MINIREVIEW

Sphingolipids as Determinants of Apoptosis and Chemoresistance in the MCF-7 Cell Model System

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An estimated 182,640 women and 1,990 men were diagnosed with breast cancer in 2008, and approximately 40,480 women and 450 men died from the disease (1). Thus, continued mechanistic studies are needed to understand the causes and develop additional therapeutics for this complicated disease. The MCF-7 cell system is one of the most recognized models for estrogen receptor (ER)-positive breast cancer and has generated ~13,000 publications cited in PubMed to date. A number of clues for biological mechanisms related to apoptotic/anti-apoptotic pathways and chemoresistance were elucidated and summarized in our previous review (2). The focus of this review is new knowledge of the central role of sphingolipid signaling in apoptotic mechanisms in estrogen receptor-positive breast cancer. The ultimate goal is to target crucial steps in survival signaling pathways that may ultimately provide additional translational solutions to the successful pharmacologic treatment of breast cancer. Exp Biol Med 234:1253–1263, 2009

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Role of Sphingolipids in Breast Cancer

Sphingolipids have been implicated in the acquisition of several oncogenic traits including insensitivity to anti-growth signals, evasion of apoptosis, sustained angiogenesis, tumor invasion, and metastasis. The sphingolipid determination of cell fate has been modeled into a proposed rheostat which is often considered in a linear signaling pathway that associates with multiple signaling cascades (Fig. 1). Hannun *et al.* (3) have published a recent review of the regulatory capabilities of sphingolipids and Wymann *et al.* (4) have published a comprehensive review of the role of lipids in disease. Ceramide, sphingosine, and sphingosine-1-phosphate (S1P) have been most often emphasized and characterized in the regulatory rheostat, though recently the topic has become more complex incorporating many other pertinent sphingolipids.

Tumor growth can be arrested and diminished by cytokines, chemotherapy, and radiation, which in addition to other stressors elicit ceramide-mediated signaling to promote cell death (5). Ceramide has been implicated as a pivotal lipid regulating the actin cytoskeleton, endocytosis, the cell cycle, and apoptosis. Once deacylated by ceramidase, ceramide forms sphingosine (3, 6). Phosphorylated by sphingosine kinase, sphingosine is a precursor for S1P, a bioactive lipid involved in cell survival, cell migration, and inflammation (3, 7). Taha *et al.* and Alvarez *et al.* have reviewed the signaling pathways of the different S1P receptors which are members of the G-protein coupled

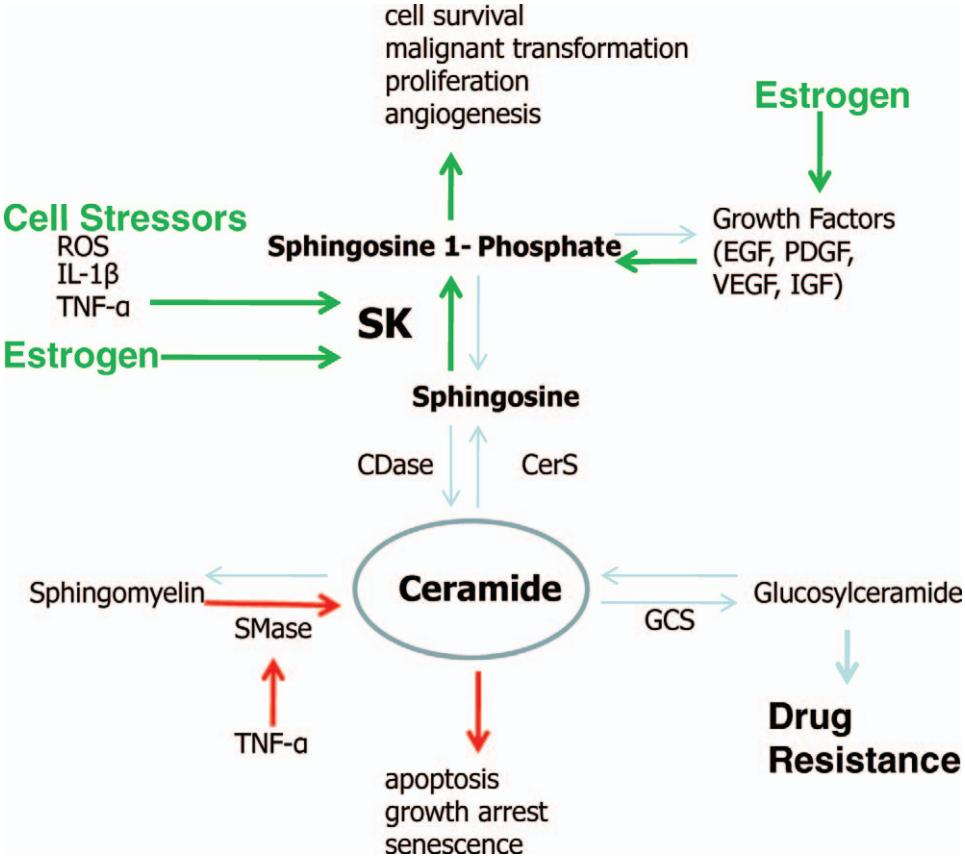

Figure 1. A road map of crucial junctions in sphingolipid signaling. The relative amount of ceramide to sphingosine-1-phosphate is regulated in response to various extracellular stimuli. Cell stressors, estrogen, and growth factors all increase sphingosine kinase activity and the amount of sphingosine-1-phosphate in order to promote cell survival, proliferation, and angiogenesis. Alternatively, cells can increase ceramide in response to TNF-α or chemotherapeutics to induce apoptosis, senescence, and growth arrest. A color version of this figure is available in the online journal.

family of receptors. FTY720 (Novartis®) is a fungal metabolite that once phosphorylated by SK acts on the S1P receptor. FTY729 has received attention as an immunosuppressant agent that is of value in renal transplantation. FTY720 internalizes the S1P receptor of lymphocytes, silencing S1P signaling and sequestering receptors in the lymph nodes while decreasing circulating T cells (8). In clinical trials, FTY720 proved somewhat disappointing due to serious adverse effects such as cardiac arrest. These studies, however, emphasize the prominent role of sphingolipids in the immune response and cell migration. Considering the systemic effects of FTY720, further studies are needed to improve delivery and specificity of pharmacological agonists/antagonists of sphingolipids to target breast cancer (9–11).

Studies of S1P, generated via two isoforms of sphingosine kinase, have provided insights into the intracellular and extracellular effects of tumor microenvironment signaling on apoptosis and chemoresistance (12). Sphingosine kinase 1 (SK1) is the predominant isoform involved in MCF-7 signaling pathways (13). Alvarez *et al.* have provided an informative table describing the activation of SK1 in a variety of different cell lines by diverse agonists and stimuli (13). The overexpression of SK1 promotes migration, proliferation, and survival in MCF-7 breast cancer cells (14, 15). Correspondingly, the downregulation of SK1 in MCF-7 cells correlates with diminished metastasis and chemoresistance (16, 17), suggesting a compelling target for therapeutic intervention. Knockdown of SK1 by siRNA induced apoptosis accompanied by effector caspase activation, cytochrome c release, and Bax oligomerization in the mitochondrial membrane of MCF-7 cells. Thus SK1 knockdown is postulated to be upstream of the mitochondrial pathway of apoptosis and is correlated with increases in ceramide levels (18). Overexpression of SK1 likely induces proliferation in MCF-7 cells in an estrogen-dependent mechanism related to activation of extracellular signal-related kinases (ERK1/2) and through the production of sphingosine-1-phosphate (S1P) (15). Through analyzing tissue samples from invasive breast carcinomas, Ruckhaberele *et al.* found a significant correlation of higher SK1 expression with estrogen receptor (ER) negativity and higher histological grade. Thus, monitoring the overexpression of SK1 which leads to higher levels of S1P may assist in the prognosis of breast cancer (17).

Exploiting Apoptosis in Breast Cancer Therapeutic Interventions

Apoptosis continues to be an exploitable means of cytotoxicity in cancer therapeutic interventions, although there are reports indicating that it doesn't always correlate with total cell kill measured by other means following anticancer therapies (19). Radiotherapy (20), all known chemotherapeutic agents (21), and immunotherapy (22, 23) have been shown to include apoptosis as a determinant for their effectiveness. Resistance to these therapies is common, and therapeutic resistance is a primary mechanism whereby tumor cells progress to a more aggressive phenotype and metastasize. Combating this innate and iatrogenically-induced therapeutic resistance is at the forefront of investigations of oncologic treatment. Aberrant anti-apoptotic signaling has been shown to be responsible for observed chemoresistance both *in vivo* and *in vitro*. Many different molecular pathways and components have been implicated as anti-apoptotic or therapeutic-resistant factors. Very recently, gene profiling studies with breast tumors from 28 patients revealed genes involved in endocytosis, Ras/ERK/AKT, JAK-STAT, and ceramide generation positively associated with the expression of estrogen receptor (ER) (24). Using biologic pathway analysis, Efroni *et al*. identified small common sets of pathways, such as Trka receptor, apoptosis response to DNA damage, telomerase, CD40L, calcineurin, and ceramide, whose differences robustly distinguish diverse tumor types from corresponding normal samples, predict tumor grade, and distinguish phenotypes such as estrogen receptor status and p53 mutation state (25). Further studies are warranted to discern the specific relationships between sphingolipids and aberrant apoptosis.

The Role of TNF-α in Inflammation and Apoptosis

Tumor necrosis factor-α (TNF-α) is a multi-functional cytokine that elicits a variety of biological responses, such as inflammation and apoptosis. Death receptors are members of the TNF ligand superfamily which are also pivotal in the organization of core apoptotic machinery. During oncogenesis the evasion of the immune system and suppression of the antitumor immune response have been correlated with aberrant expression of death receptors (26). Zhang *et al*. found in MCF-7 cells a downregulation of the death receptor CD95 (APO-1/Fas) and an increase in expression of the CD95 ligand, which was suggested to contribute to the establishment of an immune privileged site (27). Therapeutics designed to heighten the immune response to a developing tumor are being investigated in concert with considering the role of sphingolipid-mediated signaling. In MCF-7 cells apoptosis induced through the death receptor TNF receptor 1 is inhibited by the glucocorticoid-like drug, dexamethasone, which required the activation of NF-κB and the anti-apoptotic protein c-IAP1 (28). Interestingly, both TNF-α and dexamethasone have been implicated in the activation of acid sphingomyelinase (ASMase), which catalyzes the hydrolysis of sphingomyelin to apoptotic ceramide. This dichotomy is explained by Redondo *et al*. who suggest that glucocorticoids may protect against apoptotic induction by cytokines, cAMP, tumor suppression, and death signaling in glandular tissue, such as the mammary epithelia, while enhancing apoptosis of cells involved in the inflammation response including monocytes, macrophages, and T-lymphocytes (29). The investigation of the role of sphingolipids in the elimination of T cells is important since this mechanism of establishing tumor immunogenicity must be overcome to have an effective antitumor outcome. The modulation of sphingolipid profiles in different cell systems by glucocorticoids may have a compounding effect of immune suppression and apoptotic inhibition in breast cancer. In MCF-7 breast cancer cells, treatment with tamoxifen and dexamethasone increases clusterin protein levels, which have been implicated in an adaptive response to mediate chemoresistance. Clusterin overexpression has also been correlated with resistance to treatment with the anti-HER-2 antibody trastuzumab (Herceptin) and postulated to be responsible for the antiapoptotic effect of glucocorticoids (29).

Solid tumors must develop a blood supply to sustain proliferation. Because the rate of neovascularization often fails to keep pace with tumor growth, it is common for subpopulations of cells within solid tumors to experience very low oxygen and nutrient levels, as well as high levels of metabolic wastes. Hypoxic stress in tumor cells has been linked to a number of phenotypic changes fundamental to malignant progression, including DNA over replication, gene amplification and the development of resistance to chemotherapeutic agents (30). Nitric oxide (NO) has previously been shown to stabilize hypoxia inducible factor (HIF-1). HIF-1 is a transcription factor specifically activated by oxygen deprivation. In cancer NO may initiate apoptosis through the mitochondrial pathway though this intracellular messenger has also been implicated in the inhibition of cell death. Weigert *et al*. have suggested a self perpetuating mechanism where NO-sensitive apoptotic cells release sphingosine-1-phosphate which then reprograms macrophages from a killing and pro-inflammatory phenotype to an anti-inflammatory and pro-angiogenic phenotype (31). Ader *et al*. provided the first evidence that sphingosine kinase 1 modulates the transcription factor, hypoxia inducible factor 1α (HIF-1α), in several human cancer cell lineages including breast, suggesting a canonical pathway (32). The influence of these external stimuli, which have conventionally linked inflammation and angiogenesis to S1P in endothelial cell populations, is continuing to be explored in breast cancer cell populations.

Stress pathways initiated as a result of impaired inflammatory responses and vascular development have been linked to sphingolipid signaling, particularly with the involvement of ceramide. The reactive oxygen species

(ROS), superoxide anions, hydrogen peroxide, and hydroxyl radicals are generated in the mitochondria during the transport of electrons and have been implicated as second messengers that when generated in response to IL-1β, TNF-α, or lipopolysaccharide initiate a pro-inflammatory signal transduction cascade (33). The cytokines, TNF-α and IL-1, along with epidermal growth factor and platelet-derived growth factor also activate SK1 which transiently elevates levels of S1P. (3, 34) The dichotomy of proinflammatory molecules to elicit cell survival and death pathways in breast cancer may be further explained in the inter-relationships between sphingolipids.

NF-κB Transcriptional Activity and Its Role in Chemoresistance

A diverse array of agents (NSAIDS, sulfasalazine, glucocorticoids, SERMS, thalidomide, and immunosuppressive drugs) suppresses nuclear transcription factor nuclear factor-κB (NF-κB) to restore a radio- or chemotherapeutic responsive phenotype (35). In our laboratory we observed that both pharmacologic and molecular manipulation of NF-κB transcriptional activity in a drug resistant MCF-7 cell model system affected the potency and efficacy of standard chemotherapeutic agents (36). Isogenic variants of MCF-7 cells (MCF-7TN-R) demonstrated profound resistance to the apoptosis-inducing effects of tumor necrosis factor-α (TNF-α) (50 ng/ml) in contrast to the parental MCF-7 cells which after treatment with TNF-α (1 ng/ml) had a 50% reduction in cell number. The MCF-7TN-R cells also had a diminished sensitivity to several common chemotherapeutic agents (doxorubicin, etoposide) compared to the parental MCF-7 cells. Furthermore a substantial increase in NF-κB transcriptional activity was documented following treatment of the MCF-7TN-R cells with TNF-α reiterating the transcription factor's role in resistance to apoptosis (36). The reconstitution of a chemotherapeutic responsive phenotype through targeting the sphingolipid profile was proposed because the accumulation of ceramide in the mitochondria in response to TNF-α had been correlated to Bax translocation to the mitochondria and subsequent cytochrome c release and cell death (37). TNF-α induced NF-κB transcriptional activity in MCF-7TN-R cells was greatly diminished with the administration of subsequent doses of ceramide. Similar to our findings in our MCF-7 drug-resistant cell model system, Montagut *et al*. recently compared tumor specimens from 51 breast cancer patients treated with anthracyclin and/or taxane-containing neo-adjuvant chemotherapy and correlated activation of NF-κB with chemoresistance (38). The anti-apoptotic response regulated by NF-κB is also considered to be a downstream determinant of chemoresistance for many signaling pathways (36).

Mechanisms of chemotherapeutic ineffectiveness such as heightened NF-κB activity are often determined by shifts in endocrine responsiveness. Prolonged anti-estrogen exposure may cause loss of estrogen receptor (ER)-mediated signaling to apoptosis. In addition, the shift in hormone responsiveness could therefore dampen other chemotherapeutic treatments that commandeer parallel downstream mechanisms utilized by ER (39). Gu *et al*. proposed a correlation between the downregulation of interferon regulatory factor (IRF-1), and a coordinated up-regulation of its inhibitor, nucleophosmin (NPM), and NF-κB with anti-estrogen resistance. NPM reduced levels of IRF-1, potentially eliminating its ability to initiate an apoptotic caspase cascade through caspase 1 and/or caspase 7. Such an effect would likely eliminate the ability of IRF-1 to induce p21 and cooperate with wild type p53 in signaling to apoptosis (39). Zhou *et al*. found tamoxifen-resistant MCF-7 cells exhibited enhanced NF-κB and activator protein 1 (AP-1) transcriptional activity. Furthermore, the transcription profiles of four independent sets of ER-positive breast cancer revealed a correlation between high expression of NF-κB and AP-1 regulated genes to early metastatic relapse (40). Following treatment with the anti-estrogen, faslodex (ICI 182,780; ICI), in combination with the pharmacological inhibitor of NF-κB, parthenolide, a synergistic reduction in cell growth was documented in anti-estrogen-resistant MCF-7/LCC9 cells. Parthenolide has been promoted as an anti-inflammatory, anticancer, and anti-angiogenic agent that has successfully undergone phase I/II clinical trials (41, 42). Common therapeutic agents may benefit from combining strategies that decrease NF-κB activity.

Steroid and Peptide Hormone Receptors in Sphingolipid Signaling

The MCF-7 cell system is most commonly used as a model for evaluating the possible pharmacological responses of estrogen receptor (ER)-positive breast cancer. Estrogens and related ligands mediate most of their effects in breast cancer by binding to ERα, which then either mediates survival through direct activation of gene expression (genomic action) or by utilizing rapid non-genomic pathways. ER is bound to caveolin within cell membrane rafts. Interconnected associations characteristic of non-genomic signaling, which often circumvents endocrine therapy, are comparable and beginning to prove responsible with the wide array of responses initiated by sphingolipids.

Current studies are exploring how the S1P receptor associates with growth receptors and the ER in cell membrane rafts. Previously, S1P has been implicated in the transactivation of growth factor receptor tyrosine kinases such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) receptors (43). Whether the cytokines and growth factors that share similar signaling pathways with the S1P receptor are connected or parallel is still being investigated. The EGFR tyrosine kinase inhibitor, gefitinib (ZD1839, Iressa®), blocks the interaction between insulin-

like growth factor receptor (IGF-IR) and EGFR, inhibiting IGF-1-induced phosphorylation of MAPK in MCF-7 cells (44, 45). Similar studies investigating the S1P receptor in MCF-7 cells will determine whether sphingolipids at the plasma membrane play an important role in the initiation and progression of a transformed oncogenic phenotype.

The cytosolic accumulation of sphingolipids as a result of a shared growth factor signaling cascade originating from the plasma membrane and activating SK has been implicated in the malignant transformation of MCF-7 cells. Sphingosine kinase 2 (SK2) production of S1P in apoptotic MCF-7 cells leads to the development of tumor-associated macrophages that regulate NF-κB activation, change cytokine production, and reduce tumor cytotoxicity (31). Sukocheva *et al.* implicated SK1 in 17 beta-estradiol (E2)-dependent mitogenesis and demonstrated that E2-induced SK1 activity resulted in Ca2+ mobilization and ERK1/2 activation (14). These investigators then proceeded to show that in MCF-7 cells E2 induction of epidermal growth factor receptor (EGFR) was mediated by SK1 activation of S1P. Expression of EGFR is associated with poor prognosis and reduced responsiveness to anti-hormone therapy (46, 47). A current challenge in the modulation of sphingolipid signaling is locating the subcellular sites where bioactive lipids are accumulating in response to stress.

The transmission of signaling from the plasma membrane to organelles such as the mitochondria has been explored in both sphingolipid and estrogen signaling pathways. ER contributes to tamoxifen resistance through bypassing the antagonistic action of tamoxifen in the nucleus and possibly inhibiting the mitochondria-mediated apoptotic pathway of the Bcl-2 family (48). Estrogen and insulin-like growth factor (IGF-1) inhibit apoptosis in MCF-7 cells through nongenomic signaling by enhanced phosphorylation of BAD (a proapoptotic Bcl-2 family member). BAD inactivation results from activation of Ras/ERK/p90RSK and Ras/PI-3K/Akt pathways by estrogen (49). Bcl-2 has been shown to inhibit apoptosis of MCF-7 cells induced by ceramide and thapsigargin, but not by doxorubicin or tumor necrosis factor (TNF-α) (50).

These alternative pathways are often pivotal in response to stressors. In MCF-7 cells, UV increases the generation of mitochondrial ROS and subsequently induces c-Jun N-terminal kinase (JNK) activation. E2 inhibition of ROS can indirectly deregulate JNK activity. E2 acts directly on the mitochondria to inhibit mROS by up-regulating the antioxidant manganese superoxide dismutase (MnSOD). JNK activation, once stimulated by ROS, subsequently promotes the translocation and dimerization of Bax. In the mitochondria Bax inserts into the membrane to promote pore formation and the release of cytochrome c into the cytoplasm (51, 52). In a parallel pathway ceramide production subsequent to UV-induced acid sphingomyelinase activation potentially regulates Bax conformation changes at the mitochondrial membrane (53). Therefore, therapeutics that increase ceramide generation are likely to be effective at reducing the mitogenic effect of E2 in ER-positive breast cancer through antagonizing Bcl-2 proliferative signaling and activating pro-apoptotic factors. The impact of sphingolipids in specific subcellular localizations on the whole cell and homeostasis of the tumor environment are important to understand the translation of their use as chemotherapeutics.

PI3K/Akt Activation and Its Link to Chemoresistance in MCF-7 Cells

Akt/Protein kinase B (PKB), a serine-threonine protein kinase, appears promiscuous in the phosphorylation of several downstream effectors potentiating its effectiveness in mediating resistance to drug-induced apoptosis. The Akt pathway has also been found to be elevated in 40% of breast cancer patients (54). Downstream of phosphoinositide 3-kinase (PI3-K), Akt activation initiates a network of responses including positively regulating G_1/S cell cycle progression through inactivation of glycogen synthase kinase 3-β (GSK3-β) (Fig. 2). Through phosphorylation, GSK3-β is inactivated leading to an increase in cyclin D1, a key regulator of the cell cycle that is up-regulated in approximately 50% of breast cancers (55, 56). Sorafenib (BAY 43–9006), originally described as a nonspecific Raf kinase inhibitor was shown to reduce Akt phosphorylation and cyclin D1 protein levels although clinical efficacy has not been shown (57). Our laboratory has found that ceramide suppresses cyclin D1 levels in MCF-7 cells (unpublished observations). In MDA-MB-231 breast cancer cells, sorafenib and nanoliposomal ceramide synergistically enhanced apoptosis, decreased cell proliferation and effectively diminished tumor formation *in vivo* (57). In the same study Tran *et al.* elude to a preclinical evaluation of ceramide and the inhibition of Akt (57). Therapeutics initiating apoptotic and proliferative responses leading to oncogenesis are often linked to Akt signaling.

MCF-7 cells have low-level Akt activation which is pertinent when dosing with drugs such as CMEP (NSC632855, 9-chloro-2-methylellipticinium acetate), which inhibits only constitutive or ligand (heregulin)-stimulated Akt activation. In this study, drug-induced apoptosis was also correlated with a lack of mutant p53 in MCF-7 cells (58). Our laboratory observed that ceramide-induced cytotoxicity in MCF-7 cells is partly mediated by the inactivation of p53 and thus deregulation of p21 (unpublished observations). While there is literature proposing p21 contributes to p53-dependent apoptosis in response to chemotherapeutic agents, our results suggested that p21 may act as an anti-apoptotic factor in MCF-7 cells. p21 is a member of the Cip/Kip family of cyclin/cyclin-dependent kinase (CDK) inhibitors and has been cited as a G1 checkpoint protein, preventing cell cycle progression into S phase. Ceramide-induced changes in p53 and p21 levels/signaling were associated with arrest of MCF-7 cells in G_0/G_1-phase reiterating the importance of the cell cycle

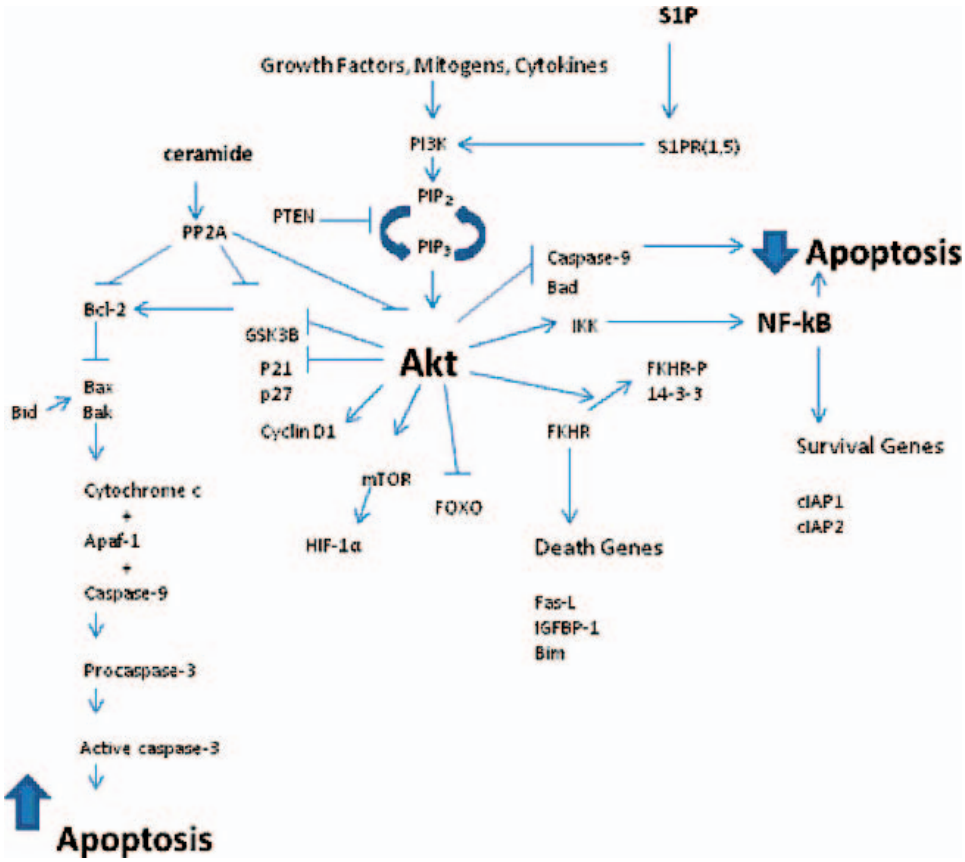

Figure 2. Role of Akt in control of apoptotic mechanisms. Sphingolipids along with growth factor receptors, particularly those involved with an oncogenic or metabolic response, may intersect at the Akt signal transduction pathway. Ceramide activates protein phosphatase-2A (PP2A) resulting in the inhibition of Akt and induction of a mitochondria-centric pathway through a caspase cascade triggering apoptosis. S1P binds to one of five S1P receptors (S1PR1–5), resulting in the activation of the PI3K-Akt pathway. Subsequently Akt promotes cell survival through inactivating pro-apoptotic components such as caspase-9, BAD, forkhead receptor (FKHR), and Forkhead Box Class O (FOXO) while activating NF-κB, a key mechanism in anti-apoptotic control. Through the inhibition of glycogen synthase kinase-3β (GSK-3β), p21 and p27, as well as decreasing the degradation of cyclin D_1, Akt promotes the progression of the cell cycle. The mammalian target of rapamycin (mTOR) is also regulated by Akt and promotes cell growth and proliferation (84). A color version of this figure is available in the online journal.

regulation in chemoresistance. Spiegel *et al*. found that sphingosine kinase-2 (SK2) is involved in p53-independent induction of p21. Furthermore, doxorubicin-induced apoptosis of MCF-7 cells was enhanced by the down-regulation of SK2 in MCF-7 cells (59). The loss of CDK inhibition mediated by p21 also leads to hyperphosphorylation of ER at serine 118, which in turn leads to the increased expression of known ER-regulated genes (60).

Akt appears important in IκB kinase (IKK)-mediated destruction of I-kB and activation of NF-κB (61, 62). Subsequently, through degrading IKKα and independent of NF-κB, Akt may influence estrogen receptor-mediated gene activation including cyclin D1, c-myc, c-fos, and estrogen receptor-binding fragment-associated gene 9 (EBAG9) (63). MCF-7 breast cancer cell lines expressing a constitutively active Akt proliferate under reduced estrogen conditions and are resistant to the growth inhibitory effects of tamoxifen, both *in vitro* as well as in *in vivo* xenograft models (64). Sunters *et al*. propose that in MCF-7 cells the apoptotic response to paclitaxel (often used when tumors establish resistance to anti-estrogen therapies) may involve FOXO3a nuclear localization as a result of decreased PI3K-Akt signaling, which is also correlated to JNK1/2 inhibition (65). The Akt pathway has been targeted by inhibiting the mammalian target of rapamycin (mTOR), which once activated by Akt, enhances the stabilization of cyclin D1 and c-myc. The mTOR inhibitor, RAD001(everoliomus) repressed estrogen-dependent growth of MCF-7 and aromatase-expressing (MCF-7/Aro) cells and proved synergistic in combination with the aromatase inhibitor, letrozole (66). A phase I study combining RAD001 and letrozole demonstrated an anti-tumor activity with side effects comparable to accepted oral therapies that sufficiently benefit patients (67).

Sphingolipid Therapeutics and Translational Significance

Ceramide and Cancer Treatment. From a pharmacological perspective, the development of new drugs that can induce apoptosis or overcome resistance mechanisms provides the impetus to continue exploring the role of sphingolipids in MCF-7 cells. Numerous anticancer drugs

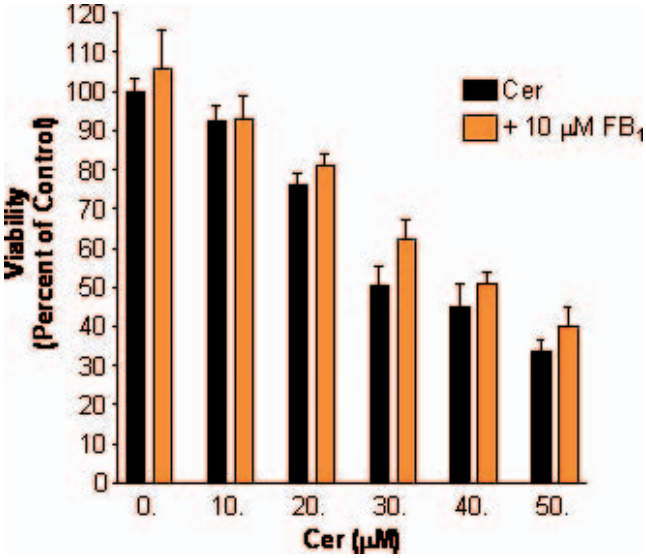

Figure 3. Inhibition of ceramide synthase does not alter Cer-mediated cell death in MCF-7 cell variants. MCF-7TN-R (TNF-α resistant MCF-7 cell line variants) were plated at 7.5×10^6 cells per 96-well plate in phenol-free DMEM. The following day, the cells were either treated with the indicated concentrations of Cer ((2S,3R)-N-octanoylsphingosine) for 24 hrs or pretreated with 10.0 μM fumonisin B_1 (FB_1) for 1 hr followed by treatment with the indicated concentrations of Cer for 24 hrs. Following incubation, viability was estimated using MTT viability assay. Data are presented as percent viability of vehicle-treated control cells. Mean values ± SE of five different experiments in quadruplicate are reported. A color version of this figure is available in the online journal.

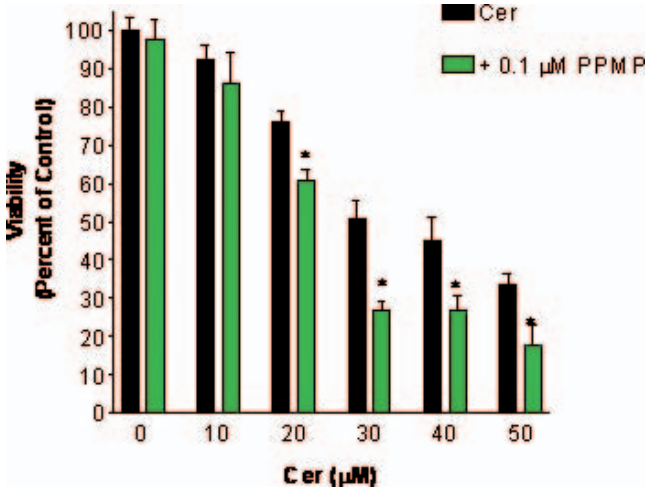

Figure 4. Inhibition of glucosylceramide synthase enhances Cer-mediated cell death in MCF-7 cell variants. MCF-7TN-R (TNF-α resistant MCF-7 cell line variants) were plated at 7.5×10^6 cells per 96-well plate in phenol-free DMEM. The following day, the cells were either treated with the indicated concentrations of Cer for 24 hrs or pretreated with 0.1 μM PPMP (DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol HCL) for 1 hr followed by treatment with the indicated concentrations of Cer for 24 hrs. Following incubation, viability was estimated using MTT viability assay. Data are presented as percent viability of vehicle-treated control cells. Mean values ± SE of five different experiments in quadruplicate are reported (* $P < 0.01$ as determined by two-way ANOVA). A color version of this figure is available in the online journal.

utilize ceramide to produce their effects. Most of these drugs increase endogenous levels of ceramide by either increasing *de novo* synthesis or inhibiting the breakdown of ceramide. Anthracyclines, anti-estrogens, retinoids, taxanes, and vinca alkaloids all stimulate ceramide accumulation. Furthermore, exogenous treatment of ceramide can act in a synergistic manner when used concurrently with some anticancer drugs, such as tamoxifen (68–74).

The alteration of ceramide metabolism in a number of different profiles has been explored highlighting the diversity and elusiveness of its downstream effects. The intracerebroventricular administration in mice of fumonisin B1 (FB1) (fungal sphingosine analog and potential inhibitor of ceramide synthase) caused neuro-degeneration in the cortex and activation of astrocytes in the hippocampal area (75). Additionally, intravenous administration of FB1 decreased cardiovascular function in horses (76). α-Galactosylceramide (α-GalCer) sensitization facilitated lipopolysaccharide (LPS)-mediated lethal systemic shock (pulmonary lesions, infiltration of inflammatory cells, and cell death) in mice and was proposed to utilize interferon (IFN)-γ and TNF-α signaling (77). Toxicity has been avoided by focusing on lipid delivery mechanisms that consider the hydrophobic interactions and subcellular localization of sphingolipids in the MCF-7 cell model. Novgorodov *et al*. attempted to tie the specificity of positively charged ceramides for the inner mitochondrial membrane and matrix space to an interaction with latent binding sites (78). In another study, C6-ceramide administered intravenously in liposomes enhanced its solubility, cell permeability, and systemic delivery, thus limiting tumor growth in a MDA-MB-231 nude mouse tumor model (79).

Premature studies of therapeutics can be detrimental reiterating the importance of a relevant and well established cell line for *in vitro* studies. The myriad of responses in response to sphingolipid targeting drugs in MCF-7 cells also emphasizes the complexity of ceramide metabolism. In our hands the inhibition of ceramide synthase did not alter ceramide-mediated cell death in the TNF-α resistant MCF-7 cell line (Fig. 3). Bielawska *et al*. targets specific subcellular compartments with B13 (acid ceramidase inhibitor) and D-erythro-MAPP (alkaline ceramidase inhibitor) analogs that affect ceramide metabolism to explain sphingolipid species expression and localization. They propose a mitochondrial-associated metabolic pathway that regulates the interconversion of ceramide and sphingosine. C_{14}-, C_{16}- and C_{18}-Cers generation, resulting from treatment with their selected analogs closely correlated with inhibitory effects on MCF-7 cell growth (80). In our preliminary experiments the inhibition of ceramidase with MAPP did not affect ceramide-mediated cell death in the MCF-7 parental or chemoresistant variant cells, supporting Bielawska's observations that more selective analogs might be therapeutically relevant. The dysregulation of ceramide metabolism as an explanation of multidrug chemotherapy resistance relies on

Table 1. Pharmacological Studies of Sphingolipid Modulators in MCF-7 Breast Cancer Cells

Name of available drugs	Mode of action	Reference citing MCF-7
2-amino-2-(2-(4-octylphenyl)ethyl)-1,3-propanediol hydrochloride (FTY720/fingolimod)	S1P(1–5) receptor agonist	Nagaoka, 2008 (85)
Fumonisin B1	Ceramide synthase inhibitor	Becker, 2005 (86)
(2S,3R)-(4E,6E)-2-octanoylamidooctadecadiene-1,3-diol (4,6-diene-Cer)	Ceramide mimic	Struckhoff, 2004 (74)
N-hexanoylsphingosine (C6-ceramide), D-erythro N-octanoylsphingosine (C8-ceramide)	Ceramide mimic	Lucci, 1999 (68) Gouaze Andersson, 2007 (87)
N,N-dimethylsphingosine (DMS)	Inhibitor of SK-1	Lavieu, 2006 (70) Nagaoka, 2008 (85)
1S,2R-D-erythro-2-N-myristoylamino-1-phenyl-1-propanol (D-erythro-MAPP) analogs	Alkaline ceramidase inhibitor	Bielawska, 2008 (80)
1R,2R-D-erythro-2-N-myristoylamino-1-nitrophenyl-propan-1,2-diol (B13) analogs	Acid ceramidase inhibitor	Bielawska, 2008 (80)
1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP)	Glucosylceramide synthase inhibitor	Gouaze, 2005 (83)

understanding the conversion and activation of short-chain ceramide species.

The metabolism of ceramide through glycosylation is another contributor to the development of drug resistance. In our laboratory DL-threo-1-phenyly-2-palmitoylamino-3-morpholino-1-propanol (PPMP), a commercially available selective inhibitor of glucosylceramide synthase (GCS), enhanced ceramide-induced cell death (Fig. 4). MCF-7 cells with TNF-α resistance (MCF-7TN-R) were more sensitive to PPMP treatment than the parental cells suggesting that TNF-α resistance in MCF-7 cells may utilize GCS to detoxify ceramide accumulation (81, 82). Previously Gouaze *et al.* proposed that lipids play a role in the establishment of multidrug resistance for doxorubicin-resistant MCF-7 cells. Gouaze *et al.* found PPMP decreased ganglioside levels, restored sensitivity to vinblastine, enhanced vinblastine uptake, and diminished expression of multidrug resistance (MDR1 gene) (83). NF-κB inhibition via ceramide can also reduce expression of MDR1 and P-glycoprotein. DMS, a selective inhibitor of sphingosine kinase, contributes to ceramide-induced MCF-7TN-R cell death. The inhibition of SK activity by DMS in MCF-7 cells impairs starvation-induced autophagy introducing a novel aspect of sphingolipid-promoted survival (70). Starvation and subsequent activation of autophagy in MCF-7 breast cancer cells may prove to be a useful model for understanding initial oncogenic progression.

Therapeutics specifically engineered to modulate sphingolipids may assist in foreseeing or deterring secondary effects. Our laboratory in collaboration with Dr. Charles Smith at UMSC in Charleston is investigating novel sphingosine kinase inhibitors in the MCF-7 cell model. We found the combination of short-chain ceramides and sphingosine kinase inhibitors to be synergistic in treating the metastatic breast cancer cell line MDA-MB-231 *in vitro* (data not shown). The combination of ceramide and sphingosine kinase inhibitors decreases the IC_{50} from micromolar to nanomolar concentrations, making combination treatment much more effective than treatment individually.

Chemoresistance and the Sphingolipid Rheostat: Challenges Remaining

Our understanding of the MCF-7 cell system, which was initially defined by studies of the estrogen receptor, is expanding to include a signaling pathway that may ultimately drive chemoresistance. Drugs that target the sphingolipid pathway in the MCF-7 cell model system (Table 1) are just beginning to show promise in the clinical setting. Clinical trials are currently underway to determine if liposomal preparations of ceramide analogs might be more efficacious in their targeting of breast tumors. A phase II study of topical ceramide lipids as treatment for cutaneous breast cancer is currently ongoing. The challenges remaining include cell impermeability, precipitation

in aqueous solutions, leading to suboptimal drug delivery. Currently, scientists are working to engineer successful drugs that can be delivered to *in vivo* targets without causing significant side effects. The finding that positively charged ceramide analogs more readily target the negatively charged mitochondria in cancer cells may lead to differential compartmental concentration within these cells with fewer side effects. In addition, the specific details of how sphingolipids work in tissue homeostasis as distinguished from specific pathophysiological situations are not entirely clear. They will be important when designing sphingolipid-based therapies. It is anticipated that these challenges will be met within the foreseeable future and will lead to a significant surge in translational success.

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