Hyman et al. 1997). Extracellularly, CD44 can bind other cell surface molecules, such as E-selectin (Dimitroff, Lee et al. 2001), as well as components of the ECM, most notably hyaluronic acid (Zoller 1995), which can act as the “soil” for circulating tumor cells (CTC’s) (Martin, Harrison et al. 2003). Intracellularly, CD44 binds ezrin, a cytoskeletal protein, leading to cell migration, interactions between tumor cells and the endothelium, tumor progression, and metastasis (Martin, Harrison et al. 2003).

1.D. **IL-8**

Interleukin 8 (IL-8), also known as CXCL8, is a pro-inflammatory cysteine-X-cysteine (CXC) cytokine that attracts neutrophils to sites of inflammation. It is translated as a 99 amino acid precursor, which is cleaved to a 77 or 72 amino acid protein capable of binding to the CXCR1 and CXCR2 receptors (Mukaida 2003).

IL-8 has been shown to play a role in the metastasis of carcinomas. Metastasis-competent melanoma cell lines secrete more IL-8 than non-metastatic cell lines. In addition, knockdown of IL-8 using siRNA inhibited lung metastasis in an *in vivo* model of melanoma (Huh, Liang et al. 2010). In melanoma, IL-8 mediates metastasis by inducing
angiogenesis (Singh and Varney 2000) and recruiting neutrophils to sites of extravasation, where they increase the binding affinity of circulating melanoma cells for endothelial cells (Huh, Liang et al. 2010). In nasopharyngeal carcinoma, IL-8 decreases distant metastasis-free survival by inducing metastasis, migration, invasion, EMT, and Akt activation (Li, Peng et al. 2012).

1.E. **Epithelial-mesenchymal Transition (EMT)**

EMT is a process that plays an important role in embryonic development (Kang and Massague 2004), wound healing, fibrosis (Roberts, Tian et al. 2006), and cancer metastasis (Kang and Massague 2004). It is characterized by a decrease in E-cadherin levels, loss of cell polarity, loss of intercellular adhesion, a morphological change from cuboid to spindle-shaped cells, and an increase in cell migration (Kang and Massague 2004). Cells that have undergone all of these modifications have transitioned from an epithelial to a mesenchymal phenotype.

A number of growth factors and cytokines can induce EMT, including interleukin 6 (IL-6) (Wendt, Balanis et al. 2014), TGFβ (Tse and Kalluri 2007), and HGF (Berx, Raspe et al. 2007).

IL-6 induces EMT by binding to its receptor, IL-6R, which causes phosphorylation and activation of JAK2, which leads to phosphorylation and activation of STAT3, which upregulates several master regulators of EMT, including Snail, Slug (Snail2), Twist
(Wendt, Balanis et al. 2014), Zeb1 (Korpal and Kang 2008), and Zeb2 (Kang and Massague 2004). These transcription factors all act to inhibit the transcription of E-cadherin. In addition to inhibiting E-cadherin via EMT master regulators, IL-6 has also been shown to increase the mesenchymal marker vimentin and to decrease cell-cell adhesion in head and neck tumor cells (Yadav, Kumar et al. 2011). Furthermore, it causes T47D human breast cancer cells to convert from a cuboid to a spindle shape (Xie, Yao et al. 2012).

Rather than activating STAT3, TGFβ acts through a separate pathway to induce EMT. When TGFβ binds to its receptors, Smad2 and Smad3 are phosphorylated, giving rise to a complex with Smad4, which is then translocated to the nucleus, where it increases the production of Snail and Zeb2, thereby down-regulating E-cadherin and causing EMT (Kang and Massague 2004, Roberts, Tian et al. 2006). In addition, activated Smad3 increases the production of TGFβ, leading to an autocrine positive feedback cycle (Tse and Kalluri 2007).

There are a number of markers that can be used to identify epithelial cells (e.g. cells that have not undergone EMT). These include E-cadherin, α-catenin, γ-catenin (Kang and Massague 2004), cytokeratin 8, cytokeratin 19 (Christiansen and Rajasekaran 2006), and epithelial cell adhesion molecule (EpCAM) (Yu, Bardia et al. 2013). Similarly, there are several markers that are used to identify mesenchymal cells (e.g. epithelial cells that have undergone EMT), which include vimentin, fibronectin, smooth muscle
actin, N-cadherin (Kang and Massague 2004), β-filamin, and talin (Christiansen and Rajasekaran 2006).

Because metastatic tumors often display epithelial characteristics (Christiansen and Rajasekaran 2006), a number of theories have been proposed to explain how EMT relates to metastasis in vivo. The first of these theories states that incomplete EMT is sufficient for metastasis to take place (Christiansen and Rajasekaran 2006). In this scenario, cells may lose E-cadherin and become more motile while still maintaining some epithelial characteristics, such as a cuboidal morphology (Christiansen and Rajasekaran 2006). A second theory is that once cells undergo EMT and reach the site of the secondary tumor, they undergo a mesenchymal-epithelial transition (MET), reverting back to an epithelial state (Christiansen and Rajasekaran 2006). A third theory hypothesizes that metastatic cancer cells do not undergo EMT at all, but instead migrate collectively, retaining epithelial characteristics the entire time (Chui 2013). A fourth model of metastasis has been termed “cell cooperativity” (Tsuji, Ibaragi et al. 2009). In this model, cells that have undergone EMT degrade the surrounding ECM, clearing a path for non-EMT cancer cells to enter the circulation. It is then these epithelial cells that colonize the foreign microenvironment to produce a secondary tumor (Tsuji, Ibaragi et al. 2009). It is possible that all four of these models can take place depending on the type of cancer and the microenvironment. Thus, EMT is sufficient but not necessary for metastasis to occur.
1.E.i. Sphingolipids and EMT

There is some evidence that sphingolipids are able to regulate EMT. Bieberich’s group showed that inhibiting the activity of PKCζ led to decreased E-cadherin levels in Madin Darby Canine Kidney (MDCK) cells and that exogenous administration of ceramide was able to rescue this effect, suggesting that ceramide may be capable of inhibiting EMT (Wang, Krishnamurthy et al. 2009). Similarly, inhibition of glucosylceramide synthase, the enzyme that converts ceramide into glucosylceramide, has been shown to decrease E-cadherin levels, increase vimentin levels, and increase cell migration in epithelial cells (Guan, Handa et al. 2009). In addition, the glycosphingolipid Gg4 was able to inhibit the effects of TGFβ on cell migration, E-cadherin, vimentin, and fibronectin (Guan, Handa et al. 2009). These data indicate that specific sphingolipids, such as ceramide and Gg4, are able to prevent EMT and may act as critical EMT checkpoints.
Chapter 2

MATERIALS AND METHODS

2.A. **Cell Lines and Treatments**

MDA-MB-231 human breast cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Cellgro) containing 10% FBS (Atlanta Biologicals), 1% GlutaMAX (Gibco), and 1% antibiotic-antimycotic (Invitrogen) at 37°C and 5% CO₂. Panc-1 human pancreatic cancer cells, B16 murine melanoma cells, and T47D human breast cancer cells were cultured in RPMI 1640 (Cellgro) containing 10% FBS (Atlanta Biologicals) and 1% antibiotic-antimycotic (Invitrogen) at 37°C and 5% CO₂. MDA-MB-468 cells were cultured in DMEM/F12 (Cellgro) containing 10% FBS and 1% antibiotic-antimycotic at 37°C and 5% CO₂. MDA-MB-468 human breast cancer cells were grown in DMEM/F12 containing 10% FBS and 1% antibiotic-antimycotic at 37°C and 5% CO₂. MDA-MB-231 (human breast adenocarcinoma) cells were obtained from American Type Culture Collection. EL cells are fibroblasts that overexpress ICAM-1 and E-selectin. They were provided by Dr. Scott Simon (UC Davis, Davis, CA). Ceramide and all other lipids were purchased from Avanti Polar Lipids. The phosphate-buffered saline (PBS) was from Cellgro, and the z-vad-fmk caspase inhibitor and pepstatin A were from Enzo Life Sciences. Scrambled siRNA and validated CD44 siRNA were from Life Technologies. 2-bromopalmitate, chloroquine, ammonium chloride, and trypan blue were purchased.
from Sigma-Aldrich. The cycloheximide was purchased from Fisher, and recombinant human IL-6 was from R&D Systems.

2.B. **Liposome Formulation and Extrusion**

Lipids, dissolved in chloroform (CHCl$_3$), were combined according to the following molar ratios: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE):1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC):C8 mPEG 750 ceramide:1,2distearyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethelene glycol)-2000] (ammonium salt):C6 ceramide (1.75:3.75:0.75:0.75:3). Ceramide-free liposomes (ghost) containing the same ratio of lipid components were used as a negative lipid control. Lipid mixtures were dried under a stream of nitrogen gas, hydrated with PBS, and then heated above lipid transition temperatures. The resulting solution underwent sonication for 1 min followed by 11 extrusions through a 100 nm polycarbonate membrane.

2.C. **MTS Cell Viability Assay**

Cytotoxicity of nanoliposomal ceramide in breast cancer cells was measured by plating 5,000 cells (MDA-MB-468 and T47D cell lines) or 10,000 cells (MDA-MB-231 cell line) into 96-well plates followed by growth for 24 hours in a humidified 37°C cell culture incubator. Next, cells were treated with PBS or CNL for 24 hours in 10% serum medium (CNL: 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 μM). After 24 hours, cytotoxicity was measured using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega).
2.D. **Anoikis Assay**

10,000 cells per well were plated into either cell culture-treated or ultra low adherent 96 well plates (Corning) and incubated at 37°C and 5% CO$_2$ overnight. Cells were then treated with ghost liposomes or CNL for 24 hours, after which time they were subjected to the Apo-ONE Caspase 3/7 Assay (Promega) per the manufacturer’s protocol in order to measure apoptosis.

2.E. **Transfection of siRNA**

100 nM siRNA was transfected into MDA-MB-231 cells using Nucleofection (Lonza) following the manufacturer’s cell-line specific protocol. Forty-eight hours after transfection, CD44 protein levels were measured using Western blot analysis, and transwell migration and anoikis assays were performed.

2.F. **Mammosphere Assay**

5,000 cells per well were grown in 6 well ultra low adherent plates (Corning) in 2 mL growth medium. Mammospheres were counted using a Nikon Eclipse phase-contrast microscope at 4X magnification. Trypan blue was used to determine cell viability. Living cells exclude Trypan blue and remain clear, whereas dead cells incorporate the Trypan blue dye and turn blue.
2.G. **Transwell Cell Migration Assay**

The transwell cell migration assay was performed using 24-well transwell inserts (Greiner Bio-one). Cells were serum starved overnight before the experiment. 200,000 MDA-MB-231 cells in 200µL of serum-free medium containing 0.002% bovine serum albumin (BSA) were added to the upper well of the transwell chamber. 600µL of medium with or without chemoattractant (10% FBS) was added to the lower chamber. Cells were allowed to settle for half an hour before being treated. After incubation for 24 h at 37°C, all cells were stained with calcein AM (BD Biosciences). Cells in the lower chamber (i.e. those that had migrated) were then detached from the transwell membrane using trypsin. Following detachment, these cells were transferred to a black 96-well plate, and fluorescence was measured with an excitation wavelength of 485nm and an emission wavelength of 520nm using a fluorescent plate reader. Increased fluorescence correlates with an increased number of cells.

2.H. **Flow Chamber Extravasation Assay**

The *in vitro* micro-fluidic device used to simulate cellular extravasation under dynamic conditions consisted of a modified chemotactic Boyden chamber. The bottom piece of the chamber was a 48-well acrylic plate (Neuro Probe, Gaithersburg, MD) and the top piece was an acrylic plate with an inlet and an outlet for flow media. The two plates were separated by a 0.02 inch thick silicone gasket (PharmElast, Trelleborg, Hudson, MA). A 7 cm x 2 cm opening was cut in the center of the gasket to form the flow field. A monolayer of EI cells (fibroblast L-cells that had been transfected to express
human ICAM-1 and E-selectin) was grown to confluence on one side of a sterilized
polyvinylpyrrolidone-free polycarbonate filter (8 μm pore size; Neuro Probe,
Gaithersburg, MD) that was coated with fibronectin (30 μg/ml, 3 h) (BD Discovery
Labware, Bedford, MA). The other side of the filter was scraped before placing it in the
chamber to remove El cells that may have grown on the bottom side of the filter. The
center 12 wells of the bottom plate were filled with soluble type IV collagen (100 μg/ml
in DMEM/0.1 % BSA) (BD Discovery Labware, Bedford, MA). The rest of the wells were
filled with medium (DMEM/0.1 % BSA). The cells being studied (untreated or cells
treated with ghost, CNL, siScr, or siCD44) (500,000 cells) were mixed in the flow medium
(DMEM/0.1 % BSA), and the chamber was placed in an incubator (37°C, 4 h). The media
was circulated through the chamber at the desired shear rate (50 or 100 s⁻¹). The
volumetric flow rate (Q) was related to the wall shear stress \( \tau_w = 6\mu Q/w^2 \), where \( \mu \) is
the fluid viscosity, \( h \) is height, and \( w \) is width of the flow field. The filter was removed
from the chamber and stained with HEMA-3 (Fisher Scientific, Pittsburgh, PA). The filter
was then attached to a microscope slide and the top side was scraped to remove the El
cell monolayer. The migrated cells were then quantified in 5 different locations and
averaged for each slide. At least 3 slides were analyzed for each case.

2.I. Western Blots

Whole-cell lysates were isolated using NP-40 lysis buffer (50 mM HEPES, 137 mM
NaCl, 2 mM Na₃VO₄, 10 mM sodium pyrophosphate, 1% NP-40, 10% glycerol, 50 mM
NaF, 1 mM EGTA, 2 mM EDTA, 2 mM β-glycerophosphate, and protease inhibitors).
Protein quantification was completed using the DC Protein Assay (Bio-Rad). Protein samples were prepared by heating at 70°C for 10 minutes after the addition of denaturing sample buffer. Proteins were separated using SDS-PAGE on a 4-12% gel (Life Technologies) and transferred to a nitrocellulose membrane (General Electric). Antibodies were diluted in 5% BSA in TBS-T. After 1 hour of blocking in 5% BSA, membranes were incubated with the primary antibody, washed in TBS-T, incubated with the horseradish peroxidase-conjugated secondary antibody, and then washed again. Protein bands were visualized using a commercially available chemiluminescence kit (Thermo Scientific).

The following antibodies were used: CD44 (Cell Signaling 3570), transferrin receptor (Life Technologies 13-6800), caveolin-1 (Santa Cruz sc-7875), EGFR (Cell Signaling 4267), E-cadherin (BD 610181), pSTAT3 (Cell Signaling 9145), STAT3 (Cell Signaling 9139), β-actin (Sigma A5441), goat anti-mouse (Santa Cruz sc-2005), and goat anti-rabbit (Santa Cruz sc-2004).

2.J. qRT-PCR

Real-time PCR was performed using TaqMan Gene Expression Assay (Life Technologies) on an ABI 7900HT qPCR instrument. The PCR reaction consisted of .5 μL 20X TaqMan Gene Expression Assay, 5 μL 2X TaqMan Gene Expression Master Mix, and 5 μL of cDNA template (100 ng) in a total volume of 10.5 μL. The assay included a negative control that lacked any reverse transcriptase, as well as each of the test cDNA’s.
2.K. **Immunofluorescence**

Cells were fixed in 4% paraformaldehyde and then washed in PBS, permeabilized in 0.1% Triton X-100, and incubated in .5% SDS for antigen retrieval. The mouse monoclonal CD44 antibody (Cell Signaling 3570, 1:400) was then applied overnight at 4°C, followed by an AlexaFluor 546 goat-anti-mouse secondary antibody (Molecular Probes A-11030, 1:500) for two hours at room temperature. Nuclei were stained with Hoechst 34580 and slides were permanently mounted. For negative controls, the primary antibody was omitted. Immunostained cells were then imaged using a confocal microscope (TCS SP8, Leica) with a 63x objective. Images were processed and quantified using Imaris 3D-4D Image Analysis software (Bitplane). At least three cells were imaged per treatment.

2.L. **Overexpression of CD44**

The retroviral backbones encoding GFP (control) or CD44s and a blasticidin selection marker (obtained from Addgene) were transfected with Lipofectamine 3000 into HEK293 cells growing on 100 mm dishes together with packaging plasmids, GAG-Pol and VSV-G. Forty-eight hours later, culture media was harvested and passed through a 0.45 micron filter to remove cell debris. Viral stocks were used undiluted right away or stored at -80°C. T47D cells were plated on 60 mm plates at a density of 10,000/cm². The next day, retroviral stocks were thawed and polybrene (Sigma) was added to them at a final concentration of 8 µg/mL. Growth media was removed from the T47D plates and replaced with approximately 1.75 mL of retroviral stock with
polybrene per plate. Plates were sealed with Parafilm, placed in a tabletop centrifuge equipped with a backed rotor, and centrifuged at 25°C for 45 minutes at 1220 g. After centrifugation, 5 mL of fresh growth media was added to the cells. Media was changed 3 h thereafter or the next morning. Three to four days after infection, cells were transferred to growth media containing 10 µg/mL blasticidin for 7 days. Based on our previous experience, all non-infected T47D cells died at this time; however, with virus-infected cells, we saw numerous surviving colonies. These colonies were pooled and the resulting cells were kept in 5 µg/mL blasticidine for an additional week.

2.M. **Statistical Analysis**

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis between two samples was performed using the student’s t-test. Comparisons of more than two groups were performed using one-way or two-way analysis of variance (ANOVA) with Tukey’s correction. A p-value of p<0.05 was considered to be statistically significant, and the experiments were repeated three times. To analyze the interaction between CNL and siCD44 on cell migration, a two-way analysis of variance (two-way ANOVA) model was set up. This part of the analysis was performed using statistical software SAS version 9.3 (SAS Institute, Cary, NC, USA). The significance level used was 0.05. For the anoikis experiments, a two-way analysis of variance (ANOVA) model was used to compare the mean activity levels among the groups (adherent/non-adherent) and treatments (no treatment, ghost, and CNL). Their interaction was
examined by an interaction plot. Pair-wise comparisons were performed to examine the interaction between treatment and group using Tukey’s test.
Chapter 3

INDUCTION OF ANOIKIS BY CERAMIDE

Anoikis, or anchorage-independent cell death, is triggered when adherent cells detach from the ECM. Resistance to anoikis is a necessary part of cancer metastasis. Given the ability of ceramide to induce apoptosis in a wide variety of cancer cell types and its ability to induce anoikis in HEK293 (Widau, Jin et al. 2010) and HeLa cells (Hu, Xu et al. 2005), I hypothesized that ceramide nanoliposomes (CNL) would be able to induce anoikis in metastatic carcinoma cells.

Initially, an MTS assay revealed a cytotoxic IC$_{50}$ of 12 µM for CNL when administered to MDA-MB-231 triple negative breast cancer cells (Fig. 3.1). Subsequently, a lower concentration (5µM) of CNL was used to investigate the ability of CNL to induce anoikis in MDA-MB-231 breast cancer cells. Metastatic cancer cells must develop resistance to anoikis in order to survive in the bloodstream or lymphatic system. For these studies, MDA-MB-231 cells were treated with 5µM of either ghost liposomes, which do not contain ceramide, or CNL under adherent or non-adherent conditions and then assayed for caspase 3/7 activity, which indicates apoptosis. Results showed that CNL was able to significantly increase apoptosis under non-adherent conditions (Fig. 3.2). This effect was prevented by z-vad-fmk, a caspase inhibitor, indicating that this was indeed caspase-dependent anoikis. As CD44 levels have been associated with anoikis resistance (Su, Lai et al. 2011), I attempted to determine
Fig. 3.1 Effect of CNL on Cell Viability in MDA-MB-231 Cells

MDA-MB-231 cells were treated with 1.56-200 µM CNL for 24 h, followed by MTS assay for cell viability. The IC$_{50}$ was 12 µM.
Fig. 3.1  Effect of CNL on Cell Viability in MDA-MB-231 Cells
MDA-MB-231 cells were treated with 5 µM ghost, 5 µM CNL, or 25 µM z-vad-fmk pan-caspase inhibitor under either adherent or non-adherent conditions for 24 h, followed by an assay for caspase 3/7 activity. N=3. *, p=.0001. Results show that the treatment (p<.0001), group (p<.0001), and their interaction (p=.0001) are all significant.
Fig. 3.2  Both CNL and Inhibition of CD44 Induce Anoikis in Breast Cancer Cells
whether CNL regulates CD44. Indeed, treatment with 5 μM CNL for 24 hours significantly reduced CD44 levels in MDA-MB-231 breast cancer cells (Fig. 3.3). I next utilized siRNA to knock down the CD44 gene (Fig. 3.4) in order to mimic the effects of ceramide. CD44 siRNA also significantly increased anoikis in MDA-MB-231 cells (Fig. 3.2), suggesting that inhibition of CD44 may mediate the ability of CNL to induce anoikis. The ability of CNL to induce anoikis in metastatic carcinoma cells was confirmed in the B16 murine melanoma cell line (Fig. 3.5).

Given the ability of CNL to increase anoikis, studies were performed to investigate if CNL could prevent mammosphere formation under non-adherent conditions. Mammospheres are thought to arise from cancer stem cells (CSC’s) and tend to be highly resistant to anoikis (Liu, Dontu et al. 2006). Similar to the caspase 3/7 assay, it was found that CNL significantly inhibited MDA-MB-231 mammosphere formation over the course of 9 days (Fig. 3.6A). The trypan blue dye exclusion technique was used to confirm mammosphere cell viability, with live cells excluding the blue dye (Fig. 3.6B-D). These data support the hypothesis that CNL induces anoikis in breast cancer cells. Since mammospheres result from CSC’s, and CD44 is a marker for CSC’s, it is possible that CNL is able to cause apoptosis/anoikis in CD44+ CSC’s, which are often drug-resistant (Donnenberg and Donnenberg 2005).

We next wanted to see if overexpression of CD44 could rescue the effect of CNL, as this would provide strong evidence that ceramide acts through CD44 to induce anoikis. Attempting to overexpress CD44 in the MDA-MB-231 cell line would be senseless because these cells already overexpress CD44. So, working with my
Fig. 3.3  CNL Decreases CD44 Protein Levels

MDA-MB-231 cells were treated with 5 μM ghost vehicle control liposomes or CNL for 24 h, followed by Western blot analysis of CD44 and β-actin (loading control). Dividing lines indicate that each lane was taken from different parts of the same gel.
Fig. 3.3  CNL Decreases CD44 Protein Levels

5 μM Ghost  -  +  -
5μM CNL  -  -  +

CD44

β-actin
siRNA results in 97% knockdown of CD44 in MDA-MB-231 cells. Cells were treated with 100 nM scrambled (Scr) or CD44 siRNA for 48 h, followed by Western blot analysis of CD44 and β-actin.
Fig. 3.4  Knockdown of CD44 Using siRNA
Fig 3.5  **CNL Induces Anoikis in Melanoma Cells**

B16 cells were treated with 10 μM ghost, 10 μM CNL, or 25 μM z-vad-fmk under either adherent or non-adherent conditions for 24 h, followed by an assay for caspase 3/7 activity. N=5. *, p=.0001. Results show that the treatment (p<.0001), group (p<.0001), and their interaction (p=.0001) are all significant.
Fig 3.5  CNL Induces Anoikis in Melanoma Cells

B16 Cells

Normalized Caspase 3/7 Activity

- Adherent
- Non-adherent

No Treatment  10 µM Ghost  25 µM Z-vad-fmk  CNL  CNL+Z-vad-fmk

No Treatment  10 µM Ghost  25 µM Z-vad-fmk  CNL+Z-vad-fmk
Fig. 3.6  **CNL Inhibits Mammosphere Formation**

MDA-MB-231 cells were treated with 5 µM ghost or CNL for 9 days, followed by mammosphere analysis using a phase-contrast microscope. N=3. Bars, SEM. Blue dye = trypan blue (dead cells). *, p=.0009.
Fig. 3.6  CNL Inhibits Mammosphere Formation

A

![Bar graph showing the effect of different treatments on the number of mammospheres.](image)

B  C  D

No Treatment 5 μM Ghost 5 μM CNL

- No Treatment
- 5 μM Ghost
- 5 μM CNL
colleagues, Dr. Andrei Khokhlatchev and Younhee Choi, we overexpressed CD44s in the T47D cell line, which is a human breast cancer cell line that does not express CD44 (Draffin, 2004) (Fig. 3.7). (See Fig. 6.1 for the IC$_{50}$ of CNL in T47D cells). Using the caspase 3/7 activity assay, we found that cells that overexpressed CD44s were less sensitive to CNL-induced apoptosis (Fig. 3.8), indicating that CNL may induce apoptosis/anoikis by down-regulating CD44.
Fig. 3.7  Overexpression of CD44

CD44s was overexpressed in T47D cells.
Fig. 3.7  Overexpression of CD44

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Fig. 3.8  **CD44 Desensitizes T47D Cells to CNL-induced Apoptosis**

T47D-GFP and T47D-CD44 cells were treated with 50 µM Ghost or 1.56-25 µM CNL for 24 h, followed an assay for caspase 3/7 activity. *, p<.05 compared to T47D-GFP at the same dose. N=5
Fig. 3.8  
CD44 Desensitizes T47D Cells to CNL-induced Apoptosis

![Graph showing Caspase 3/7 activity for T47D-GFP and T47D-CD44. The x-axis represents different concentrations of CNL (0, 1.56, 3.12, 6.25, 12.5, 25 μM) and a control of 50 μM Ghost. The y-axis represents Caspase 3/7 Activity. Black bars represent T47D-GFP and grey bars represent T47D-CD44. Asterisks indicate statistically significant differences.]

Caspase 3/7 Activity

- T47D-GFP
- T47D-CD44

50 μM Ghost
1.56 μM CNL
3.12 μM CNL
6.25 μM CNL
12.5 μM CNL
25 μM CNL

0
500
1000
1500

Twelve 12
INHIBITION OF BREAST CANCER CELL MIGRATION BY CERAMIDE

I next investigated if CNL could block MDA-MB-231 cell migration when fetal bovine serum (FBS) was used as a chemoattractant under static conditions (i.e. in the absence of fluid flow). To measure cell migration, I used a transwell system with serum-free medium in the upper chamber and 10% FBS in the lower chamber, which acted as a chemoattractant to induce cell migration in the MDA-MB-231 cell line. I found that CNL inhibited cell migration in this cell line at a concentration that does not induce apoptosis after both 6 hours (Fig. 4.1A) and 24 hours (Fig. 4.1B) of treatment. This effect was independent of PKCζ (Fig. 4.2) and cRAF (Fig. 4.3). Because CD44 expression is associated with cancer cell migration and extravasation (Zohar, Suzuki et al. 2000, Zoller 2011), I assessed if the knockdown of CD44 with siRNA could mimic the effect of ceramide to reduce cancer cell migration. Compared to CNL, similar results were obtained by knocking down CD44 using siRNA (siCD44) (Fig. 4.1A). This effect is likely due to less CD44 being able to interact with the cytoskeleton through ezrin, leading to less cell migration. In addition, exogenous administration of IL-8 was able to partially rescue the effect of ceramide (Fig. 4.4). Importantly, I found that ceramide significantly reduces the secretion of IL-6 and IL-8 from MDA-MB-231 cells (Fig. 4.5), indicating that ceramide inhibits breast cancer cell migration by acting on both CD44 and IL-8.
**Fig. 4.1 Ceramide Slows Breast Cancer Cell Migration**

A and B, MDA-MB-231 cells were treated with 5 µM ghost vehicle control or CNL for 6 h (A) or 24 h (B) in a transwell cell migration assay.
Fig. 4.1  Ceramide Slows Breast Cancer Cell Migration

A  t=6 h

B  t=24 h
Fig. 4.2 Ceramide Inhibits Breast Cancer Cell Migration Independently of PKCζ

MDA-MB-231 cells were treated with 5 µM ghost vehicle control, 5 µM CNL, or 20 µM PKCζ pseudosubstrate (PKCζ inhibitor) for 24 h in a transwell cell migration assay.
Fig. 4.2  Ceramide Inhibits Breast Cancer Cell Migration Independently of PKCζ
Fig. 4.3 Ceramide Inhibits Breast Cancer Cell Migration Independently of cRAF

MDA-MB-231 cells were treated with 5 µM ghost vehicle control, 5 µM CNL, or 10 µM cRAF inhibitor for 24 h in a transwell cell migration assay.
Fig. 4.3  Ceramide Inhibits Breast Cancer Cell Migration Independently of cRAF
Fig. 4.4  Exogenous IL-8 Partially Rescues the Effect of Ceramide on Breast Cancer Cell Migration

MDA-MB-231 cells were treated with 5 μM ghost, 5 μM CNL, 300 ng/mL IL-6, or 300 ng/mL IL-8 for 24h in a transwell cell migration assay. N=3. Bars, SEM. *, p<.05.
Exogenous IL-8 Partially Rescues the Effect of Ceramide on Breast Cancer Cell Migration

*
Fig. 4.5  **CNL Partially Inhibits the Secretion of IL-6 and IL-8 From Breast Cancer Cells.**

**A-C,** MDA-MB-231 cells were treated with 5 μM CNL for 24h. Cell culture media was then collected and subjected to a MesoScale multiplex sandwich immunoassay. TNFα served as a negative control. N=3. Bars, SEM. *, p<.05.
Fig. 4.5  CNL Partially Inhibits the Secretion of IL-6 and IL-8 From Breast Cancer Cells.
In order to measure cell migration under more physiologically relevant conditions, in collaboration with Dr. Cheng Dong and colleagues of the Pennsylvania State University, we used a flow migration chamber to measure the ability of MDA-MB-231 breast cancer cells to migrate through EI endothelial cells under physiological fluid shear rates. EI cells are fibroblasts that overexpress ICAM-1 and E-selectin, thus mimicking endothelial cells. Ligands to ICAM-1 and E-selectin include αvβ3 integrin and CD44. However, only CD44 is expressed in MDA-MB-231 cells as determined by flow cytometry (data not shown). The flow migration assay requires both adhesion to endothelial cells and cell migration, and thus serves as a model of extravasation (the exit of metastatic cancer cells from the bloodstream). Our previous work demonstrated that shear rate has a much greater effect on adhesion and migration than shear stress (Liang, Slattery et al. 2005, Slattery, Liang et al. 2005, Liang, Slattery et al. 2008). Thus, we varied shear rate in our flow migration experiments. Similar to our transwell results, CNL blocked the ability of breast cancer cells to migrate under flow conditions (Fig. 4.6). This effect was greater with higher concentrations of CNL (5 μM) and higher shear rates (100 sec⁻¹). In addition, inhibiting CD44 using siRNA had an effect similar to that of CNL. Down-regulation of CD44 decreased extravasation, an effect that was enhanced by increasing the shear rate (Fig. 4.6). These results indicate that CNL and CD44 siRNA block breast cancer cell migration through endothelial cells under physiological fluid flow conditions.
Fig. 4.6  
**CNL Inhibits Breast Cancer Cell Extravasation Under Physiological Fluid Flow Conditions.**

MDA-MB-231 cells were treated with 1-5 μM CNL for 30 minutes or 100 nM scrambled (Scr) or CD44 (siCD44) siRNA for 48 h, followed by an assay for extravasation under fluid flow shear rates of 50 sec$^{-1}$ or 100 sec$^{-1}$. N=3. Bar, SEM. *, p<.05 compared to No Treatment, Ghost, and siScr for that shear rate.
Fig. 4.6  CNL Inhibits Breast Cancer Cell Extravasation Under Physiological Fluid Flow Conditions.
Chapter 5

LYSOSOMAL TARGETING OF CD44 BY CERAMIDE

Given that down-regulation of CD44 mimicked the effects of CNL to induce anoikis and reduce extravasation, I next examined whether CNL could reduce CD44 expression directly. To determine if CNL-induced anoikis and CNL-reduced extravasation is dependent upon CD44, I initially treated MDA-MB-231 cells with 20 μg/mL cycloheximide in order to determine the half-life of CD44. I collected cell lysates at 0, 1, 2, 3, 14, 24, and 48 hours, followed by Western blot analysis of CD44 to determine protein levels of CD44. I found that CD44 has a half-life of about seven hours in MDA-MB-231 cells (Fig. 5.1). CD44 expression decreased in a dose-dependent manner after treatment with CNL for 24 hours as shown by Western blot (Fig. 5.2). Such a decrease in CD44 would prevent the breast cancer cells from binding to EI endothelial cells and extravasating, thus mediating the ability of CNL to prevent extravasation in this system. CD44 protein levels were also decreased after a shorter six hour treatment with CNL compared to untreated or ghost-treated cells (Fig. 5.3). To rule out the possibility that ceramide is having a global effect on membrane proteins, I analyzed three other membrane-bound proteins: caveolin-1, the epidermal growth factor receptor (EGFR), and the transferrin receptor. CNL treatment had no effect on the levels of these proteins in MDA-MB-231 cells, indicating that CNL specifically targets CD44 without affecting other membrane proteins (Fig. 5.4). To see if CNL affects CD44 in
Fig. 5.1 The Half-life of CD44 in MDA-MB-231 Cells is About 7 Hours

Cells were treated with 20 µg/mL cycloheximide for 0-48 h, followed by Western blot analysis for CD44. N=3. Bar, SEM.
The Half-life of CD44 in MDA-MB-231 Cells is About 7 Hours
Fig. 5.2  **CNL Inhibits CD44 at the Protein Level in a Dose-dependent Manner.**

MDA-MB-231 cells were treated with increasing concentrations of ghost or CNL for 24 h, followed by Western blot analysis for CD44. N=3.
Fig. 5.2  
CNL Inhibits CD44 at the Protein Level in a Dose-dependent Manner.

CD44  
β-actin  
MDA-MB-231  
NT 5 G 10 G 1 CNL 2 CNL 5 CNL 10 CNL

CD44/β-actin Denstometry

No Treatment  5uM Ghostr  10uM Ghostr  1uM CNL  2uM CNL  5uM CNL  10uM CNL

*
Fig. 5.3  

CNL Inhibits CD44 at the Protein Level After 6 h of Treatment

MDA-MB-231 cells were treated with ghost or CNL for 6 h, followed by Western blot analysis for CD44. N=3.
Fig. 5.3  

CNL Inhibits CD44 at the Protein Level After 6 h of Treatment

![Western Blot Images]  

CD44  
β-actin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD44 Density ( arbitrary units )</th>
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<tbody>
<tr>
<td>No Treatment</td>
<td>100</td>
</tr>
<tr>
<td>5 mM Ghost</td>
<td>150</td>
</tr>
<tr>
<td>5 mM CNL</td>
<td>50</td>
</tr>
</tbody>
</table>

* Indicates statistical significance compared to the No Treatment group.
Fig. 5.4  **CNL Does Not Affect Caveolin-1, EGFR, or Transferrin Receptor Levels in MDA-MB-231 Cells**

MDA-MB-231 cells were treated with 5 µM CNL for 24 h, followed by Western blot analysis of caveolin-1 (A), EGFR (B), and the transferrin receptor (C). N=3. Bars, SEM.
Fig. 5.4  CNL Does Not Affect Caveolin-1, EGFR, or Transferrin Receptor Levels in MDA-MB-231 Cells
other breast cancer cell lines, I decided to examine the MDA-MB-468 cell line, which is similar to the MDA-MB-231 cell line in that it is a human triple negative breast cancer cell line that expresses high levels of CD44. I first did an MTS cell viability experiment with CNL in MDA-MB-468 cells, and I found that the IC₅₀ was 43 µM (Fig. 5.5). To prevent cytotoxicity, I used a concentration of 10 µM in my subsequent experiment, in which I found that treating MDA-MB-468 cells with CNL for 24 hours led to a significant decrease in CD44 protein levels (Fig. 5.6), indicating that the effect of ceramide on CD44 is not specific to MDA-MB-231 cells, but is a more widespread phenomenon. The decrease in CD44 protein levels after treatment with CNL was further confirmed in Panc-1 human pancreatic cancer cells via Western blot (Fig. 5.7). Panc-1 cells were treated with 13 µM CNL for 48 h because the IC₅₀ of CNL on Panc-1 cell viability was previously determined to be 26 µM for a 48 h treatment (Jiang, DiVittore et al. 2011). CD44 inhibition was further confirmed by immunofluorescence on MDA-MB-231 cells that had been treated with 5 µM CNL for 24 hours (Fig 5.8). While untreated and ghost-treated cells had high levels of CD44 that was found mainly at the plasma membrane, quantification of the images revealed that CNL-treated cells had significantly lower expression of CD44 (Fig. 5.8).

There is evidence that CD44 can be palmitoylated and targeted to lipid rafts (Thankamony and Knudson 2006). To test whether CNL induces palmitoylation of CD44 as an early event in its degradation, we used 2-bromopalmitate (2BP), which irreversibly inhibits palmitoyl acyltransferases. Although 2BP by itself actually increased CD44 levels, it was unable to rescue the effect of CNL, indicating that the ability of CNL to
**Fig. 5.5 Effect of CNL on Cell Viability in MDA-MB-468 Cells**

MDA-MB-468 cells were treated with 1.56-200 µM ghost or CNL for 24 h, followed by MTS assay for cell viability. The IC\textsubscript{50} of CNL was 43 µM.
Fig. 5.5  Effect of CNL on Cell Viability in MDA-MB-468 Cells

![Graph showing the effect of CNL on cell viability in MDA-MB-468 cells. The graph plots the MTS Assay (MTS Assay % Cell Viability) against the log of liposome concentration (log [liposome]). The graph compares CNL and Ghost treatments.]
Fig. 5.6  CNL Inhibits CD44 in MDA-MB-468 Cells

MDA-MB-468 cells were treated with 10 µM CNL for 24 h, followed by Western blot analysis of CD44. N=5. Bars, SEM. *, p<.05 compared to no treatment and ghost.
Fig. 5.6  CNL Inhibits CD44 in MDA-MB-468 Cells

5 μM Ghost +
5 μM CNL +

CD44

beta-actin

CD44/β-actin Densitometry

No Treatment  5 μM Ghost  5 μM CNL

*
Fig. 5.7  CNL Inhibits CD44 in Human Pancreatic Cancer Cells

Panc-1 cells were treated with 13 µM CNL for 48 h, followed by Western blot analysis of CD44. N=3. Bars, SEM. *, p<.05 compared to ghost.
Fig. 5.7  CNL Inhibits CD44 in Human Pancreatic Cancer Cells

Panc-1

CD44

β-actin

![Graph showing CD44/β-actin densitometry for different treatments: No Treatment, 15μM Gost, 13μM CNL. The graph indicates a significant decrease in CD44 levels with CNL treatment.](image)
Fig. 5.8  CNL Decreases CD44 Levels

A, MDA-MB-231 cells were treated with 5 µM ghost or CNL for 24 h, followed by indirect immunofluorescence of CD44. Red = CD44, blue = nuclei. B, The images shown in A were quantified using Imaris imaging software. N=3. *, p<.05 compared to ghost.
Fig. 5.8  CNL Decreases CD44 Levels

A

CD44  Nuclei  Combined

No Treatment

5 µM Ghost

5 µM CNL

B

Relative CD44 Intensity/Cell

0 50 100

No Treatment  5 µM Ghost  5 µM CNL
decrease CD44 levels is not dependent on palmitoylation of CD44 (Fig 5.9). Similarly, the proteasome inhibitor MG132 was also unable to rescue the effect of ceramide on CD44, indicating that ceramide does not target CD44 to the proteasome (Fig. 5.9). In addition, the effect of CNL on CD44 was not seen at the RNA level, as shown by RT-PCR (Fig. 5.10). Treatment of MDA-MB-231 cells with 5 μM CNL for 24 hours had no effect on CD44 RNA levels.

To further understand the effect of CNL on CD44 expression, we used chloroquine, which inhibits lysosomal activity by preventing endosomal and lysosomal acidification. Western blot protein analysis revealed that chloroquine was able to inhibit the loss of CD44 caused by CNL, suggesting that CNL targets CD44 to the lysosome for degradation (Fig. 5.11). We confirmed the role of the lysosome by using other lysosomal inhibitors, namely pepstatin A in combination with E64D, which inhibit lysosomal proteases; as well as ammonium chloride, which prevents endosomal and lysosomal acidification. Similar to the results obtained with chloroquine, inhibition of the lysosome with these agents prevented CNL from inhibiting CD44 (Fig. 5.12), indicating that CNL targets CD44 to the lysosome for degradation. Ceramide has previously been shown to cause ubiquitination of the HERG K⁺ channel, leading to its internalization and lysosomal degradation (Chapman, Ramstrom et al. 2005). CNL could be acting through a similar mechanism in regards to CD44, whereby increased ceramide levels at the plasma membrane could cause CD44 to be localized to lipid rafts, ubiquitinated, internalized, and degraded by the lysosome.
Fig. 5.9  Neither Palmitoylation nor the Proteasome are Required for the CNL-induced Decrease in CD44 Levels

MDA-MB-231 cells were pre-treated with 7.5 µM MG132 (proteasome inhibitor) or 10 µM 2BP (palmitoyl transferase inhibitor) for 0.5 h, followed by treatment with 5 µM ghost or CNL for 14 hours. Western blot analysis for CD44 was then performed.

N=3.
Neither Palmitoylation nor the Proteasome are Required for the CNL-induced Decrease in CD44 Levels
Fig. 5.10  CNL Does Not Affect CD44 at the RNA Level

MDA-MB-231 cells were treated with 5 µM CNL for 24 h, followed by RT-PCR analysis of CD44, using β-actin as an endogenous control. N=9. Bars, SEM. *, p<.05 compared to dose-matched control.
Fig. 5.10  CNL Does Not Affect CD44 at the RNA Level
Fig. 5.11  Chloroquine Rescues the Effect of CNL on CD44

MDA-MB-231 cells were pre-treated with 50 µM chloroquine for 1 h, followed by treatment with 5 µM ghost or CNL for 6 h. Western blot analysis for CD44 was then performed. N=3. Bars, SEM. *p<.05 compared to ghost.
Fig. 5.11  Chloroquine Rescues the Effect of CNL on CD44
Fig. 5.12 Lysosomal Inhibitors Rescue the Effect of CNL on CD44

MDA-MB-231 cells were pre-treated with 10 µg/mL pepstatin A in combination with 10 µM E64D or 50 mM NH₄Cl for 1 h, followed by treatment with 5 µM ghost or CNL for 6 h. Western blot analysis for CD44 was then performed. N=3. Bars, SEM. *, p<.05.
Fig. 5.12  Lysosomal Inhibitors Rescue the Effect of CNL on CD44
Chapter 6

CERAMIDE INHIBITS IL-6-INDUCED EPITHELIAL-MESENCHYMAL TRANSITION

IL-6 has previously been shown to induce EMT in head and neck cancer and breast cancer cells (Yadav, Kumar et al. 2011, Xie, Yao et al. 2012). Given the ability of ceramide to block a decrease in E-cadherin protein levels in MDCK cells (Wang, Krishnamurthy et al. 2009) and the ability of complex glycosphingolipids to block EMT (Guan, Handa et al. 2009), I tested the ability of CNL to prevent IL-6-induced EMT in human breast cancer cells.

First, I performed an MTS cell viability assay in the T47D human breast cancer cell line and determined the IC$_{50}$ of CNL to be 23 µM (Fig. 6.1). Thus, for my following experiments, I used a non-toxic concentration of 10 µM.

By treating T47D cells with 50 ng/mL human recombinant IL-6 for 24 hours, I was able to see a significant decrease in E-cadherin protein levels. Pre-treatment with 5µM CNL for 1 hour was able to prevent this (Fig. 6.2). In fact, concentrations of CNL ranging from 5-25 µM were all able to completely prevent IL-6-induced E-cadherin inhibition (Fig. 6.3). In addition, pre-treatment with CNL was able to prevent IL-6 from reducing E-cadherin RNA levels (Fig. 6.4), as well as RNA levels of the epithelial marker
Fig. 6.1 Effect of CNL on Cell Viability in T47D Cells

T47D cells were treated with 1.56-200 µM CNL for 24 h, followed by MTS assay for cell viability. The IC\textsubscript{50} was 19 µM.
Fig. 6.1  Effect of CNL on Cell Viability in T47D Cells
Fig. 6.2  **CNL Inhibits an IL-6-induced Decrease in E-cadherin Protein Levels**

T47D cells were pre-treated with 10 µM CNL before being treated with 50 ng/mL IL-6 for 24 h, followed by Western blot analysis for E-cadherin. N=3.
Fig. 6.2  
CNL Inhibits an IL-6-induced Decrease in E-cadherin Protein Levels

![Graph showing E-cadherin and β-actin levels with treatment conditions: No Treatment, 10 μM Ghost, 10 μM CNL, 50 ng/mL IL-6, CNL + IL-6.](image)
Fig. 6.3  Dose-response Experiment on the Effect of CNL on E-cadherin Levels

T47D cells were pre-treated with 5-25 µM CNL before being treated with 50 ng/mL IL-6 for 24 h, followed by Western blot analysis for E-cadherin. N=3. *, p<.05 compared to No Treatment.
Fig. 6.3  Dose-response Experiment on the Effect of CNL on E-cadherin Levels

![Image of Western Blot](image)

**E-cadherin**

**β-actin**

<table>
<thead>
<tr>
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<th>Density</th>
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<tbody>
<tr>
<td>No Treatment</td>
<td></td>
</tr>
<tr>
<td>50 ng/mL IL-6</td>
<td></td>
</tr>
<tr>
<td>5 μM CNL + IL-6</td>
<td></td>
</tr>
<tr>
<td>10 μM CNL + IL-6</td>
<td></td>
</tr>
<tr>
<td>20 μM CNL + IL-6</td>
<td></td>
</tr>
<tr>
<td>25 μM CNL + IL-6</td>
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</table>

*Significant difference compared to control*
Fig. 6.4  **CNL Prevents an IL-6-induced Decrease in E-cadherin RNA Levels**

T47D cells were pre-treated with 10 μM CNL for 1 h before being treated with 50 ng/mL IL-6 for 24 h, followed by RT-PCR analysis of E-cadherin, using β-actin as an endogenous control. *N=9. Bars, SEM. *, p<.05.
Fig. 6.4  
CNL Prevents an IL-6-induced Decrease in E-cadherin RNA Levels

Diagram showing the effect of CNL on E-cadherin RNA levels under different treatment conditions.
EpCAM (Fig. 6.5). Furthermore, treatment with 50 ng/mL IL-6 every 3 days for 10 days led to a loss of cell-cell adhesion and a transition from a cuboidal cell morphology to a more spindle-shaped morphology. This was prevented by treatment with 5 µM CNL for 1 hour prior to treatment with IL-6 (Fig. 6.6). These results were seen for up to 13 days (Fig. 6.7).

IL-6 elicits many of its effects, including induction of EMT, by activating STAT3. For this reason, we investigated whether CNL could prevent the activation of STAT3 by IL-6. When IL-6 activates STAT3, STAT3 becomes phosphorylated at tyrosine 705. Thus, we measured pSTAT3 (Y705) protein levels via Western blot. In the absence of exogenous IL-6, STAT3 was in an inactive state in T47D cells. Upon treatment with 50 ng/mL IL-6 for 24 hours, STAT3 became phosphorylated at Y705. Pre-treatment with 10 µM CNL for 1 hour was able to partially block this activation (Fig. 6.8), providing a possible mechanism for the ability of CNL to prevent IL-6-induced EMT.
Fig. 6.5  **CNL Prevents an IL-6-induced Decrease in EpCAM RNA Levels**

T47D cells were pre-treated with 10 μM CNL for 1 h before being treated with 50 ng/mL IL-6 for 24 h, followed by RT-PCR analysis of EpCAM, using β-actin as an endogenous control. N=9. Bars, SEM. *, p<.05.
Fig. 6.5  CNL Prevents an IL-6-induced Decrease in EpCAM RNA Levels
Fig. 6.6 Pre-treatment with CNL Prevents an IL-6-induced Morphology Change after 10 Days of Treatment

T47D cells were pre-treated with 5 µM CNL for 1 hour before being treated with 50 ng/mL IL-6. This process was repeated every 3 days for 10 days, after which time light microscopic images were taken using a 10x objective.
Fig. 6.6  Pre-treatment with CNL Prevents an IL-6-induced Morphology Change after 10 Days of Treatment
Fig. 6.7  Pre-treatment with CNL Prevents an IL-6-induced Morphology Change after 13 Days of Treatment

T47D cells were pre-treated with 5 µM CNL for 1 hour before being treated with 50 ng/mL IL-6. This process was repeated every 3 days for 13 days, after which time light microscopic images were taken using a 10x objective.
Fig. 6.7  Pre-treatment with CNL Prevents an IL-6-induced Morphology Change after 13 Days of Treatment

No Treatment  
5 µM Ghost  
5 µM CNL

50 ng/mL IL-6  
CNL + IL-6
Fig. 6.8  CNL Partially Blocks IL-6-induced Activation of STAT3

T47D cells were pre-treated with 5-10 µM CNL for 1 h before being treated with 50 ng/mL IL-6 for 24 h, followed by Western blot analysis of pSTAT3 (Y705), total STAT3, and β-actin (loading control). N=3. *, p<.05 compared to IL-6-treated cells.
Fig. 6.8  CNL Partially Blocks IL-6-induced Activation of STAT3

- pSTAT3
- STAT3

Graph showing the comparison of pSTAT3/STAT3 levels across different treatments:

- No Treatment
- 50 ng/mL IL-6
- 5 μM CNL + IL-6
- 10 μM CNL + IL-6

* Indicates statistical significance.
Chapter 7

DISCUSSION

In this dissertation, I have shown that ceramide is able to induce anoikis, target CD44 to the lysosome, inhibit cell migration under both static and physiological fluid flow conditions, and inhibit EMT in breast cancer cells. In this final chapter, I will discuss which of my findings agree with previously published work and which do not. I will also talk about future directions, including \textit{in vitro}, \textit{in vivo}, and clinical studies that would complement the data provided here. Finally, I will briefly discuss the possibility of using CNL as an anti-cancer therapeutic in the clinic.

7.A. \textit{Anoikis}

Ceramide has previously been shown to induce anoikis in HEK293 (Widau, Jin et al. 2010) and HeLa cells (Hu, Xu et al. 2005). In this dissertation, I have extended these findings to metastasis-competent breast cancer and melanoma cell lines. In addition, I have discovered a new mechanism by which ceramide is able to prevent anoikis resistance: targeting of anti-anoikic CD44 to the lysosome.

In this dissertation, I have shown that ceramide induces caspase-dependent anoikis in metastatic breast cancer and melanoma cells. However, it would also be interesting to know whether ceramide induces caspase-independent anoikis. This could be ascertained by treating metastatic breast cancer cells with CNL, followed by isolation
of mitochondrial and cytoplasmic fractions, followed by Western blot analysis for Bit1, which is released from the mitochondria during caspase-independent anoikis. Increased Bit1 release from the mitochondria would be further evidence that ceramide is inducing anoikis and not just apoptosis, as mitochondrial Bit1 release is specific to anoikis.

In addition to showing that ceramide induces anoikis, I have also shown that knockdown of CD44 in breast cancer cells induces anoikis. This implicates CD44 as an important inhibitor of anoikis in metastatic breast cancer cells, which agrees with the findings of other labs that CD44 is involved in anoikis resistance in colon cancer cells (Lakshman, Subramaniam et al. 2004, Su, Lai et al. 2011). I used caspase 3/7 activity as a measure of apoptosis and anoikis. Future confirmatory experiments could use other ways to measure anoikis, such as annexin V staining, followed by flow cytometry. In addition, it would be interesting to see if CD44 plays a role in anoikis resistance in the B16 murine melanoma cell line. To test this, CD44 siRNA could be used to knock down CD44 in the B16 cell line, followed by a caspase 3/7 activity assay under both adherent and non-adherent conditions to measure anoikis.

Besides showing that ceramide induces anoikis in breast cancer cells, I have also shown this effect in melanoma cells, indicating that it could be a more general phenomenon. However, confirmatory experiments in a large number of normal and cancerous cell types should be done to substantiate this.

I confirmed my anoikis data by examining the ability of ceramide to block mammosphere formation. Mammospheres are clumps of anoikis-resistant mammary cells that are thought to arise from a single cancer stem cell (CSC). Since I found that
ceramide completely inhibits the formation of mammospheres and that it inhibits CD44, which is a stem cell marker (Prince, Sivanandan et al. 2007), it is possible that ceramide is able to induce apoptosis and anoikis in CSC’s. This could be tested by using fluorescence-activated cell sorting (FACS) to isolate CD44+/CD133+/CD24− (CSC population) and CD44+/CD133+/CD24− cells from murine breast cancer tumors or breast cancer cell lines, followed by cell viability, apoptosis, and anoikis assays with CNL. CSC’s are typically resistant to chemotherapy. However, because of its ability to inhibit CD44 and mammosphere formation, I would hypothesize that CNL would be able to effectively kill CSC’s, especially under non-adherent conditions, such as are found in the bloodstream and lymphatic system.

Although I have shown that ceramide induces anoikis in the MDA-MB-231 and B16 cell lines, it is possible that it also induces autophagy, which is a process by which organelles are enclosed within an autophagosome, which then fuses with a lysosome, leading to degradation of the organelles and either cell survival or cell death, depending on the cause and extent of autophagy. Given the ability of ceramide to induce autophagy (Daido, Kanzawa et al. 2004, Scarlatti, Bauvy et al. 2004) and the considerable crosstalk between apoptosis and autophagy (Young, Kester et al. 2013), it is possible that ceramide is inducing both anoikis and autophagy in the cell lines that I examined. This could be assessed by using electron microscopy to look for autophagic vacuoles; by using Western blots to probe for LC3-I and LC3-II, which are markers of autophagy; and by measuring TORC1 activity, which is typically decreased during autophagy. Since ceramide has been shown to induce cell death through autophagy...
(Daido, Kanzawa et al. 2004, Young, Kester et al. 2013), I would hypothesize that ceramide induces autophagy in the MDA-MB-231 and B16 cell lines.

**7.B. Cell Migration**

Independent of its ability to induce apoptosis and anoikis, ceramide has previously been shown to be able to prevent neurotensin-induced cell migration in breast cancer cells (Heakal and Kester 2009). In addition, a recent study found that overexpressing ceramide synthase 6 (CerS6) in mesenchymal breast cancer cells increased the level of C16:0 ceramide and inhibited cl-CD95L-induced cell migration. Furthermore, inhibiting CerS6 via shRNA in epithelial breast cancer cells decreased C16:0 ceramide levels and induced cell migration (Edmond, Dufour et al. 2014). In this dissertation, I have extended these findings to the more general case of breast cancer cell motility in the absence of neurotensin and cl-CD95L, as well as the more physiologically relevant model of breast cancer cell extravasation under fluid flow conditions, which requires not only cell motility, but also adhesion of breast cancer cells to endothelial cells. In addition, I have provided a heretofore unknown mechanism by which ceramide is able to inhibit cell migration, namely through down-regulation of CD44 (Fig. 7.1).

Previous work showed that ceramide is able to prevent migration and invasion in metastatic breast cancer cells by inhibiting MMP9 (Heakal and Kester 2009). Interestingly, there are multiple papers that describe a link between MMP9 and CD44.
Fig. 7.1  Model of Ceramide in Cell Migration and Anoikis

Ceramide induces anoikis by inhibiting CD44 and inhibits cell migration by inhibiting CD44 and IL-8.
Fig. 7.1  Model of Ceramide in Cell Migration and Anoikis
CD44 and MMP9 interact (Yu and Stamenkovic 1999), and when MMP9 is knocked down with siRNA, cell surface levels of CD44 decrease (Desai, Ma et al. 2009). This raises the possibility that ceramide, MMP9, and CD44 constitute a single pathway.

In this thesis, I studied cell migration and extravasation using both static and fluid flow models. Since the static migration and flow migration assays measure different things, it is useful to compare results from both assays, though there were some differences in the way that the two experiments were carried out. First of all, 10% FBS was used as the chemoattractant in the static migration experiments, whereas soluble collagen IV was used as the chemoattractant in the flow migration experiments. Second, the cells used in the static migration experiments were serum starved overnight prior to treatment, while the cells in the flow migration experiment were not serum starved. Despite these differences, I feel that insights can still be gained from comparing results from the two assays. Static migration measures only cell motility, while flow migration measures both adhesion of cancer cells to the endothelium and cell motility. At a 6 h time point, 5 µM CNL slowed breast cancer cell migration under static conditions by approximately 35%, and CD44 siRNA also slowed static migration by approximately 35% (Fig. 4.1A). At a 24 h time point, CNL slowed static migration by 21% (Fig. 4.1B). Under a physiological flow rate of 50 sec\(^{-1}\), 1 µM CNL decreased migration by 56%, 5 µM CNL decreased migration by 80%, and siCD44 decreased migration by 26% (Fig. 4.6). Under a physiological flow rate of 100 sec\(^{-1}\), 5 µM CNL completely abolished cell migration, while CD44 siRNA decreased migration by 58% (Fig. 4.6). Since CNL has a greater effect on flow migration than on static migration, our data indicate that
ceramide is affecting both cell motility and adhesion, with the greatest effect seen at the highest shear rate and the highest concentration of CNL. Increased shear forces decrease the amount of time that circulating tumor cells are in contact with endothelial cells, thus decreasing the likelihood that they will adhere to the endothelium and extravasate. Ceramide is likely slowing motility by reducing the amount of CD44 that can interact with the cytoskeleton through ezrin, while the effects of ceramide on extravasation are likely due to both decreased interactions of CD44 with ezrin a loss of the ability of cancer cells to bind E-selectin and adhere to endothelial cells.

In addition to showing that ceramide is able to prevent the migration of metastasis-competent breast cancer cell lines under both static and physiological fluid flow conditions, I have also shown that knockdown of CD44 has a similar effect. This finding agrees with other studies that have found a role for CD44 in cell migration in fibroblasts, macrophages, metastatic breast cancer cells (Bourguignon, Zhu et al. 2000, Zohar, Suzuki et al. 2000), melanoma cells (Thomas, Etoh et al. 1993), mesenchymal stem cells (Zhu, Mitsuhashi et al. 2006), and ovarian cancer cells (Bourguignon, Zhu et al. 2001). Although I have shown that both ceramide and knockdown of CD44 inhibit breast cancer cell migration under fluid flow conditions, knockdown of CD44 did not have as much of an effect as ceramide. This is likely due to ceramide acting through mechanisms in addition to CD44, including inhibition of MMP9 (Heakal and Kester 2009) and IL-8 (Fig. 4.4).

In both the static and flow models of cell migration, we measured the migration of many cells at once (200,000 cells and 500,000 cells, respectively). To further confirm
the ability of ceramide to prevent cell migration, it would be interesting to use time-lapse microscopy to track the movement of single cells. It would also be interesting to use an *in vivo* zebrafish model of tumor dissemination. In this model, fluorescently-labeled human cancer cells are injected into the perivitelline cavity of a zebrafish embryo and tumor development and invasion are monitored using a fluorescent microscope. Given the results presented in Chapter 4, I would hypothesize that ceramide-treated cells would move and disseminate less than untreated or ghost-treated cells. I would also expect inhibition of CD44 to decrease cell movement and dissemination. Furthermore, I would hypothesize that exogenous administration of IL-8 would rescue the effect of ceramide.

While I have shown that ceramide induces anoikis and inhibits cell migration *in vitro*, it will be essential to study the effect of ceramide on these processes *in vivo*. One way to do this would be to inject luciferase-expressing murine breast cancer cells into balb/c mice, followed by treatment with CNL. Lungs, livers, brains, and bones could be examined for metastasis using bioluminescence, Bouin’s staining, and immunohistochemistry (IHC). These cells would have to go through all of the steps of the metastatic cascade, including cell migration and resistance to anoikis, in order to metastasize. My hypothesis would be that CNL-treated animals would have fewer metastases than ghost-treated animals.

A second animal model that could be used would be to inject MDA-MB-231 cells into the lateral tail vein of immune-compromised mice, followed by treatment with CNL. These cells would have to be resistant to anoikis and would have to extravasate in order
to form metastases. Since intravenously-injected MDA-MB-231 cells metastasize to the lungs, the lungs could be removed and examined for metastases using Bouin’s staining and IHC. Again, I would hypothesize that CNL-treated mice would have fewer metastases than ghost-treated mice.

Ceramide has been shown to activate a number of downstream effectors, including PKCζ and cRaf. To rule out a role for PKCζ or cRaf in the effect of ceramide on cell migration, I used the PKCζ pseudosubstrate, which blocks PKCζ activity, and a small molecule inhibitor of cRaf. Neither of these inhibitors rescued the effect of ceramide, indicating that ceramide inhibits cell migration independently of PKCζ and cRaf. One potential problem with this approach is that these inhibitors are non-specific. The PKCζ pseudosubstrate inhibits both PKCζ and PKCι, and small molecule inhibitors are notoriously non-specific. To definitively rule out a role for PKCζ and cRaf, it would be helpful to use siRNA.

Although the ability of ceramide to inhibit cell migration did not depend on PKCζ or cRaf, it was partially rescued by the administration of exogenous IL-8. In addition, I showed that ceramide reduced IL-8 secretion from metastatic breast cancer cells. Taken together, these data indicate that ceramide reduces cell migration by inhibiting IL-8 secretion and the ensuing pro-migratory autocrine signalling that takes place. This agrees with previous work done by our lab and others, showing that ceramide prevents IL-8 secretion from corneal epithelial cells (Sun, Fox et al. 2008), as well as activating protein phosphatase 2A (PP2A) in the respiratory endothelium, thereby preventing IL-8 production (Cornell, Hinkovska-Galcheva et al. 2009).
7.C. **Down-regulation of CD44**

In my work, I have shown that ceramide may inhibit cell migration via down-regulation of IL-8 and CD44. In fact, to the best of my knowledge, my work is the first to show that ceramide regulates CD44. By using three different methods to inhibit the lysosome, I have shown that ceramide targets CD44 to the lysosome for degradation, independent of palmitoylation or proteasomal degradation. This agrees with a previous report showing that CD44 can be ubiquitinated and targeted to the lysosome (Bartee, Eyster et al. 2010). In addition, ceramide has been shown to cause the ubiquitination of the HERG K⁺ channel, which also resides in the plasma membrane, leading to its lysosomal degradation (Chapman, Ramstrom et al. 2005). Thus, it seems probable that ceramide causes CD44 to be ubiquitinated, internalized, and degraded by the lysosome.

Thankamony and Knudson found that CD44 is localized to lipid rafts before being internalized (Thankamony and Knudson 2006). This agrees with our findings, as ceramide is found at high concentrations in lipid rafts (Fox, Houck et al. 2007). Thus, the ceramide delivered by CNL is likely inducing lipid raft formation, leading to internalization of CD44. The decrease in CD44, a pro-metastatic transmembrane protein, helps to explain how CNL can prevent extravasation. In addition, CD44^hi^/CD24^lo^ mammary epithelial cells have been shown to be resistant to anoikis (Gauger, Hugh et al. 2009), suggesting that CD44 may help breast cancer cells evade anoikis.
In my work, I have focused on the standard form of CD44 (CD44s). However, there are 10 alternatively spliced CD44 variant exons that result in dozens of CD44 isoforms (Naor, Wallach-Dayan et al. 2008). Certain CD44 variants have been associated with breast cancer. For example, CD44v1, CD44v2, CD44v3, and CD44v4 are all overexpressed in metastatic tissue from breast cancer patients, as is CD44s (Iida and Bourguignon 1995). In addition, CD44v3, CD44v5, and CD44v6 have been found to decrease survival in breast cancer patients (Kaufmann, Heider et al. 1995). Similarly, CD44v6 contributes to tumor progression in colorectal cancer patients (Wielenga, Heider et al. 1993), and CD44v7 has been implicated in cell migration in rat fibrosarcoma cells (Katagiri, Sleeman et al. 1999). It would be informative to see if ceramide targets these variants to the lysosome, in addition to CD44s. To test this, breast cancer cells could be treated with CNL in combination with different lysosomal inhibitors, followed by IF and Western blot analysis for the CD44 variants of interest. Since the CD44 variants consist of CD44s plus one or more variable regions, I would hypothesize that ceramide would be able to target them to the lysosome as well. However, their 3D structures could prevent this.

Though I have shown that ceramide reduces CD44 levels in vitro, it will also be important to see if such an effect occurs in vivo. One way to do this would be to inject immune-compromised mice with MDA-MB-231 cells and then to treat the mice with CNL. Hyaluronan could be conjugated to the outside of the liposome in order to target CD44-expressing tumor cells. Hyaluronan-conjugated liposomes have been successfully used by other labs to target tumors in mice (Peer and Margalit 2004). After a tumor
forms, it could be removed and IHC aimed at detecting CD44 could be performed. Given my in vitro data, I would hypothesize that tumors from CNL-treated mice would have less CD44 than tumors from ghost-treated mice.

Though in vitro and animal data are important, it will be even more important to test these findings in the clinic. One way to do this would be to use mass spectrometry and IHC to measure the levels of ceramide and CD44 in breast cancer patient tumor biopsies. I would hypothesize that there would be an inverse relationship between ceramide and CD44 levels.

I have shown that ceramide reduces CD44 protein levels in metastatic breast cancer cells after both 6 and 24 hours of treatment with CNL, a finding that I confirmed in a pancreatic cancer cell line. In addition, the reduction in CD44 levels was seen using both Western blot analysis and IF. The fact that CD44 RNA levels were unaffected indicated that this was a post-transcriptional effect. This led me to treat cells with a proteasome inhibitor (MG132) and a palmitoyl transferase inhibitor (2-bromopalmitate), neither of which were able to rescue ceramide-induced CD44 downregulation.

Although the ability of ceramide to reduce CD44 was independent of the proteasome and palmitoylation, chloroquine, an inhibitor of the lysosome, was able to rescue the effect of ceramide, suggesting that ceramide targets CD44 to the lysosome for degradation. This was confirmed by using two other means of inhibiting the lysosome: treatment with pepstatin A in combination with E64D; and treatment with ammonium chloride. One way to further confirm that ceramide targets CD44 to the
lysosome would be to perform IF for lysosomes and CD44. The lysosomal dye would have to be added to live cells, followed by fixation and staining for CD44. I would hypothesize that untreated cells would retain CD44 at the plasma membrane, whereas CNL-treated cells would have CD44 co-localized with lysosomes.

Given that ceramide reduces CD44 levels, it would be interesting to see if ceramide directly interacts with CD44. This could be tested using electron paramagnetic resonance (EPR) spectroscopy or optical spectroscopy (Smith 2012).

7.D. Epithelial-mesenchymal Transition

Resistance to anoikis, cell migration, and overexpression of CD44 all promote breast cancer metastasis. A fourth metastasis-related process that is regulated by ceramide is EMT, which is characterized by a decrease in E-cadherin levels. Previous studies have shown that ceramide is able to rescue decreased E-cadherin levels caused by inhibition of PKCζ (Wang, Krishnamurthy et al. 2009) and that specific glycosphingolipids are able to inhibit TGFβ-induced EMT (Guan, Handa et al. 2009). Recently, Edmond et al. found that CerS6 is expressed at lower levels in mesenchymal cells compared to epithelial cells (Edmond, Dufour et al. 2014). CerS6 catalyzes the production of C14 and C16 dihydroceramide from sphinganine (Levy and Futerman 2010). These dihydroceramide species can then be converted to C14 and C16 ceramide (Ogretmen and Hannun 2004). In this dissertation, I have broadened our understanding of how sphingolipids are able to regulate EMT by showing that ceramide can prevent IL-6-induced EMT by blocking the activation of STAT3 (Fig. 7.2).
Ceramide is able to prevent IL-6-induced EMT by inhibiting IL-6 secretion and STAT3 activation.
Fig. 7.2  Model of Ceramide in EMT

IL-6 → JAK → pSTAT3 → EMT

Ceramide
Since EMT is sufficient for metastasis, it will be important to see if there is a correlation between ceramide levels and breast cancer metastasis. One study in head and neck squamous cell carcinoma (HNSCC) patients found that low levels of C18 ceramide in the primary tumor correlated with lymphovascular invasion and nodal metastasis (Karahatay, Thomas et al. 2007). It would be interesting to see if such a correlation holds true for breast cancer patients as well. Do breast cancer patients with higher levels of ceramide in their primary tumor have fewer metastases and increased survival? I would hypothesize that they do, although it could depend on which ceramide species one is talking about. Recently, observations have been made that different ceramide species have different biophysical properties in membranes and induce different effects, which can be cell type-dependent (Grosch, Schiffmann et al. 2012). For instance, C16:0, C24:0, and C24:1 ceramides induce apoptosis in most cell types, but are pro-tumorigenic in HNSCC cells (Karahatay, Thomas et al. 2007, Grosch, Schiffmann et al. 2012). On the other hand, C18:0 ceramide appears to always be apoptotic (Grosch, Schiffmann et al. 2012). These differences can be explained by the biophysical and biochemical properties of the different ceramide species. Specifically, C16:0 ceramide readily interacts with cholesterol, aiding in the formation of lipid rafts, which leads to downstream signalling, while C22:0, C24:0, and C24:1 ceramides have less affinity for cholesterol, and thus block lipid raft formation. In addition, the ratio of long chain (C14-C20) to very long chain (C22-C26) ceramides determines whether autophagy will take place (Grosch, Schiffmann et al. 2012). Furthermore, specific ceramide species activate specific effectors. For example, C16:0 ceramide activates PKCζ (Muller, Ayoub
et al. 1995) and PP2A (Grosch, Schiffmann et al. 2012). Thus, the ceramide species and the cell type in question will determine the resulting phenotype.

I have shown that ceramide is able to prevent an IL-6-induced decrease in the epithelial marker E-cadherin in epithelial breast cancer cells. This makes sense in terms of ceramide being able to prevent IL-6 secretion (see Chapter 4). However, since IL-6 was added exogenously in my experiments, it is likely that there is another mechanism at play, namely that ceramide is able to inhibit STAT3 activation. The effect on E-cadherin was dose-dependent and seen at both the RNA and protein levels. In addition, ceramide was able to rescue the effect of IL-6 on another epithelial marker, EpCAM, as shown by RT-PCR, a finding that would be strengthened by Western blot analysis.

In addition to changes in epithelial and mesenchymal markers, another hallmark of EMT is an increased ability of cells to migrate. To address this aspect of EMT, it would be interesting to test the ability of ceramide to affect cell migration in T47D cells that have been treated with IL-6. Previous research has demonstrated that IL-6 is able to induce cell migration in a Boyden chamber system in T47D cells (De Luca, Lamura et al. 2012). Given the ability of CNL to prevent cell migration in the MDA-MB-231 cell line and to prevent the loss of epithelial markers and cell-cell adhesion in the T47D cell line, I would hypothesize that pre-treatment with CNL would prevent IL-6 from inducing cell migration in the T47D cell line.

The idea that ceramide inhibits STAT3 activation disagrees with a previous paper that showed that ceramide activates STAT1 and STAT3 in fibroblasts (Maziere, Conte et al. 2001). This discrepancy could be due to cell-specific effects or it could depend on the
ceramide species that are being produced. Also, the authors of the previous paper immunoprecipitated phosphor-tyrosine and then immunoblotted STAT3, whereas I used a pSTAT3 antibody. It is unclear whether one protocol is superior to the other.

Despite the anti-EMT, anti-migratory, and pro-anoikic functions of CNL that I have described in this dissertation, the question of whether or not CNL could be a viable treatment for metastatic breast cancer remains an open one. Vincristine, morphine, verteporfin, amphotericin B, cytarabine, doxorubicin, and daunorubicin are all available in liposomal formulations that have been approved by the United States Food and Drug Administration (Fan and Zhang 2013). Furthermore, CNL itself has been shown to be non-toxic in rats (Zolnik, Stern et al. 2008), and a relatively high dose of 72 mg/kg showed therapeutic efficacy with no side effects in a murine model of breast cancer (Stover, Sharma et al. 2005). On the other hand, orally bioavailable drugs that can be given as pills are better tolerated by patients compared to drugs that must be injected intravenously, as would be the case with CNL. For this reason, an orally available small molecule inhibitor of sphingosine kinase, ceramide kinase, or glucosylceramide synthase could be a more effective way of increasing ceramide levels and preventing tumor growth and metastasis. Nevertheless, given its safety and efficacy in rodents and the fact that several liposomal drugs are already on the market, CNL could be a successful new drug for the treatment of breast cancer, including late stage breast cancer.


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VITA

EDUCATION/DEGREES AWARDED

2015  PhD in Cell and Molecular Biology (Dr. Mark Kester, mentor)
      Pennsylvania State University College of Medicine, Hershey, PA

2004  BS in Neurobiology, Physiology, and Behavior, with high honors
      University of California Davis, Davis, CA

PUBLICATIONS

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