Ceramide synthesis and metabolism as a target for cancer therapy

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Abstract

Sphingolipids, which include ceramides and sphingosine, are essential structural components of cell membranes that also have messenger functions that regulate the proliferation, survival, and death of cells. Exogenous application of ceramide is cytotoxic, and exposure of cells to radiation or chemotherapy is associated with increased ceramide levels due to enhanced de novo synthesis, catabolism of sphingomyelin, or both. Ceramide can be metabolized to less toxic forms by glycosylation, acylation, or by catabolism to sphingosine, which is then phosphorylated to the anti-apoptotic sphingosine 1-phosphate. Glucosylceramide synthase overexpression has been shown to enhance resistance to doxorubicin, suggesting that inhibition of ceramide metabolism or catabolism might enhance cancer chemotherapy. Several anticancer agents, including the cytotoxic retinoid, fenretinide (4-HPR), have been shown to act, at least in part, by increasing tumor cell ceramide via de novo synthesis. Combinations of 4-HPR and modulators of ceramide action and/or metabolism demonstrated increased anti-tumor activity in pre-clinical models with minimal toxicity for non-malignant cells, and were effective in a p53-independent manner against tumor cell lines resistant to standard cytotoxic agents. Phase I trials of ceramide metabolism inhibitors in combination with 4-HPR and with other cytotoxic agents are in development. Thus, pharmacological manipulation of sphingolipid metabolism to enhance tumor cell ceramide is being realized and offers a novel approach to cancer chemotherapy.
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1. Introduction

Membrane sphingolipids contain a long-chain sphingoid base (such as sphingosine), an amide-linked long-chain fatty acid, and one of several polar head groups. The head groups define the various sphingolipid classes, with a hydroxyl group found in ceramides (Fig. 1), phosphorylcholine in sphingomyelin (SM), and carbohydrates in the various glycosphingolipids. Because SM is concentrated in the outer leaflet of the plasma membrane and provides a barrier to the extracellular environment [1], it was long assumed to serve only structural roles. However, accumulating evidence has pointed toward an important role for sphingolipids in cellular damage and death-response signaling. The rapid activation of

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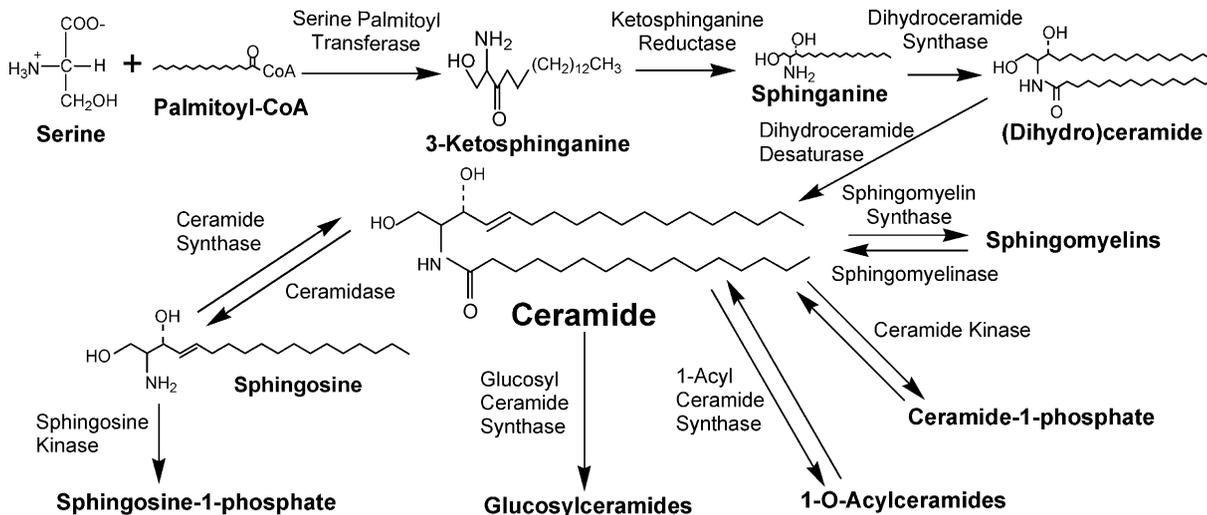
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Fig. 1. Major synthetic and metabolic pathways for ceramide. Increased ceramide leading to cytotoxicty can come from de novo synthesis due to stimulation of serine palmitoyltransferase and/or dihydroceramide synthase, or by degradation of sphingomyelins via spingomyelinases. Metabolism of ceramide by glycosylation or acylation, appear to 'shunt' ceramide into less toxic forms, as does catabolism via ceramidase. Phosphorylation of sphingosine derived from ceramide stimulates pro-life metabolic pathways and acts to oppose certain cytotoxic actions of ceramides.

sphingomyelinase (SMase) in response to 1,2-diacyl-glycerols, but not phorbol esters [2], suggested the existence of a SM-based signaling pathway [3,4]. Subsequently, a SM-based signaling pathway was shown to be active in certain receptor-mediated responses [5], providing evidence that ceramide serves as a second messenger [6]. Sphingolipid metabolites, including ceramide, sphingosine, and sphingosine 1-phosphate (S1P), are now recognized as messengers playing essential roles in cell growth, survival, and death [7,8]. Here we will briefly review sphingolipid metabolism and the current evidence that ceramide generation and catabolism is a potential target for cancer chemotherapy.

2. Synthesis of ceramide

Fig. 1 shows that intracellular ceramide can be formed either by de novo synthesis, or through the SMase-dependent catabolism of SM, in various separate cellular compartments. The generation of ceramide from both catabolism of SM and by de novo synthesis have been implicated in the response to cytotoxic agents, and it is possible that cell death

signaling by ceramide may be enhanced by the summation of cellular ceramide levels that are derived from both pathways.

2.1. Generation of ceramide by sphingomyelinase

Membrane neutral magnesium-dependent SMase (nSMase) and acid SMase (aSMase) are rapidly activated by diverse stress stimuli resulting in increased ceramide levels over a period of minutes to hours [7,8]. The alkaline SMase activity found in intestinal mucosa and bile has, as yet, no defined role in signal transduction. The aSMase was originally considered a strictly lysosomal enzyme because of its pH optimum at 4.5–5.0. However, an aSMase isoform has been identified in vesicles near the plasma membrane [9] and was shown to be secreted extracellularly [10–12]. A plasma membrane form of aSMase has also been observed in microdomains enriched in SM (caveolae) where IL-1β stimulates ASMase activity [12]. Thus, there may be three pools of aSMase: acidic lysosomal aSMase responsible for SM metabolism, secretory aSMase associated with inflammation and stress responses, and a receptor-activated aSMase. Recent evidence

supports that this last form of aSMase translocates to the outer cell membrane after activation by various cell surface receptors, such as CD95 (fas) and CD40, and hydrolyzes membrane SM into ceramide [13–15]. The liberated ceramide associates into ceramide-rich domains, or 'rafts', in the cell membrane, which facilitates the clustering of activated receptor molecules [16,17]. It is speculated that such receptor clustering may stabilize or promote receptor-mediated apoptosis signaling [18]. Ionizing-radiation-induced apoptosis is also dependent on ceramide increase, although whether this involves aSMase exclusively, or possibly also a nuclear nSMase, is still under investigation [19–21].

The nSMases (pH optimum 7.4) are only now being defined at the molecular level [7], but it is clear from the residual activity seen in aSMase knockout mice that one or more distinct genes encode such enzymes [22]. Activity of nSMase is inhibited by glutathione and is therefore, de-repressed under conditions of oxidative stress when glutathione is depleted [23]. Agents such as TNF-α, which decrease glutathione, can lead to prolonged ceramide generation [23]. Arachidonic acid may also enhance nSMase activity [24], making it possible that cyclooxygenase inhibitors could induce apoptosis via an increase in nSMase activity [25]. Ceramide derived from nSMase appears central to ischemia/reperfusion cell death in cardiomyocytes [26], and nerve growth factor (NGF) induces nSMase-dependent cell death associated with Jun kinase (JNK) activation in hippocampal neurons [27]. Additionally, nSMase activity has been implicated in cell death induced by such diverse agents as nitric oxide, low density lipoproteins, and T cell receptor (TCR) cross-linking [28–30]. Recently, nSMase activity has also been implicated in cell membrane raft formation in neural cell apoptosis [31].

To date, two mammalian nSMases, nSMase1 and nSMase2, have been identified and cloned. nSMase1 was identified by Stoffel and co-workers by aligning multiple bacterial SMases which matched a protein sequence in the *Saccharomyces cerevisiae* database [32]. Subsequent biochemical characterization of nSMase1 demonstrated that this enzyme is not likely a bona fide SMase as it exerts primarily lyso-platelet-activating factor-phospholipase C (lyso-PAF-PLC) activity [33]. Cloning of nSMase2 was carried out using a more sophisticated profile-based database search that considered sequence errors caused by truncated coding regions and frameshift errors [34]. Characterization of nSMase2 revealed that it is expressed in a brain-specific manner, and that it manifests optimal activity under neutral conditions, requiring Mg^{2+}, Triton X-100, and phosphatidyl-serine. Moreover, in contrast to nSMase1, nSMase2 does not exhibit lyso-PAF-PLC activity [35]. Thus, the enzymatic properties of nSMase2 are consistent with the properties of partially purified nSMase from rat [36] or bovine brain [37]. Finally, the role of nSMase2 in cell growth was also investigated. These studies revealed that increased nSMase2 activity inhibit growth of MCF-7 cells [35]. Further characterization of the molecular properties of nSMase2 is necessary to define the role of this enzyme in sphingolipid metabolism, stress signaling, and cell growth and cell death.

2.2. Generation of ceramide by de novo biosynthesis

De novo ceramide biosynthesis requires the coordinated action of serine palmitoyltransferase and ceramide synthase to generate ceramide (Fig. 1). This process begins with the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine [7,8], which is then reduced to the sphingoid base sphinganine (dihydrosphingosine), and acylated by ceramide synthase to generate dihydroceramide. The introduction of a trans-4,5 double bond by a desaturase converts the acylated sphinganine to acylated sphingosine (ceramide). Alternately, this pathway may re-utilize sphingosine released by sequential degradation of more complex sphingolipids for ceramide synthesis (indeed, recent evidence suggests that exogenous, cell-penetrant, short chain ceramides recycle into long chain ceramides via this pathway [38]). The de novo ceramide synthetic pathway can be stimulated by drugs and ionizing radiation and usually results in a prolonged ceramide elevation [39,40].

3. Metabolism of ceramide

Once generated, ceramide can accumulate in the cell or may be converted into a variety of metabolites (Fig. 1). Phosphorylation by ceramide kinase [7,8]

generates ceramide 1-phosphate, while deacylation by either neutral or acid ceramidase [41] yields sphingosine, which may then be phosphorylated by sphingosine kinases (SKs) to S1P. Two distinct mammalian SKs have been cloned. The two enzymes differ in temporal patterns of appearance during development, are expressed in different tissues, and possess distinct kinetic properties [42,43], implying that they may perform different cellular functions.

Ceramide may also be converted into SM by transfer of phosphorylcholine from phosphatidylcholine to ceramide via SM synthase [7,8]. Alternatively, it can be glycosylated by glucosylceramide synthase (GCS) in the golgi to form glucosylceramide, which can be further modified into more complex glycosphingolipids. Ceramide can also be acylated by the lesser-known 1-O-acylceramide synthase (ACS) and placed into a putative, stress-buffering, acylceramide 'holding pool' [44–48]. The relative importance of GCS versus ACS in protecting cells from ceramide stress is not currently known, although data suggest that ACS may play as great, or a greater role, than GCS (B. Maurer, unpublished results).

It is now clear that ceramides generated by de novo synthetic enzymes and the various-activated SMases exist in distinct cellular pools. This distinction was not always appreciated in the past, which may have somewhat confused the interpretation of data reported in the literature. It is not currently fully understood the extent to which, and the manner in which, these pools may or may not interact to effect common end-signaling events (such as mitochondrial disruption, JNK activation, and/or apoptosis), or may be commonly accessible by the various catabolic enzymes. An example of this is the report that ceramide generated in Jurkat cells by nSMase during apoptosis induced by ligation of the CD95 receptor or exposure to gamma-radiation was not glycosylated by GCS, whereas de novo synthesized or exogenous, short-chain ceramides were glycosylated [49]. Such data suggest that not all intracellular ceramide pools are equivalent, and it remains to be demonstrated the potential that each ceramide pool has for cytotoxic enhancement. To date, the strongest evidence for a synergizable cytotoxic ceramide pool is probably that derived from the study of de novo synthesis stimulated by fenretinide, as described in Section 6.

4. Ceramide and S1P as second messengers that regulate apoptosis

While ceramide has been proposed as a messenger for events as diverse as differentiation, senescence, proliferation, and cell cycle arrest, most research has focused on its role in apoptosis [7,8,50]. Evidence supporting ceramide as a message for apoptosis induction is based on data from many cell systems and on several kinds of studies. Firstly, agonist-and stress-induced increases in ceramide levels precede biochemical and morphologic manifestations of apoptosis in many systems. Secondly, increasing cellular ceramide by addition of natural ceramide, exogenous SMase, or pharmacologic agents that interfere with enzymes of ceramide metabolism can induce or increase apoptosis or other forms of cell death. Lastly, genetic models, including Niemann–Pick Disease cells, *ASMase − /− mice, and GCS − / − mice*, manifest abnormalities in stress responses that would be predicted from the biochemical and cell biological studies. Other sphingolipid metabolites (except sphingoid bases, usually in non-physiologic doses, and the ganglioside GD_3 in select cells) are not cytotoxic [7,8], so it appears that ceramide per se is necessary and likely sufficient for some forms of cell stress-associated cell death (apoptotic or otherwise).

The existence of a 'S1P Rheostat' has been proposed as an explanation for how ceramide can have in different cells a wide variety of diverse and often opposing functions [51]. Spiegel and co-workers based this proposal on the observation that ceramide and its metabolite S1P can have opposing effects on biologic outcome. While ceramide is often anti-proliferative and pro-death, S1P has been implicated as a second messenger that promotes cellular proliferation and survival [52]. S1P has also been implicated in protection against ceramide-mediated cell death [51]. Thus, it has been suggested that the balance between these two sphingolipid messengers may be an important factor determining survival or death of mammalian cells [52]. The recent discovery of a set of cell surface S1P receptors (also known as the EDG receptors) has further complicated the interpretation of S1P dynamics [53]. It is not yet clear to what extent S1P functions via the EDG receptors, and to what extent S1P acts directly as an intracellular signal. The available data suggest that, by

either mode, S1P antagonizes ceramide-mediated biologic responses. Ceramide is also reported to activate various protein kinase C (PKC) isoforms, which may contribute to the S1P rheostat by, for instance, activating the pro-life transcription factor NFkappaβ [54–56].

4.1. Shunting of ceramide to less-toxic sphingolipids

As sphingosine levels in mammalian cells are often one to two orders of magnitude lower than those of ceramide, the generation of substantial amounts of S1P requires additional sphingosine production. Typically, S1P is produced by coordinate activation of SMase to generate ceramide, ceramidase to generate sphingosine from ceramide, and by SK [53]. In fact, activation of ceramidase may be so robust in some cells that the ceramide levels never rise substantially, suggesting that SM has been converted stoichiometrically to sphingosine. For this reason, activation of the SM pathway can never be ruled out by a failure to detect ceramide increases, unless measurements of SM, sphingosine, and S1P levels are also available. Similarly, activation of GCS [57] and ACS [44–48] can shunt ceramide into glucosyl- and acyl- ceramides, decreasing the cytotoxicity from ceramide accumulation, and requiring that glucosyl-ceramide and acylceramide measurements may be required to detect SM pathway activation.

4.2. Cellular localization of enzymes that generate or degrade ceramide

Other metabolic pathways may contribute to the diverse action of ceramide in different cellular systems. Traditionally, the ceramide backbone of sphingolipids was thought to be generated by de novo synthesis in the endoplasmic reticulum and the head groups added in the Golgi apparatus, after which the newly synthesized lipids are shuttled to various organelles. Similarly, sphingolipid-degrading enzymes were thought to be localized to the endosomal-lysosomal system. However, recent studies have detected isoforms of both the synthetic and the degradative enzymes in alternative compartments, where they appear to play distinctive roles. For instance, a mitochondrial form of ceramide synthase is activated by ionizing radiation treatment of HeLa cells and appears requisite for efficient mitochondrial commitment to the apoptotic process (R. Kolesnick, unpublished results). Likewise, both ASMase and SM synthase are targeted in part to the plasma membrane, with the former specifically targeted to membrane rafts. One form of acid ceramidase may be a secreted enzyme, while a form of neutral ceramidase may be mitochondrial [58]. Thus, localization of enzymes involved in both synthesis and degradation of ceramide likely affect the type of response generated in sphingolipid signaling.

5. Ceramide and cancer chemotherapy

As cellular stress is known to increase ceramide levels in cells, it is not surprising that increased ceramide has been observed in response to a number of cancer chemotherapeutic agents, including etoposide, vincristine, daunorubicin, doxorubicin, fludarabine, paclitaxel, PSC 833, fenretinide, and irinotecan, or after radiation treatment (reviewed in Ref. [57]). Chemotherapy-mediated increases in tumor cell ceramide can result from stimulation of the ceramide de novo synthetic pathway, an increase in SMase activity, or speculatively, by a disruption of ceramide catabolism.

Manipulation of ceramide levels may enhance the effectiveness of some cancer therapies. For example, exogenous ceramide enhanced paclitaxel-mediated apoptotic death of Tu138 head and neck cancer cells in vitro and preliminary data with Tu138 xenografts in nude mice showed synergistic reduction in tumor growth when treated with paclitaxel and ceramide (personal communication, H. Wanebo).

The potent ceramidase inhibitor B13 increased the ceramide content of tumor cells and induced tumor cell apoptosis, without affecting the ceramide level or survival of normal liver cells [59]. B13 also prevented growth of two aggressive human colon cancer cell lines metastatic to the liver. These findings suggest that ceramidase inhibition offers a promising therapeutic strategy for selective toxicity toward malignant cells, and suggest that pre-clinical studies of ceramidase inhibitors in combination with chemotherapy known to increase ceramide should be undertaken.

Increased glucosylceramide levels are found in drug-resistant tumors [57], suggesting that shunting

ceramide to a glycosylated form may decrease the cytotoxic effects of high levels of ceramide stimulated by the chemotherapy. Overexpression of GCS via an inducible retroviral vector has been shown to increase resistance to doxorubicin in an MCF-7 breast cancer cell line, and treating multi-drug resistant tumor cell lines with GCS antisense enhanced the response to doxorubicin [60]. Thus, glycosylation of ceramide via GCS may be one mechanism by which tumor cells can enhance their ability to survive some chemotherapy, and inhibiting GCS activity might potentiate the cytotoxicity of drugs that increase ceramide (see also Section 6.3). Similarly, preventing the acylation of ceramide by inhibiting ACS may increase the efficacy of ceramide-generating chemotherapies [61].

Blockade of S1P biosynthesis provides another possible means to amplify ceramide-mediated death signals. Both safingol (L-*threo*-dihydrosphingosine), an in vitro SK inhibitor [62], and dimethylsphingosine [63], are potential SK inhibitors that could decrease the formation of S1P from degraded ceramide. Safingol has been shown to synergistically enhance tumor cell killing by the ceramide-generating retinoid fenretinide (see Section 6). Schwartz and co-workers conducted an incomplete phase I clinical trial of safingol in patients with advanced cancer as both a single agent, and in combination with doxorubicin [64]. With a fixed dose of doxorubicin (45 mg/m^2), safingol (given as a 1 h bolus infusion) could be escalated to micromolar doses without dose-limiting toxicity and without altering the pharmacokinetics of doxorubicin. Although the initial phase I trial of safingol ended prior to achieving a maximally tolerated dose (MTD) (due to drug supply problems), and entered too few patients to assess activity, the modest toxicity seen was encouraging. New formulations for safingol are being developed via the National Cancer Institute Rapid Access to Intervention Development program, and clinical trials of safingol in combination with fenretinide (see Section 6) and other drugs are planned for the near future.

Thus, it has been demonstrated that pharmacological inhibition of ceramide catabolism may potentiate the activity of ceramide-increasing, anticancer therapies. The relative effectiveness of the inhibition of any specific catabolic ceramide pathway on increasing the cytotoxicity of the different pools of ceramide (i.e. from de novo synthesis, SMases, radiation, etc.) has yet to be completely assessed, and the effect of inhibiting several catabolic ceramide pathways, such as ceramide-1-phosphate formation, have yet to be explored.

Conversely, blocking ceramide generation, if it can be done in normal tissues preferentially over tumor cells, has the potential to protect some tissues from side effects of cancer therapy. The lethal gastrointestinal syndrome, which limits the efficacy of radiation and chemotherapy [65], apparently results from apoptotic damage to the endothelial cells of the microvasculature of the small intestine, and, indirectly, from crypt stem cell dysfunction and tissue necrosis [66]. Genetic inactivation of ASMase prevents this toxicity, as does treatment with the endothelial cell survival factor bFGF, which acts in part by suppressing ASMase activity. These studies suggest a potential approach to protecting the gut and increasing the therapeutic ratio.

6. Fenretinide, a ceramide-generating retinoid

Maximizing the cytotoxic synergy of ceramide catabolism inhibitors will depend on effectively inducing ceramide stress in tumor cells (i.e. driving ceramide production in an effective pool). A synthetic retinoid made in the late 1960s, *N*-(4-hydroxyphenyl) retinamide, or fenretinide (4-HPR), has been reported to inhibit the growth of neuroblastoma, and many other cell lines in vitro at 1–10 M concentrations in a dose-dependent manner [67]. In contrast to 13-cis-RA and all-trans-RA, 4-HPR does not induce maturational changes, but can cause both apoptosis and a p53- and caspase-independent necrosis [68].

6.1. Mechanism of action for 4-HPR

4-HPR cytotoxic activity does not appear to be mediated by retinoid receptors. Studies in breast cancer cell lines have demonstrated that 4-HPR has a low binding affinity for nuclear retinoic acid receptors (RARs) compared with trans-retinoic acid [69], and only minimally activates the RAR element and retinoid X receptor response elements [70]. Generation of reactive oxygen species in the cytotoxicity achieved by 4-HPR has been implicated

in cell lines from leukemia [71], cervical carcinoma [72], and neuroblastoma [68]. A dose and time-dependent increase in ceramide was observed in neuroblastoma cell lines [68], including a highly alkylator and etoposide-resistant cell line [73], and in cell lines from lymphoblastic leukemias [74], Ewing family tumors [75], and prostate and breast cancer (B.J.M. and C.P.R., unpublished). Fenretinide increased tumor cell ceramide up to ten-fold or more in vitro via de novo synthesis at drug concentrations clinically achievable in plasma [61]. In a neuroblastoma cell line, 4-HPR increased the activities of both serine palmitoyltransferase and ceramide synthase [76]. Fenretinide was minimally toxic to, and did not increase ceramide in non-malignant cells, including fibroblasts [61] and EBV-transformed lymphoblastoid cell lines [74]. Together, these properties make 4-HPR a particularly attractive agent upon which to base a ceramide-modulating chemotherapy.

6.2. Fenretinide clinical trials

The toxicity of 4-HPR given orally at low doses in chemoprevention clinical trials was minimal with no hematologic toxicity reported [77]. The major clinical toxicity of 4-HPR has been decreased night vision, due to decreased plasma retinol levels [77]. Recently, phase I clinical trials of high-dose, oral 4-HPR in adult and pediatric solid tumors have been completed. In pediatrics, the MTD of oral 4-HPR given for 7 days, every 3 weeks, was 2475 mg/m^2/day, which achieved 4-HPR plasma levels of $6-10 \, \mu M$ with minimal systemic toxicity [78]. In adults, the recommended 4-HPR phase II oral dose was 1800 mg/m^2/day due to the large number of capsules required at higher doses, and the apparent saturation of drug bioavailability [79]. The plasma levels ($6-10 \, \mu M$) achieved in the Children's Oncology Group pediatric phase I trial are comparable to 4-HPR concentrations that were highly effective against 13-cis-RA-resistant neuroblastoma cell lines in pre-clinical studies in vitro [80]. However, the relationship between 4-HPR plasma levels, drug delivered to tumor cells, and the cytotoxic activity observed in pre-clinical models remains to be defined. Due to the poor bioavailability and large capsule size of the current 4-HPR oral formulation (particularly a problem with young children), alternate formulations of 4-HPR are needed. As it is likely that obtaining levels of fenretinide in tumor cells sufficient to drive optimal ceramide generation for primary cytotoxic chemotherapies will require intravenous delivery, intravenous formulations of fenretinide have been developed and are entering phase I clinical trials with support from the NCI RAID program (to C.P.R.) [61]. To facilitate the ease of 4-HPR delivery for particularly 4-HPR- and/or ceramide- sensitive tumors, and for more convenient use in maintenance chemotherapy approaches, the development of a new oral formulation with a greater bioavailability is also being supported by the NCI RAID program (to B.J.M.).

6.3. Combinations of 4-HPR and ceramide catabolism modulators

Drugs that inhibit GCS and ACS (such as PPMP) prevent the shunting of ceramide into nontoxic metabolites, and PPMP synergistically enhanced the cytotoxicity of 4-HPR in cell lines from various types of cancer [61,74]. Safingol (L-*threo*-dihydrosphingo-sine), a putative SK inhibitor, also synergistically increased 4-HPR cytotoxicity in tumor cell lines, including those with altered p53 status and high alkylator resistance. Interestingly, safingol is acylated by ceramide synthase into a stereochemically variant, L-*threo* ceramide that cannot be glucosylated by GCS [61]. Significantly, 4-HPR + safingol only minimally increased native (D-*erythro*) ceramide in normal fibroblasts and blood mononuclear cells in vitro, and toxicity to normal fibroblasts and bone marrow myeloid progenitors was slight, potentially indicating a favorable therapeutic index [61]. Whether safingol synergized 4-HPR cytotoxicity as the dihydrosphin-gosine form, or as L-*threo* ceramide, has not been determined. As predicted for a cytoxic mechanism based upon ceramide levels, PPMP was able to further increase the cytotoxicity of 4-HPR + safingol [61]. Formulations of both safingol and PPMP suitable for clinical use in combination with 4-HPR are currently being developed with the support of the NCI (to C.P.R. and B.J.M.).

Table 1
A summary of enzymes that are potential drug targets in the sphingomyelin pathway

Enzyme	Drug	↑ or ↓ enzyme	Ref.
Serine palmitoyl-transferase	4-HPR, PSC 833	↑	[76,90,91]
Ceramide synthase	4-HPR, daunorubicin	↑	[39,76]
Ceramidase	B13	↓	[59]
Sphingosine kinase	safingol	↓	[61]
Glucosylceramide synthase	PPMP, PPPP, butyldeoxynojirimycin	↓	[61,74, 92–94]
1-O-Acylceramide synthase	PPMP	↓	[61,74,92]

Shown also are drugs known to stimulate (↑) or inhibit (↓) enzymatic activity.

7. Potential therapeutic targets in sphingolipid pathways

Table 1 lists enzymes of sphingolipid pathways that are potential targets for ceramide-based therapies and their targeting drugs that are currently in pre-clinical or clinical development. Ceramidase inhibitors and inhibitors of ceramide glycosylation and/or acylation exist or are in early pre-clinical development. As detailed above, pre-clinical data point towards using ceramide-generating drugs in combination with secondary agents that inhibit ceramide catabolism or metabolism to potentiate the effects of ceramide-increasing anti-neoplastic drugs. It is not clear to what extent catabolism inhibitors that potentiate the cytotoxicity of de novo synthesized ceramide can potentiate ceramide derived from SM breakdown. Depending on the precise cellular compartment of its generation, these same ceramide catabolism inhibitors could also potentially be used to increase the cytotoxic effects of ceramides induced by ionizing radiation therapy. Indeed, multiple investigators have demonstrated the dependence of radiation-induced apoptosis on ceramide increases [81–85] and that radiation sensitivity may be augmented by addition of exogenous sphingoid bases or modulators of endogenous ceramide production [86–89]. Pre-clinical studies identifying optimal combinations of existing drugs, and the development of new drugs for the wealth of targets

in the various sphingolipid pathways, should lead to an exciting new generation of clinical trials.

8. Conclusions

Until recently, cancer chemotherapy has focused primarily on targeting DNA, microtubule disruption, or on targeting critical cellular proteins or metabolites involved in DNA synthesis and repair. The goal of such pharmacological interventions has been to cause lethal damage to malignant cells with tolerable toxicity to normal cells and organs. Cancer drug development is now turning toward potentially more selective approaches to inducing tumor cell death or cytostasis, and signal transduction inhibitors have advanced from clinical trials to finding approved therapeutic indications. A growing body of literature points toward the response to cellular stress as being one function of sphingolipids, suggesting that ceramide-related pathways are a fertile area for identification of therapeutic targets. Malignant cells appear to be hypersensitive to the effects of ceramide perturbation and/or generate ceramide more selectively in response to drugs, such as fenretinide, and pre-clinical studies suggest that pharmacological induction of ceramide may have a favorable therapeutic index. The ability of ceramide and ceramide-generating drugs to induce cytotoxicity in cancer cells lacking p53 function suggests that ceramide-based therapeutics may be active in some settings where resistance to DNA damaging agents is high. Combining ceramide generation with inhibition of ceramide catabolism may provide an even more powerful approach to killing cancer cells with the potential for modest collateral toxicity to normal tissues.

Further research is needed, including full molecular characterization of the enzymes involved in the generation and metabolism of ceramide and developing a more complete understanding of the complex pathways that regulate sphingolipid synthesis. As ceramide generation and metabolism are likely controlled differently among various types of cells, and are potentially altered in multi-drug-resistant cells, signaling pathways and pharmacological manipulation of those pathways will need to be studied across many cell types, and comparisons between chemotherapy-sensitive and drug-resistant

cancer cells are needed. The events down-stream from ceramide generation that result in cell death also remain to be completely elucidated, and may vary depending on the type of cell or the prior exposure of the cell to cytotoxic stress. Continued work in these important areas could identify potential therapeutic targets, and should increase our understanding of how to best pursue those targets already identified. With drugs that target ceramide now entering clinical trials, and more such agents in pre-clinical development, we can anticipate learning more about the potential for sphingolipids as a cancer therapeutic target in the near future.

Acknowledgements

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