Sphingolipid targets in cancer therapy

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Abstract
Considerable progress has been made recently in our understanding of the role of ceramide in the induction of apoptotic cell death. Ceramide is produced by cancer cells in response to exposure to radiation and most chemotherapeutics and is an intracellular second messenger that activates enzymes, leading to apoptosis. Because of its central role in apoptosis, pharmacologic manipulation of intracellular ceramide levels should result in attenuation or enhancement of drug resistance. This may be achieved through direct application of sphingolipids or by the inhibition/activation of the enzymes that either produce or use ceramide. In addition, attention should be given to the subcellular location of ceramide generation, because this has been shown to affect the biological activity of sphingolipids. This review summarizes the sphingolipid biosynthetic pathway, as it relates to the identification of important targets for drug discovery, and the development of novel agents capable of enhancing chemotherapy.

Introduction
Cell death occurs via two distinct pathways, necrosis and apoptosis, the choice depending on the type of cell and the specific injury. Necrosis is a process by which cells die due to failure of membrane integrity, both plasma and organelle membranes, and is usually followed by an inflammatory response. Apoptosis, on the other hand, proceeds in an orderly process through the activation of specific metabolic pathways and is usually not associated with an inflammatory response. Apoptosis is now recognized as the major pathway by which cytotoxic agents induce cell death. It can be initiated through receptor-mediated and non–receptor-mediated processes, both of which eventually lead to the activation of effector caspases, the proteases responsible for dismantling the cell.

It has become apparent that both receptor-mediated and non–receptor-mediated processes use ceramide as an intracellular, proapoptotic signaling molecule. Since the late 1980s, when the first reports linking hydrolysis of sphingomyelin to signal transduction appeared, knowledge of sphingolipids as second messengers has expanded considerably. It is now clear that ceramide has a central role in both apoptotic and mitogenic pathways. Administration of water-soluble ceramides to cells in vitro can initiate apoptosis. On the other hand, reducing intracellular ceramide levels leads to drug resistance. Ceramide is often generated in response to chemotherapeutics and radiotherapeutics via hydrolysis of sphingomyelin by activated sphingomyelinase (SMase).

Modulation of sphingolipid-induced apoptosis has been proposed as a means to increase the sensitivity of tumors to various therapeutic agents (1, 2). Sphingosine kinase, ceramidase, and glycosylceramide synthase, among other enzymes important to sphingolipid metabolism, are being studied as potential new drug targets. However, other aspects of sphingolipid signaling pathways are equally attractive as a means of enhancing drug-induced apoptosis. These include up-regulation of ceramide synthesis and increasing the level of intracellular sphingomyelin through the use of exogenously administered sphingomyelin. This review summarizes the current understanding of ceramide metabolism as it relates to the development of new strategies and/or reagents for the treatment of cancer.

Sphingolipids in Cell Death
Ceramide-Mediated Apoptosis
Ceramide has been shown to be a mediator of cellular stress (reviewed in refs. 3–5). There are numerous agents that can induce accumulation of ceramide within the cell, including cytokines (tumor necrosis factor-α and Fas), environmental stress (heat and hypoxia), radiation (UV and ionization), and chemotherapeutics (doxorubicin, 1-β-D-arabinofuranosylcytosine, etoposide, PSC-833, vincristine, and many others). The generation and accumulation of ceramide can result from an increase in de novo synthesis, as has been shown by the use of cytokines and/or drugs (PSC-833 and doxorubicin) or through the hydrolysis of sphingomyelin by SMases (cytokines, doxorubicin and most other chemotherapeutics, and radiation). Which of these two pathways is used is largely cell type and stimulus specific.
That ceramide is a second messenger of apoptosis is indicated by numerous studies showing a rapid (2–30 minutes) increase in ceramide levels before the onset of apoptosis and the activation of caspases, although some studies note a sustained level over longer time periods, suggesting that ceramide may also play a role in the execution of apoptosis (6). Induction of apoptosis also can be achieved using exogenously administered, water-soluble ceramides.

Comparing lipids that vary in key structural elements, particularly, the C4-C5 double bond, N-acylation, and phosphorylation of the O1 hydroxyl group, it is observed that these elements profoundly affect the biological activity of ceramide (Fig. 1). The C4-C5 double bond is essential for induction of apoptosis, because dihydroceramide is not apoptotic. Deacylated ceramide (sphingosine) is also proapoptotic, indicating that the acyl group is dispensable for apoptosis induction. However, phosphorylation of either ceramide or sphingosine results in bioactive lipids that have mitogenic capabilities. Diacylglycerol is mitogenic, indicating a strict requirement for the serine core in ceramide for its apoptotic function. Recent evidence also suggests that N-acyl chain length influences the induction of apoptosis by ceramide, with N-palmitoyl-sphingosine (C16-ceramide) being the most potent inducer of apoptosis compared with ceramides with other chain lengths (7). In addition, we have observed that sphingomyelins with differing N-acyl chain lengths are capable of providing varying degrees of chemosensitization.1 In this regard, sphingomyelin derived from egg yolk, which is rich in C16-sphingomyelin, is more effective compared with milk-derived or brain-derived sphingomyelin, which contain significantly less C16 and more of the longer acyl chains.

Modulating the expression and activity of enzymes directly involved in ceramide production or use affects therapeutic efficacy (1, 2). Blocking ceramide production through the use of the ceramide synthase inhibitor fumonisin B1 has been reported to attenuate the apoptotic response in several cell lines (8, 9). Altering the activity of SMase in cells can confer apoptotic resistance or sensitivity, depending on whether activity, and hence the ability to produce ceramide from sphingomyelin, is increased or decreased, respectively. Conversely, cells from patients with Niemann-Pick disease, a lysosomal storage disease that results from a genetic deficiency in SMase with an acidic pH optimum (aSMase), have a reduced apoptotic response that can be restored through transfection of the aSMase gene (10). Reducing the expression of aSMase through the generation of knockout mice also reduces the apoptotic response (10).

Increasing intracellular ceramide content, through increased de novo synthesis or inhibition of ceramide use, has the effect of increasing drug sensitivity and apoptosis. For instance, stimulation of de novo ceramide synthesis with PSC-833 (11) increases production of ceramide and provides a greater apoptotic response. Similarly, ceramide levels and apoptosis are also increased through inhibition of ceramidase using B13 or (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (12, 13), or inhibition of glycosylceramide synthase (GCS), which converts ceramide into nonapoptotic glucosylceramide, using tamoxifen or DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (14).

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1 Unpublished results.

Figure 1. The biosynthetic pathway of sphingolipids. NOE, N-oleylethanolamine; MAPP, (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol; NBDJ, N-butyldoxygoejirimycin; PPMP, DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; DMS, dimethylsphingosine; PC, phosphatidylcholine.
Biology of Sphingomyelinases

Activation of SMase is the predominant pathway for the generation of ceramide in response to chemotherapy (3, 5). Although activation of de novo ceramide synthesis has been reported, it is less common (8). Five SMases have been described in mammalian cells, differing in pH optima, cation dependency, and intracellular location (15). SMase was first identified in the lysosomes as an enzyme with an acidic pH optimum and was thought to be responsible for cellular membrane turnover. This enzyme, now known as aSMase, is found primarily in the lysosomes and to a lesser degree in the caveolae, sphingolipid-rich microdomains of the plasma membrane that are involved in receptor-mediated signaling. One view is that ligand binding to specific membrane receptors activates aSMase with subsequent generation of ceramide (16). This aSMase-generated ceramide might then promote the clustering of raft domains and thus the signal-transducing receptors in these domains. Clustering of signal receptors has been shown to be necessary for their function.

Acidic SMase−/− cells from Niemann-Pick patients and cells from aSMase knockout mice retain a SMase activity with a neutral pH optimum (neutral SMase, nSMase). Several forms of nSMase exist, including a plasma membrane-bound, Mg2+-dependent and a cytosolic, Mg2+-independent form as well as nuclear and mitochondrial forms (4, 15, 17). Although two putative nSMase genes have been cloned, the molecular identity of these proteins remains unclear. nSMase can be activated by phosphatidylinerse, DTT, and Mg2+ and can be inhibited by glutathione and EDTA. Inhibition of nSMase by glutathione links this enzyme to the cells’ redox status, an important point because many drugs, as well as radiation, increase intracellular reactive oxygen species as part of their presumed mode of toxicity (18). nSMase is activated by a wide range of drugs and therapeutics, including tumor necrosis factor, Fas, radiation, most nonalkylating agents, hypoxia, heat shock, and serum deprivation. Lastly, a SMase with a basic pH optimum (bSMase) is present in the lumen of the intestine and is likely involved in digestion (20). bSMase has been identified in the lysozomes as an enzyme with an acidic pH optimum and was thought to be responsible for cellular membrane turnover. This enzyme, now known as aSMase, is found primarily in the lysosomes and to a lesser degree in the caveolae, sphingolipid-rich microdomains of the plasma membrane that are involved in receptor-mediated signaling. One view is that ligand binding to specific membrane receptors activates aSMase with subsequent generation of ceramide (16). This aSMase-generated ceramide might then promote the clustering of raft domains and thus the signal-transducing receptors in these domains. Clustering of signal receptors has been shown to be necessary for their function.

Targets of Ceramide

Once generated, ceramide interacts with a variety of intracellular targets, including kinases, phosphatases, and proteases, to activate downstream effector molecules (3, 20, 21). Ceramide interacts with the ceramide-activated protein kinase (also known as kinase suppressor of Ras) to activate c-Raf and the extracellular signal-regulated kinase 1/2 signal transduction pathway. Ceramide activates protein kinase Cζ, which, in turn, activates the stress-activated protein kinase pathway. Activation of this pathway leads to multiple effects, including enhanced alternative splicing through activation of hnRNP A1 and inhibition of protein synthesis via the RAX-PKR (double-stranded RNA–dependent protein kinase)/eIF2α pathway (22). Phosphatases PP2A and PPI are activated by ceramide (23, 24) and dephosphorylate bcl-2, Akt/protein kinase B, retinoblastoma protein, c-Jun, and the nuclear serine/arginine-rich domain proteins. Ceramide recruits PP2A, through a regulatory B subunit, to mitochondria where it activates the Bcl-2 phosphatase to also inactivate bcl-2 (25). Activation of phospholipase A2 by ceramide has been shown (26). The protease cathepsin D is activated by ceramide and participates in the activation of caspase-3 after translocation from the lysosomes (27).

Site of Ceramide Production

The conventional view, until recently, was that sphingomyelin was strictly a structural component of the cell; thus, sphingomyelin in organelles not associated with secretory/ endocytotic vesicles was due to contamination. The discovery that ceramide is a potent intracellular second messenger suggests that nuclear and mitochondrial sphingomyelin may, in fact, play a role in the normal physiology of the cell. Although intra-membrane and inter-membrane movements of lipids occur as part of normal metabolism, it now seems that the intracellular site of ceramide production is critical to the initiation of the apoptotic response (2, 4). Bacterial SMase, added to cell culture media, readily cleaves externally accessible sphingomyelin to ceramide. However, it does not lead to an apoptotic response (28). On the other hand, intracellular expression of the enzyme does lead to apoptosis. Several investigators have concluded that caveolar-bound SMase, or plasma membrane inner leaflet-associated SMase, is responsible for increased ceramide levels after exposure to cytokines and radiation (29). Others point to the mitochondria as the site of ceramide generation and induction of apoptosis (4, 30, 31). Addition of ceramide to isolated mitochondria results in the release of cytochrome c, the production of reactive oxygen species, and the inhibition of respiratory complex III (32, 33). Mitochondria contain sphingomyelin and several enzymes of sphingolipid metabolism, including ceramide synthase (34), SMase (3), and ceramidase (35). Birbes et al. (31) targeted active or inactive forms of bacterial SMase to specific subcellular compartments (mitochondrial, nuclear, endosomal, plasma membrane, and cytoplasmic) in Molt-4 cells. Only when the active SMase was targeted to mitochondria could apoptosis be initiated. The induction of apoptosis could be blocked by bcl-2, indicating that ceramide production is upstream from the bcl-2 control point.

Sphingolipids have also been identified in intracellular compartments other than the mitochondria and compartments of the endocytic pathway. Sphingomyelin is present in the nucleus and associated with areas of active transcription (36). Digestion of intact nuclei with RNase resulted in a decrease of sphingomyelin from 4.5% of the total phospholipid to 0.8% (37). Similarly, digestion of nuclear sphingomyelin with SMase resulted in increased RNase sensitivity. In addition, both sphingomyelin synthase and SMase are present; thus, it seems that an equilibrium between sphingomyelin and ceramide exists within the nucleus. There is also a higher ratio of short-chain ceramides (C16:0 + C18:0) to long-chain ceramides (C24:0 + C24:1) within the chromatin, when compared with nuclear envelope or nuclear matrix (36). Although the
specific role of nuclear ceramide (or sphingosine) remains unclear, ceramide has been implicated in regulating the activities of protein kinase Cζ and PP2A, and in turn, Akt, all of which are present in the nucleus. Although the site of ceramide production and action is not firmly established, the sum of the studies suggests that distinct intracellular pools of sphingomyelin exist for signaling the initiation of apoptosis. Thus, modulation of the sphingolipid content within these pools should influence the sensitivity of cells to environmental stress.

Sphingolipid Involvement in Multidrug Resistance

Multidrug resistance is characterized by resistance to multiple drugs, some of which may not have been used as the initial therapeutic, and is common in relapsed disease and significantly limits further chemotherapeutic options (38). Experimentally, multidrug resistance can be achieved in cultured cells by continuous exposure to drug or by transfection of one of several genes, such as those for GCS, P-glycoprotein, or MRPI. P-glycoprotein and MRPI are ATP-dependent drug efflux proteins of the plasma membrane and have been shown to be capable of effluxing sphingolipids, including glycosylceramide and sphingomyelin (39, 40), thus decreasing potential intracellular sphingomyelin and ceramide pools and affecting the intracellular distribution of bioactive sphingolipids.

Bioavailability and Intracellular Distribution of Sphingolipids

Ceramide Synthesis

De novo synthesis of ceramide occurs on the cytosolic face of the endoplasmic reticulum with the pyridoxal phosphate-dependent condensation of serine and palmitic acid and is followed by NADPH-dependent reduction of 3-dehydrophinganine to d-erythro-phinganine (Fig. 1; ref. 3, 5). Acylation of sphinganine by ceramide synthase leads to dihydroceramide and, with introduction of a trans-4,5 double bond, to ceramide. Alternatively, ceramide may be generated through the acylation of sphingosine, a product of sphingolipid recycling. Acylation uses fatty acid-CoA of varying chain length, the biological importance of which is unclear. Two forms of ceramide synthase have been described: microsomal (cytosolic face) and mitochondrial. These differ in that the microsomal form seems to prefer acyl chains of ≥18 carbons and sphinganine versus sphingosine, whereas the mitochondrial form prefers acyl chains of 16 or 18 carbons and does not discriminate between sphinganine and sphingosine. Dihydroceramide desaturase is the enzyme responsible for introduction of the C4-C5 double bond and is also found on the cytosolic face of the endoplasmic reticulum. The exact cofactors involved have not been unequivocally identified, but it seems that, like all other known desaturases, dihydroceramide desaturase uses iron and cytochrome b5. The enzyme requires that sphinganine be acylated, with preference for shorter acyl chains, and accepts dihydrophingomyelin as a substrate. Ceramide lies at the center of all further sphingolipid metabolism.

Glucosylceramide Synthesis

Glucose or galactose can be attached to ceramide in the first step toward synthesis of higher-order glycosphingolipids. GSC is the primary ceramide glycosylase and is found in the Golgi, whereas galactosylceramide synthase is found in the Golgi and endoplasmic reticulum. After glucosylation, glucoceramide is transferred to the luminal leaflet of the Golgi, where cell-specific glycolipid-glycosyltransferases act to produce the >200 known gangliosides. Cabot et al. examined both agonists and antagonists of GCS expression and reported that increased GCS activity results in multidrug resistance, whereas decreased GCS expression and/or activity resulted in a buildup of ceramide and enhanced chemosensitivity (41). However, recent reports using inhibitors of GCS having greater specificity for GCS suggest that this enzyme may not contribute significantly to apoptosis resistance. These reports used derivatives of deoxygalactonojirimycin, which are more specific for GCS than 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, to show that in the same cell lines where 1-phenyl-2-decanoylamino-3-morpholino-1-propanol effectively reverses resistance, deoxygalactonojirimycin had no effect on resistance, although it inhibited GCS (42). Results with other cell lines suggest that if GCS is important to apoptosis resistance, at the very least it is likely to be cell line dependent.

Sphingomyelin Synthesis

Sphingomyelin is produced through the transfer of a phosphocholine head group from phosphatidylcholine to ceramide by sphingomyelin synthase (phosphatidylcholine/ceramide-PCh transferase), after transport of ceramide to the cisternae of the Golgi (43). Sphingomyelin synthase is an integral membrane protein and uses membrane-bound ceramide and phosphatidylcholine. The major site of sphingomyelin synthesis is not yet clear, because greater quantities of the enzyme reside in the plasma membrane than in the Golgi. However, the rate-limiting factor in the Golgi seems to be the availability of ceramide substrate. Inhibitors of GCS stimulate the synthesis of sphingomyelin, suggesting that sphingomyelin and ganglioside synthases compete for the same pool of substrate ceramide. An alternative pathway to the formation of sphingomyelin exists through the addition of phosphoethanolamine to ceramide followed by single methyl transfer to form the choline head group. This reaction may be more important in tissues that have low levels of sphingomyelin synthase, which, surprisingly, includes brain, a sphingomyelin-rich tissue.

For most cell types, total sphingomyelin ranges from 2% to 15% of total lipid phosphate, with notable exceptions being erythrocytes and nervous tissue, which have greater amounts. Subcellular fractionation and other methods have been employed to determine the levels of sphingomyelin in each compartment. The amount of sphingomyelin within the plasma membrane of cultured fibroblasts has been estimated at 40% to 90% of total cellular sphingomyelin (44). However, varying amounts of sphingomyelin have been reported in the endosomes, inner leaflet of the plasma membrane, Golgi, nucleus, and mitochondria. It has been observed that portions of the endoplasmic reticulum are in...
close apposition to the Golgi, nuclear, and mitochondrial membranes and may facilitate intermembrane transfer of lipids to these organelles (45). Within rat liver cells, most sphingomyelin is located in endocytic-associated and secretory-associated organelles (46). As a percent of total lipid in each compartment within rat liver cells, the amount of sphingomyelin is ~20% plasma membrane, 20% lysosomal, 15% endosomal, 10% Golgi, 5% microsomal, 5% nuclear, and 1% mitochondrial. In the past, sphingomyelin outside of the endocytic pathway was considered an artifact or contaminant of organelle isolation. It is now apparent that the subcellular location of sphingolipids is important for their function and activity within the cell.

**Sphingosine and Sphingosine-1-Phosphate Synthesis**

Deacylation of ceramide produces sphingosine, which can then be phosphorylated to sphingosine-1-PO₄ (S1P). Sphingosine comprises 0.01 to 0.04 mol % of total phospholipids and has been described as having effects on diverse signaling pathways, including inhibition of phosphatidate phosphorylase and protein kinase C, and stimulation of apoptosis, diacylglycerol kinase, and phospholipase D (47). However, many of these effects may be a consequence of sphingosine phosphorylation or reacylation to ceramide. S1P has mitogenic effects, both as an extracellular signaling molecule and as an intracellular second messenger. On the surface of the cell, there exists a family of high-affinity S1P receptors that transmit their signal through G-protein–coupled receptors to several signal transduction pathways. Intracellularly, sphingosine kinase is activated by platelet-derived growth factor, epidermal growth factor, tumor necrosis factor-α, and nerve growth factor receptors. Two sphingosine kinases have been found in mammalian cells and differ in their tissue expression, catalytic properties, and substrate preferences. Sphingosine kinase is activated through phosphorylation and translocation to the plasma membrane (48). An intracellular balance between ceramide and S1P may control whether a cell proliferates or undergoes apoptosis. However, the lack of demonstrable intracellular targets has led some to postulate that S1P activity may arise from ceramide. S1P has mitogenic effects, both as an extracellular signaling molecule and as an intracellular second messenger. On the surface of the cell, there exists a family of high-affinity S1P receptors that transmit their signal through G-protein–coupled receptors to several signal transduction pathways. Intracellularly, sphingosine kinase is activated by platelet-derived growth factor, epidermal growth factor, tumor necrosis factor-α, and nerve growth factor receptors. Two sphingosine kinases have been found in mammalian cells and differ in their tissue expression, catalytic properties, and substrate preferences. Sphingosine kinase is activated through phosphorylation and translocation to the plasma membrane (48).

**Ceramide-1-Phosphate and Sphingosylphosphocholine Synthesis**

Ceramide may be phosphorylated to ceramide-1-PO₄ in the plasma membrane (52). Ceramide kinase activity has been isolated from several cell lines and tissues and is Ca²⁺-stimulatable, in contrast to diacylglycerol kinase, which is Mg²⁺ dependent. Ceramide-1-PO₄ can stimulate cytosolic phospholipase A₂ to initiate signaling through arachidonic acid release, stimulate DNA synthesis and cell division, and inhibit aSMase, which may have implications for the induction of apoptosis in cells. A ceramide-1-PO₄ phosphatase was found to be associated with the plasma membrane (53).

In addition, sphingomyelin may also be deacylated to the lysosphingomyelin sphingosylphosphocholine. Sphingosylphosphocholine has been described to have a wide range of functions, including mitogenic activity, induction of increased intracellular calcium and stress fiber formation, activation of potassium currents, and modulation of cell migration (54). The S1P-binding EDG receptors are also activated by sphingosylphosphocholine, albeit at a much higher concentration (low nanomolar versus low micromolar, respectively), and may mimic intracellular S1P in its ability to affect Ca²⁺ levels. Much higher affinity is displayed toward receptors, which also bind lysosphosphatidylcholine, whereby intracellular Ca²⁺ levels and extracellular signal-regulated kinase activation are increased.

**Implications of Subcellular Distribution on Ceramide Production and Therapy Resistance**

It is now clear that sphingolipids and sphingolipid-associated metabolic enzymes are distributed broadly throughout the cell in multiple compartments. Importantly, these lipids and enzymes are found in compartments with known involvement in intracellular signaling, particularly the nucleus, plasma membrane, and mitochondria. It is quite conceivable that alterations in subcellular distribution or activity of either specific lipid(s) and/or enzyme(s) could result in apoptotic resistance. For instance, ceramide levels could be maintained below the threshold needed to activate apoptosis by reducing de novo ceramide synthesis or Smae activity, increasing ceramidase, GCS, or sphingomyelin synthase activities, or changing the availability of substrates needed for ceramide synthesis. Alternatively, constitutive activation of sphingosine kinase could affect the balance between mitogenic and apoptotic lipids in favor of cell growth. Thus, there are potentially several aspects of sphingolipid metabolism through which clinical efficacy could be improved via appropriate pharmacologic manipulation.

**Sphingolipid Metabolism as a Target for Cancer Therapy**

Table 1 summarizes aspects of sphingolipid metabolism, as well as known modulators of these pathways, that could be

<table>
<thead>
<tr>
<th>Target</th>
<th>Known modulators</th>
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<tr>
<td>Serine palmitoyl-CoA transferase</td>
<td>PSC 833</td>
</tr>
<tr>
<td>Sphingomyelinase</td>
<td>Most chemotherapeutics</td>
</tr>
<tr>
<td>S1P lyase</td>
<td>None known</td>
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<tr>
<td>S1P phosphatase</td>
<td>None known</td>
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<tr>
<td>Endogenous sphingolipids</td>
<td>Sphingosine, ceramide, sphingomyelin</td>
</tr>
<tr>
<td>Ceramidase</td>
<td>B13, MAPP</td>
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<tr>
<td>Ceramide glycosylation</td>
<td>NBDJ, PPMP</td>
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<tr>
<td>Sphingomyelin synthase</td>
<td>D609</td>
</tr>
<tr>
<td>Sphingosine kinase</td>
<td>Myriocin, dimethylsphingosine</td>
</tr>
<tr>
<td>Ceramide kinase</td>
<td>None known</td>
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Table 1. Potential sphingolipid-related targets for improving antitumor therapeutic efficacy
Dietary sphingolipids Several sphingolipids Reduces formation of aberrant colonic crypts and in vitro most of these targets are manipulated increase efficacy. In this regard, when the activities of or reduces mitogenic sphingolipids should, in theory, any method or molecule that increases proapoptotic factors considered as potential targets and agents for improving therapeutic efficacy in the treatment of cancer. Essentially, any method or molecule that increases proapoptotic factors or reduces mitogenic sphingolipids should, in theory, increase efficacy. In this regard, when the activities of most of these targets are manipulated in vitro, the expected effect on apoptosis and cell death is generally observed. Translation of these results to in vivo models is currently under way in several laboratories, and the data, thus far, support the notion that sphingolipid metabolism will be a useful target for therapeutic intervention.

An important caveat regarding many of the inhibitors and modulators of sphingolipid metabolism identified to date is that they have other biological effects, which complicate, or are likely to complicate, their use in patients. For example, PSC 833 is a cyclosporin A analogue that inhibits P-glycoprotein and increases de novo synthesis of ceramide through increased serine palmitoyl-CoA transferase activity (11). P-glycoprotein is an ATP-dependent, multidrug resistance–conferring drug efflux pump that is overexpressed in highly drug resistant cell lines and is associated with a poor prognosis in several cancer types. P-glycoprotein also has important functions in the physiology of normal cells as a transporter of xenobiotics and other molecules. Early trials with PSC 833 clearly showed inhibition of P-glycoprotein function in both normal and malignant tissues that simultaneously and dramatically changed the pharmacokinetics of the cytotoxic drugs and which, unfortunately, led to higher systemic toxicity (55). Myriocin and its derivatives are serine-palmitoyl transferase inhibitors (56). However, these compounds, first identified as immunosuppressants, can act as potent SIP receptor inhibitors, after phosphorylation by sphingosine kinase 2, to reduce the migration of lymphocytes from secondary lymphoid tissues (57). As discussed previously, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol was reported to increase drug sensitivity by inhibition of glucosylceramide synthase but now seems to act through some other, yet to be identified, mechanism (42).

Table 2 summarizes studies on the effects of sphingolipids on tumor growth in humans or murine models. The only agent for which there is currently published data from clinical trials is safingol (1-threo-dihydrosphingosine). Safingol was originally identified as a protein kinase C inhibitor and subsequently found to also inhibit sphingosine kinase. In a limited phase I trial with doxorubicin (58), safingol did not significantly alter the pharmacokinetics of doxorubicin. Of 17 patients enrolled, four had minor responses (3 of 6 pancreatic cancers and 1 of 3 sarcomas). However, response could not be correlated with the dose received by the patients; therefore, future studies will need to determine its true clinical value.

The other agents in Table 2 have been studied in mouse models. Dimethylsphingosine, a sphingosine kinase inhibitor, inhibited the growth of tumors of the epidermoid carcinoma cell line, KB-3-1, in vivo, as well as their multidrug resistance derivative, KB-C2 multidrug resistance, with evidence of increased apoptosis in both tumor models (59). However, when dimethylsphingosine was combined with doxorubicin, only additive effects were observed, as opposed to the synergistic response expected from simultaneously inhibiting sphingosine kinase and stimulating ceramide production. Trimethylsphingosine was as effective as dimethylsphingosine in reducing the growth of human gastric tumor xenografts (60). However, sphingosine, by itself, had no statistically significant effect on tumor growth.

Because active ceramidase would be expected to reduce the cellular levels of ceramide and thus impart an increase
in drug resistance, ceramidase inhibitors have been investigated. B13 completely prevented the establishment and growth of two aggressive human colon cancer cell lines (13). In these studies, the tumor cells were injected into the hepatic portal vein of mice followed 2 hours later by the first of five doses of B13 administered every 3 days; the effect of B13 on established tumors was not reported. Importantly, these investigators also found no increase in ceramide content within the liver nor was apoptosis observed in normal tissues. It seems that in general, normal cells are not as sensitive to sphingolipid modulation as are cancer cells.

Because ceramide itself is a toxic molecule (LD₅₀ = 10 mg/kg in BALB/c mice; ref. 61), efforts have been made to formulate it into less toxic liposomes before administration to xenograft tumor-bearing mice. Comparing pegylated liposomes of phosphatidylcholine, phosphoethanolamine, and C₆-ceramide to pegylated liposomes lacking ceramide, Kester et al. showed reduced toxicity of ceramide (LD₅₀ = 100 mg/kg in BALB/c mice) and significant antitumor effects in syngeneic (410.4 in BALB/c mice) and xenogeneic mammary tumor models (MDA-MB-231 in nude mice; ref. 61). Other studies have also shown that liposomal ceramide is less toxic than free ceramide and can reduce growth of J774 ascites tumor (62).

Our approach to increasing tumor ceramide levels has been by increasing substrate for activated SMase(s). Based on reports in the literature indicating that sphingomyelin levels within tumor cells are altered (63, 64), we reasoned that a perturbation in metabolism could alter not only the absolute levels of sphingolipids but also their subcellular distribution. Changing the subcellular distribution could reduce the amount of sphingomyelin in the pool used for signaling apoptosis. Thus, insufficient amounts of substrate sphingomyelin would limit the production of ceramide due to a lack of substrate sphingomyelin for the drug-activated SMase(s). Based on reports in the literature indicating that sphingomyelin levels within tumor cells are altered (63, 64), we reasoned that a perturbation in metabolism could alter not only the absolute levels of sphingolipids but also their subcellular distribution. Changing the subcellular distribution could reduce the amount of sphingomyelin in the pool used for signaling apoptosis. Thus, insufficient amounts of substrate sphingomyelin would limit the production of ceramide due to a lack of substrate sphingomyelin for the drug-activated SMase(s). To overcome this, we administered small (<100 nm) sphingomyelin micelles to cells in culture and tumor-bearing mice. We have shown that colonic, pancreatic, melanoma, and lymphoma cell lines in culture respond to nontoxic levels of exogenous sphingomyelin with increased chemo sensitivity, and that this interaction was synergistic (65, 66). An examination of the sphingomyelin effect in Panc1 pancreatic cells revealed that gemcitabine alone did not cause a significant increase in cellular ceramide levels, mitochondrial depolarization, or apoptosis but did activate aSMase. However, when gemcitabine was augmented with nontoxic levels of sphingomyelin, ceramide levels, mitochondrial depolarization, and apoptosis increased significantly compared with each treatment arm alone but caused no further activation of either aSMase or nSMase (66). These data suggest that in these cells and under the conditions examined, aSMase is activated by gemcitabine, but that apoptosis is attenuated due to a lack of sphingomyelin in the pool of substrate used to produce ceramide.

The ability of sphingomyelin to improve chemotherapeutic efficacy in xenograft models of colonic and pancreatic cancer was also examined (67). In both models, we found that inclusion of sphingomyelin improved chemotherapeutic efficacy. Inclusion of sphingomyelin with 5-fluorouracil therapy of GW-39 human colonic tumor xenografts was associated with increased ceramide production and apoptosis. Of critical importance, we found no evidence of increased toxicity to normal tissues, including liver, kidney, and WBC, after five daily i.v. injections of 10 mg sphingomyelin; preliminary evidence suggests that repeated cycles of sphingomyelin at this dosage are well tolerated in mice. The finding that sphingomyelin interacted in a synergistic manner with chemotherapy in vivo is further evidence that manipulating sphingomyelin substrate pools is a viable target for therapeutic intervention.

In a series of studies of sphingolipids as cancer preventive agents, Merrill and colleagues examined the ability of dietary sphingomyelin to reduce the occurrence of chemically induced intestinal cancers in mice (19, 68). They fed female CF1 mice a diet supplemented with 0.025% to 0.1% (w/w) sphingomyelin after the mice had ingested the colon cancer-inducing chemical 1,2-dimethylhydrazine. Control animals fed the supplemented diet had no adverse effects. The animals treated with DMH and fed the sphingomyelin-supplemented diet had significantly less aberrant colonic crypts and adenocarcinomas compared with animals treated with 1,2-dimethylhydrazine but which remained on standard rodent diet (<0.005% sphingomyelin, w/w). Additional experiments showed that dietary glycosphingolipids could produce similar results (68). It was postulated that intestinal SMase(s) and endoglycosidase(s) released ceramide, which was then responsible for the observed effects.

Conclusions

Although the study of bioactive sphingolipids is still relatively young, the recent expansion in knowledge has allowed us to question how therapeutics may be designed to take advantage of sphingolipid signaling pathways. It is now established that rational modulation of sphingolipid metabolism can lead to improved efficacy in human tumor xenograft models. An important point, which has not yet been clearly evaluated, is whether altered sphingolipid metabolism plays a role in cancer growth and resistance to therapy in the clinical setting. Increases and decreases in sphingolipid levels have been noted between tumor and normal tissues. These differences seem to be a function of tumor type and site of growth and are not consistent even within a given tumor type. However, none of these studies examined the effect on sphingolipid signaling pathways. Because of their biophysical properties, sphingolipids are less capable of moving freely from one intracellular compartment to another. Thus, molecules, such as ceramide, will likely have their biological effect (if any) only within the compartment in which they are generated. It is likely that many of the conflicting reports in the literature could be reconciled through increased knowledge of the relationship between intracellular location and function.
The modulation of which sphingolipid metabolite will prove most valuable for the treatment of cancer has not been determined. Safingol combined with doxorubicin, whereas being well tolerated and without effect on doxorubicin pharmacokinetics, has not yet been investigated to determine its true clinical value. The other agents listed in Table 2 are still at the preclinical stage of development, where cures in mouse models are common. Furthermore, currently available agents are not specific for sphingolipid metabolism, thus obscuring the true relationship between sphingolipid levels and the results obtained with them. However, because apoptosis is critical to the effectiveness of chemotherapy and ceramide is a key signaling molecule, it is very likely that increasing the apoptotic potential of cancer cells through rational manipulation of sphingolipid levels will lead to improved treatments. To realize this, we will need a better understanding of sphingolipid biology in human cancers and more specific reagents with which to manipulate sphingolipid levels in vivo.

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Mol Cancer Ther 2006;5(2). February 2006


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Mol Cancer Ther 2006;5:200-208.

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