Targeting Ceramide Metabolism—a Strategy for Overcoming Drug Resistance

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Inherent or acquired drug resistance, which frequently characterizes cancer cells, is caused by multiple mechanisms, including dysfunctional metabolism of the lipid second messenger ceramide. Ceramide, the basic structural unit of the sphingolipids, plays a role in activating cell death signals initiated by cytokines, chemotherapeutic agents, and ionizing radiation. Recent discoveries about the metabolism of ceramide suggest that this agent may have an important influence on the effectiveness of various cancer therapeutics. In particular, the cytotoxic effect of chemotherapy is decreased when generation of ceramide is impaired but is increased when the degradation of ceramide is blocked. Herein, we review the mechanisms of resistance to chemotherapeutic agents in terms of ceramide metabolism. [J Natl Cancer Inst 2001;93:347–57]

CERAMIDE FORMATION

Ceramide, the basic unit of the lipid sphingomyelin, can be produced de novo or by the hydrolysis of sphingomyelin. Ceramide is produced de novo by ceramide synthase via N-acylation of sphinganine and the addition of a double bond (Fig. 1). Ceramide can also be produced when neutral or acidic sphingomyelinas are activated to cleave the bond between ceramide and the phosphoric acid of sphingomyelin (Figs. 1 and 2). The two long-chain hydrocarbon moieties in ceramide are responsible for the lipid character of the molecule. The addition of galactose to ceramide initiates production of sulfatides (not shown), whereas the addition of glucose generates glucosylceramide, the ganglioside precursor (Fig. 1).

CERAMIDE CELL SIGNALING AND APOPTOSIS

Most research on ceramide-mediated signaling focuses on pathways initiated by the hydrolysis of sphingomyelin (23–25). Additionally, the production of ceramide de novo may initiate signaling pathways (26). The production of ceramide is the result of diverse stimuli that include growth factor deprivation (27–29), cytokines (30–33), ionizing radiation (34,35), heat shock (36), chemotherapy and other cytotoxic agents (26,37–39), and various environmental factors, such as stress (40,41) and even diet (42). These stimuli initiate ceramide-mediated signaling pathways. In addition, exposure of cells to receptor-specific ligands, such as 1,25-dihydroxyvitamin D₃ (43), tumor necrosis factor-α (TNF-α) (39), CD28, or CD95/APO-1/Fas ligand (44), may initiate ceramide-mediated signaling pathways.

Ceramide-mediated cell signaling has been shown to contribute to cell cycle arrest, terminal cell differentiation, and apoptosis (43–48), as well as to cell proliferation (49). Ceramide and other sphingolipids act as second messengers modifying a number of target proteins that induce a cascade of enzymatic and transcriptional activity. One ceramide target protein that has been characterized extensively is ceramide-activated protein kinase (50,51). Other downstream target proteins include ceramide-activated protein phosphatases (52,53), the small guanosine triphosphate-binding protein raf-1, and the atypical protein kinase Cζ (54). These proteins appear to contribute to proliferation and, possibly, to inflammation by recruiting the mitogen-activated protein kinase pathway (55), which has been described in detail (56). These proteins also may recruit the stress-activated protein kinase/c-JUN N-terminal kinase (SAPK/JNK) pathway (56). The SAPK/JNK pathway stimulates activity of AP-1 nuclear factors (e.g., c-Jun) that promote transcriptional activation of various genes and that appear necessary to apoptosis (57). Studies have shown that disrupting the SAPK/JNK/AP-1 pathway with antisense oligonucleotides to either JNK-1 or c-jun (57) or with dominant negative c-jun mutants blocks ceramide-mediated apoptosis (58).

Ceramide-induced cell death proceeds by at least two pathways. One pathway is transcriptionally dependent, and the other is transcriptionally independent. The transcriptionally dependent pathway is mediated by the interaction of members of the TNF superfamily of receptors (i.e., TNF-α and CD95/APO-1/Fas re-
and their specific ligands. In the CD95 receptor pathway, ceramide is generated by acid sphingomyelinase in a complex series of steps. This involves several adapter molecules, such as the TNF receptor 1-associated death domain and the Fas-associated death domain (FADD or MORT1). Death domains of each of these adapter molecules are capable of binding with and potentially activating downstream protease caspsases that affect apoptosis [reviewed in (44)]. This type of transcription-dependent ceramide signaling may be important in determining the cytotoxic response to certain chemotherapeutic agents. Activation of CD95/APO-1/Fas signaling by ceramide has been shown to mediate doxorubicin-induced apoptosis.

The transcription-independent pathway is characterized by the direct activation of acid sphingomyelinase by environmental stresses, such as ionizing radiation and oxidative damage. The subsequent production of ceramide, in turn, activates the SAPK/JNK apoptotic pathway. In addition, the transcriptionally independent formation of ceramide also may affect apoptosis-related proteins of the Bcl-2 family. Ceramide may alter the relationship between proapoptotic (i.e., Bax and Bad) and antiapoptotic (i.e., Bcl-2 and Bcl-XL) members of the Bcl-2 family of proteins that are associated with the mitochondrial membrane. Inhibitory proteins, such as Bcl-2, when overexpressed in cells, have the capacity to block ceramide-mediated cell death without altering ceramide generation.

Both the transcriptionally dependent and independent pathways activate downstream molecules of the interleukin 1β-converting enzyme family of caspase proteases, which affect apoptosis. A number of caspases may be activated in the various ceramide signaling pathways. Caspase-8 is the particular caspase associated with the TNF receptor-mediated signal and interacts directly with the adapter molecules FADD/MORT1. Caspase-9 and caspase-2 are mitochondrial-associated proteases and may be activated in either pathway. Both caspase-8 and caspase-9 recruit caspase-3. Ultimately, multiple caspases are activated, leading to proteosome-directed DNA fragmentation and cell death.

The ceramide pathway is a complex system of Fig. 1. Glycosphingolipid metabolism and sites of drug interaction. Ceramide can be generated de novo via ceramide synthase (cer syn) by the addition of fatty acid to sphinganine and through degradation of sphingomyelin by sphingomyelinase (spm ase). The addition of galactose to ceramide yields sulfatides (not shown), and the addition of glucose to ceramide yields glucosylceramide, precursor of gangliosides. Agents that increase ceramide levels and their proposed sites of interaction are shown in boxes. The drugs shown on the left are believed to increase cellular ceramide levels by the de novo synthesis route, although not solely through ceramide synthase, because serine palmitoyltransferase is also involved. Agents listed at the top right have been shown to enhance ceramide formation by activation of sphingomyelinase. Drugs listed at the bottom promote ceramide elevation by hindering glycosylation via the glucosylceramide synthase (gcs) route (ketoconazole, our unpublished data). 4-HPR = N-(4-hydroxyphenyl)retinamide; Ara-C = 1-β-D-arabinofuranosylcytosine (cytarabine); TNF-α = tumor necrosis factor-α; PPMP = 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; NB-DNJ = N-butyldeoxynojirimycin; DT₃₅s-GM-CSF = a fusion toxin consisting of a truncated diphteria toxin linked to human granulocyte-macrophage colony-stimulating factor.

Fig. 2. Chemical structure of sphingomyelin. Sphingomyelin consists of a long-chain hydrophobic ceramide moiety and a phosphocholine polar head group. The aliphatic chains are generally 16–24 carbons long, saturated or unsaturated, and may contain a hydroxy radical.
signal reinforcement and magnification that has not been defined completely. There may be cross-talk at various levels of either the transcriptionally dependent or independent pathways, and nuclear transcription factors, phospholipases, reactive oxygen intermediates, and intracellular calcium may play relevant roles (48).

CERAMIDE AND RESPONSE TO CHEMOTHERAPY

A number of clinically important cytotoxic agents (Table 1) appear to be effective because of their ability to activate ceramide-mediated pathways in cancer cells. Drugs can impact ceramide metabolism by promoting ceramide synthesis de novo (Fig. 1, left), by activating sphingomyelinase (Fig. 1, top right), and/or by blocking glucosylceramide formation (Fig. 1, bottom). In each case, the result is an enhanced ceramide-governed cytotoxic response. The drugs are of a diverse nature and include the anthracyclines doxorubicin (Adriamycin) and daunorubicin, the vinca alkaloids vincristine and vinblastine, antiestrogens such as tamoxifen, the novel synthetic retinoid N-(4-hydroxyphenyl)retinamide (4-HPR), and the taxane paclitaxel (Taxol).

Table 1. Agents that elicit ceramide generation

<table>
<thead>
<tr>
<th>Agent*</th>
<th>System</th>
<th>Reference No(s.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daunorubicin</td>
<td>Jurkat E6.1; HL-60; U-937; P388, human and murine leukemia</td>
<td>(26,69,70)</td>
</tr>
<tr>
<td>Doxorubicin (Adriamycin)</td>
<td>MCF-7, breast cancer</td>
<td>(71)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>MCF-7-AdrR, doxorubicin-resistant breast cancer</td>
<td>(72)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>ALL-697, leukemia</td>
<td>(65)</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>KB-3-1, epidermoid carcinoma</td>
<td>(73)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Jurkat, leukemia</td>
<td>(96)</td>
</tr>
<tr>
<td>GW 1843</td>
<td>Molt-4, leukemia</td>
<td>(98)</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>MCF-7, MCF-7-AdrR</td>
<td>(72,81)</td>
</tr>
<tr>
<td>1,25-(OH)2 Vit D3</td>
<td>HL-60; HaCaT, keratinocytes</td>
<td>(43,99)</td>
</tr>
<tr>
<td>DT&lt;sub&gt;64&lt;/sub&gt;-GM-CSF</td>
<td>HL-60</td>
<td>(88)</td>
</tr>
<tr>
<td>4-HPR</td>
<td>CHLA-90; SMS-LHN, neuroblastoma; HL-60</td>
<td>(83,84)</td>
</tr>
<tr>
<td>SDZ PSC 833</td>
<td>MCF-7-AdrR; MCF-7; KB-V-1, epidermoid carcinoma</td>
<td>(73,81,137)</td>
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<td>Suramin</td>
<td>MCF-7; DU-145; PC-3, prostate cancer</td>
<td>(91)</td>
</tr>
<tr>
<td>Ara-C</td>
<td>HL-60</td>
<td>(37)</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>B-CLL, leukemia</td>
<td>(101)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>MCF-7; MDA-MB-468, breast cancer</td>
<td>(78)</td>
</tr>
<tr>
<td>Irinotecan (CPT-11)</td>
<td>4B1, murine fibroblasts; HT-29, colon cancer</td>
<td>(102); unpublished data</td>
</tr>
<tr>
<td>Oxalad acid</td>
<td>CHP-100, neuroepithelioma</td>
<td>(149)</td>
</tr>
<tr>
<td>Photodynamic treatment</td>
<td>LS178Y, mouse lymphoma</td>
<td>(150)</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>HL-60</td>
<td>(103)</td>
</tr>
<tr>
<td>Ionizing radiation</td>
<td>BL30A, bovine artery endothelial cells; Burkitt’s lymphoma cells</td>
<td>(104,105)</td>
</tr>
</tbody>
</table>

*1,25-(OH)2 Vit D₃ = 1,25-dihydroxyvitamin D₃; DT<sub>64</sub>-GM-CSF = a fusion toxin consisting of a truncated diphtheria toxin linked to human granulocyte-macrophage colony-stimulating factor; 4-HPR = N-(4-hydroxyphenyl)retinamide; Ara-C = 1-β-D-arabinofuranosylcytosine (cytarabine).

Some of the cytotoxic properties of vinca alkaloids (vincristine and vinblastine), widely used in the treatment of leukemia patients, may be due to their ability to increase cellular ceramide. Exposure of ALL-697 leukemia cells to vincristine causes apoptosis after a sustained increase in ceramide (65). The related alkald vinblastine also works by increasing cellular ceramide levels. Vinblastine concentrations as low as 1.5 nM cause a concomitant increase in ceramide levels and cell death in KB-3-1 human epidermoid carcinoma cells. However, concentrations as high as 1.0 μM have no effect on ceramide levels in the vinblastine-resistant counterpart KB-V-1 cells (73), again suggesting that drug resistance may be related to ceramide metabolism.

Paclitaxel inhibits microtubule depolymerization and is effective against a number of different solid tumors, including ovarian and breast cancers. Paclitaxel induces programmed cell death in a number of different cell types, including leukemia and breast cancer cells (74–76). Studies have shown that the coadministration of paclitaxel with exogenous ceramide substantially inhibits cell proliferation and elicits apoptosis in a synergistic fashion in Jurkat T cells (75) and Tu138 head and neck squamous cell cancer (77). Moreover, studies have shown that the effects of paclitaxel are linked to the de novo synthesis of ceramide in MDA-MB-468 and MCF-7 breast cancer cells (Fig. 3, A, MCF-7 cells) (76,78) and that paclitaxel-dependent cytotoxicity was abrogated by blocking ceramide production with 1-cycloserine, an inhibitor of ceramide synthesis (78).

Triphenylethylene antiestrogens, such as tamoxifen, block conversion of ceramide to glucosylceramide (79,80) and, thereby, promote increases in cellular ceramide. This activity is independent of estrogen receptor status. Although increases are moderate (35%), the combination of tamoxifen with agents, such as doxorubicin (72) or the cyclosporin A analogue SDZ PSC 833 (81), has a synergistic effect on ceramide formation. For example, exposure of MCF-7-AdrR cells to tamoxifen, SDZ PSC 833, or tamoxifen plus SDZ PSC 833 increased ceramide levels by 35%, 50%, and 110%, respectively (81).
Fig. 3. Dose–response profiles for ceramide formation induced by various agents in human cancer cells. The representative cell lines include breast cancer (MCF-7), prostate cancer (LNCaP), vincristine-resistant leukemia (HL-60/VCR), and ovarian carcinoma (SKOV-3). Cells were exposed to agents at the concentrations indicated for 24 hours with [3H]palmitic acid in the medium as the ceramide metabolic precursor and ceramide were measured. Data are expressed as the percent of control. 4-HPR = N-(4-hydroxyphenyl)retinamide; DT388-GM-CSF = a fusion toxin consisting of a truncated diphtheria toxin linked to human granulocyte–macrophage colony-stimulating factor.

The synthetic retinoid 4-HPR elicits apoptosis in human prostate carcinoma cells through genesis of reactive oxygen species, nuclear retinoic acid receptors, or apoptosis-related genes (82). In addition, 4-HPR increases the level of intracellular ceramide in highly drug-resistant human neuroblastoma cell lines (83) and in the prostate cancer cell line LNCaP (Fig. 3, B, and our unpublished data). Because the expression of p53 protein is not affected by 4-HPR, the retinoid may form the basis for a novel p53-independent chemotherapy regimen targeting the ceramide pathway. In leukemia cells, 4-HPR but not retinoic acid activates ceramide formation and induces apoptosis (84). Simultaneous treatment of leukemia cells with 4-HPR and the ceramide synthase inhibitor fumonisin B1 decreases ceramide elevation and blocks induction of apoptosis.

DT388-GM-CSF, a fusion toxin consisting of a truncated diphtheria toxin linked to human granulocyte–macrophage colony-stimulating factor (GM-CSF) (85), is toxic to acute myeloid leukemia progenitors but the GM-CSF receptor but not to normal marrow cell progenitors (86). The fusion toxin modulates drug resistance in vincristine-resistant HL-60 cells (87). Although one mechanism of action is inactivation of the expression of multidrug resistance proteins, we have shown that DT388-GM-CSF also is a potent agonist of ceramide formation in HL-60/VCR cells (Fig. 3, C). In these cells, ceramide is generated via hydrolysis of cellular sphingomyelin, suggesting a sphingomyelinase-governed response. In addition, we have shown that ceramide formation is followed by activation of caspase-9 and caspase-3 in this cell system (88).

Suramin, a polysulfonated naphthylurea introduced in the 1920s for the treatment of African trypanosomiasis, is synergistic with TNF-α and doxorubicin in human prostate cancer cells (89). Synergy was noted at drug concentrations achieved clinically. A subsequent study (90) showed that bcl-2-transfected prostate cancer cells were resistant to apoptosis induced by doxorubicin; use of a doxorubicin/suramin combination circumvented this resistance. Gill and Windebank (91) reported that suramin disrupts glycolipid metabolism and elicits apoptosis after elevation of ceramide in various cancer cell models. In animal studies, suramin slows the growth of DU145 prostate cancer xenografts in nude mice (92), and it is currently in clinical trials for breast and prostate cancers (93–95).

Several other cytotoxic agents have been shown to elevate cellular ceramide levels. Tepper et al. (96) reported a threefold elevation of ceramide levels in Jurkat T cells whose DNA has been damaged by exposure to the topoisomerase II inhibitor etoposide. Etoposide increases cellular ceramide levels de novo via activation of serine palmitoyltransferase (97). GW1843, a thymidylate synthase inhibitor in clinical development, causes Molt-4 leukemic T cells to undergo apoptosis with activation of both acidic and neutral sphingomyelinases and ceramide production (98). In this study, the kinetics of ceramide formation were consistent with its role in signaling apoptosis. 1,25-Dihydroxyvitamin D3 stimulates hydrolysis of sphingomyelin in leukemia cells (43) and in keratinocytes (99). It also inhibits the growth of prostate adenocarcinoma cells (100), but it is not known whether ceramide is involved in growth inhibition. Two nucleoside analogues, fludarabine and 1-β-D-arabinofuranosylcytosine, promote ceramide generation in leukemia cells. Exposure of HL-60 cells to 1-β-D-arabinofuranosylcytosine causes a time-dependent and dose-dependent increase in ceramide (37) that follows activation of neutral sphingomyelinase. Treatment of B cells (from patients with chronic B-cell lymphocytic leukemia) with fludarabine results in ceramide elevation and in killing by both apoptotic and nonapoptotic mechanisms (101). The topoisomerase I inhibitor irinotecan (CPT-11) increases ceramide levels in 4B1 mouse fibroblasts (102) and in HT-29 human colon cancer cells (our unpublished data). CPT-11 cytotoxicity is blocked by treatment with the ceramide synthesis inhibitor fumonisin B1 ([102]; our unpublished data). Treatment of HL-60 cells with sodium nitroprusside, a nitric oxide donor, is associated with activation of magnesium-dependent neutral sphingomyelinase, generation of ceramide, and apoptosis (103).
To our knowledge, this work is the first to show a relationship between sphingolipid metabolites and nitric oxide-mediated cell death signaling; however, the response may be cell type specific.

**Ceramide and Response to Radiotherapy**

In addition to playing an important role in cell death signaling in response to chemotherapeutic agents, ceramide also may be important to radiation-induced cell death. Exposure of bovine aortic endothelial cells to ionizing radiation induces apoptotic signaling as a result of hydrolysis of sphingomyelin to ceramide by a neutral sphingomyelinase (104). Subsequent studies in HL-60 and U-937 cells showed that DNA fragmentation induced by ionizing radiation is accompanied by a decrease in Bcl-2 messenger RNA (mRNA) levels. Exposure of cells to ceramide had the same impact on Bcl-2, suggesting that modulation of Bcl-2 gene expression may be a target of ceramide-mediated apoptosis after exposure to ionizing radiation.

Cellular resistance to ionizing radiation also may be linked to impaired ceramide production or defective ceramide signaling (105,106). Michael et al. (105) demonstrated a direct relationship between resistance to radiation-induced apoptosis and defective ceramide signaling in Burkitt’s lymphoma. Acquired defects in ceramide cell death signaling may contribute to the development of radioresistant thymoma cell lines (107). Ceramide also has been shown to participate in molecular events that govern UV radiation-induced apoptosis (108). Moreover, defective radiation-induced ceramide generation and cellular resistance to radiation treatment in sphingomyelinase knockout mice and in patients with Niemann–Pick disease (characterized by a congenital deficiency in sphingomyelinase) can be corrected by transfection of the human acidic sphingomyelinase gene (34).

**Ceramide Metabolism and Multidrug Resistance**

Although there is controversy surrounding the role of ceramide in programmed cell death (65,109,110), the resistance of cancer cells to chemotherapy can be reversed by targeting the metabolism of ceramide. Our research strongly indicates that dysfunctional ceramide metabolism contributes to multidrug resistance and that enhancement of the ceramide response enhances cellular response to chemotherapy. Specifically, ceramide glycosylation by the enzyme glucosylceramide synthase (GCS), which forms the noncytotoxic metabolite glucosylerceramide and has been noted in some multidrug-resistant cell lines (18,19), may be an important pathway for bypassing apoptosis.

The cytotoxic potential of many cancer drugs is related to activation of signal transduction pathways that lead to apoptosis (111–113). Disruption of this process renders tumor cells drug resistant. TNF-α-resistant MCF-7 breast cancer cells have been characterized by the inability of their neutral or acidic sphingomyelinases to generate ceramide (20). Using rhabdomyosarcoma cells, Bourteele et al. (114) showed that TNF-α-induced apoptosis is preceded by a multiphasic increase in intracellular ceramide and inhibition of two ceramide-metabolizing enzymes, GCS and sphingomyelin synthase. In addition, acid ceramidase, which catalyzes ceramide breakdown, is overexpressed in prostate tumor tissue and in prostate tumor cell lines (115). This enzyme may play a role in later stage hormone-insensitive, chemotherapy-refractory prostate cancer. Overexpression of acid ceramidase also protects cells from TNF-α-induced death, whereas pharmacologic suppression of acid ceramidase by N-oleylethanolamine restores ceramide accumulation and sensitivity to cytokines (116). These results suggest that the ability of cells to limit ceramide metabolism contributes to chemosensitivity.

Evidence is mounting that the accumulation of a glycosylated form of ceramide, glucosylerceramide, may play an important role in the development of drug resistance. Glucosylerceramide is an intermediate metabolite in the synthesis and degradation of the more complex gangliosides (Fig. 1, right). A number of drug-resistant cancer cell lines accumulate this noncytotoxic metabolite (18,19). Drug-resistant MCF-7/AdR breast cancer and KB-V-1 cutaneous cancer cells have higher levels of glucosylerceramide than their drug-sensitive counterparts, MCF-7 and KB-3–1 (19). The human ovarian adenocarcinoma cell line NIH:OVCAR-3, established from a patient resistant to doxorubicin, melphalan, and cisplatin, also expresses high levels of glucosylerceramide (19). Moreover, analysis of tumors from selected cancer patients who failed to respond to chemotherapy treatment demonstrated elevated glucosylerceramide levels (19). These findings suggest that elevated glucosylerceramide levels in cancer cells or in tumors represent a marker for a drug-resistant phenotype.

The level of activity of GCS, which converts ceramide to glucosylerceramide, may determine the multidrug resistance phenotype in cancer cells. To better characterize the influence of GCS on multidrug resistance, we used a retroviral “tetracycline-on” expression system to introduce the GCS gene into drug-sensitive MCF-7 cells. The resulting cell line MCF-7/GCS expresses an 11-fold higher level of GCS activity, is resistant to doxorubicin and exogenous ceramide (21), and is also resistant to TNF-α-induced cell death (22). Lipid metabolism studies confirmed that TNF-α causes an increase in ceramide in MCF-7 cells but causes an increase in glucosylerceramide in resistant MCF-7/GCS cells. In addition, TNF-α exposure increases caspase activity in MCF-7 cells but not in MCF-7/GCS. Furthermore, resistance to doxorubicin and TNF-α in MCF-7/GCS cells is related to hyperglycosylation of ceramide and not to changes in the levels of P-glycoprotein, Bcl-2, or TNF receptor-1 expression (21,22). These results support the theory that GCS activity and the accumulation of cellular glucosylerceramide are important to the development of chemotherapeutic resistance in cancer cells.

**Chemosensitization and Reversal of Drug Resistance by Regulating Ceramide Metabolism**

P-glycoprotein is the most widely studied contributor of multidrug resistance. Binding of the calcium channel blocker verapamil and other multidrug resistance modulators to P-glycoprotein inhibits the pump activity of P-glycoprotein and thereby reverses drug resistance (117,118). Among the P-glycoprotein-inactivating compounds are calcium channel blockers, cyclosporin A and its analogue SDZ PSC 833, and tamoxifen (119,120). However, additional mechanisms of action have been postulated for these agents because clinical efficacy does not always correlate with multidrug resistance phenotype (121). Radin and colleagues (122–124) described application of glycosphingolipid metabolism in cancer treatment and described how inhibitors of GCS may act as chemotherapeutic agents (125). Several classic P-glycoprotein drugs retard the conversion of ceramide to glucosylerceramide (72,79,80,126) (Fig. 1).
Tamoxifen as used in the Dartmouth regimen for the treatment of melanoma (127) and evaluated for the treatment of pancreatic carcinoma and malignant gliomas (128,129) blocks glucosylceramide formation in vinblastine-resistant cells (79). In doxorubicin-resistant cells, clinically relevant concentrations of tamoxifen, verapamil, and cyclosporin A markedly decrease glucosylceramide levels with 50% inhibitory concentrations of 1.0, 0.8, and 2.3 μM, respectively (80). When MCF-7-AdR cells were supplemented with ceramide in the presence of tamoxifen, decreased viability and apoptosis occurred (72). However, exposure of MCF-7-AdR cells to the tamoxifen analogue triphenylethylene, which is missing the dimethylethanolamine moiety, does not inhibit ceramide glycosylation or sensitize cells to doxorubicin. These and additional results (130) suggest a structural and a stereoselective requirement of tamoxifen analogues to be effective (e.g., cis-tamoxifen has no chemotherapy-sensitizing activity).

Several other hormone-modulating agents that are capable of altering drug resistance also retard cellular synthesis of glucosylceramide. Toremifene, an antiestrogen that is chemically and pharmacologically similar to tamoxifen, is also effective (126,131) and is being evaluated for reversing multidrug resistance in renal cell cancer patients (132). The antiprogestine mifepristone (RU486) inhibits proliferation and induces apoptosis in breast cancer cells (133). Mifepristone also inhibits glucosylceramide production (126) while sensitizing MCF-7-AdR cells to the toxic effects of doxorubicin (72). Of interest, the combination of mifepristone and tamoxifen has been shown to elicit greater cell death than either agent alone when tested on MCF-7 breast cancer cells (134). The antifungal ketoconazole, which is structurally similar to tamoxifen, overcomes resistance to doxorubicin and vinblastine (135) and inhibits glucosylceramide synthesis (our unpublished data).

SDZ PSC 833 is a cyclosporin-based multidrug resistance modulator that appears to act via a P-glycoprotein mechanism (136). However, we have also shown that this agent is an effective inducer of ceramide formation (137). Investigators (138) have reported the antiproliferative effects of SDZ PSC 833 in drug-resistant cells, and our group (73,81) demonstrated that this agent’s ability to increase cellular ceramide is associated with a progressive decline in cell survival. SDZ PSC 833 is active in the 1- to 5-μM range; in drug-sensitive MCF-7 breast cancer cells, SDZ PSC 833-induced ceramide generation is associated with decreased cell survival (137), independent of the expression of P-glycoprotein (139). SDZ PSC 833 increases vinblastine sensitivity in both P-glycoprotein-rich and P-glycoprotein-poor cancer cells (73). An example of the influence of SDZ PSC 833 on ceramide metabolism is shown in Fig. 3, D, with the human ovarian carcinoma cell line SKOV-3. Cells with enhanced capacity for ceramide glycosylation (18) are more resistant to SDZ PSC 833 than wild-type cells (81). A metabolic study (81) shows that drug-resistant cells initially generate ceramide in response to SDZ PSC 833, but this ceramide is converted to glucosylceramide. Studies with fumonisin B₁ (73,81,137) and analyses of sphingomyelin decay indicate that SDZ PSC 833 activates de novo synthesis of ceramide in cancer cells. Examination of intracellular ceramide metabolites showed that doxorubicin-resistant MCF-7-AdR breast cancer cells converted ceramide to glucosylceramide, whereas drug-sensitive MCF-7 cells contained only free ceramide and no detectable glucosylceramide (126).

1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP), 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), and 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) are structural analogues of the natural sphingolipid substrate of GCS and are potent competitive enzyme inhibitors (140). PPMP effectively inhibits glucosylceramide synthesis (50% inhibitory concentrations = 0.9 μM) in MCF-7-AdR cells (80). A PPMP concentration that maximally inhibits glucosylceramide synthesis has no effect on cell viability but does sensitize cells to doxorubicin (80). Nicholson et al. (141) showed preferential killing of drug-resistant cell lines by PPMP and its analogue PPPP. Spinedi et al. (142) showed that PDMP suppressed glucosylceramide synthesis and markedly potentiated the apoptotic effect of C₂₀-ceramide in CHP-100 neuroepithelioma cells. The authors concluded that activation of glucosylceramide synthesis is a cellular mechanism for escape from ceramide-induced apoptosis.

The imino sugars N-butyldeoxynojirimycin and N-butyldeoxyoxagalctonojirimycin also inhibit GCS (143,144) and are currently in clinical trials for treatment of Gaucher’s disease (a glycosphingolipid lysosomal storage disease). The ability to block ceramide glycosylation makes the imino sugars promising therapeutic agents for the treatment strategy shown in Fig. 1.

Studies indicate that the coadministration of ceramide metabolism modulators enhances levels of ceramide. Investigators have shown that cytotoxicity of the novel synthetic retinoid 4-HPR can be enhanced by adding modulators of ceramide metabolism (145). Exposure of CHLA90 cells to 4-HPR produced a fivefold increase in ceramide levels, a fourfold increase in glucosylceramide levels, and a 2.5 order of magnitude increase in cell killing (83). When PPMP was added, inhibition of GCS completely prevented the increase in glucosylceramide, yielded a sevenfold increase in ceramide levels, and produced a synergistic multifold greater enhancement in cytotoxicity (145). Adding tamoxifen to treatment with SDZ PSC 833 in MCF-7/AdR breast cancer cells increasingly inhibits the metabolism of ceramide and further diminishes cell survival. When treatment with tamoxifen is combined with doxorubicin, ceramide levels increase fivefold to 26-fold and cell survival drops to zero (81). It appears that drug combinations eliciting the greatest ceramide increase may be the most cytotoxic.

A number of pathways affecting the metabolic fate of ceramide are activated in response to various chemosensitizing agents. Nevertheless, in some cases, the mechanisms by which ceramide metabolism is affected by these agents is not completely clear. In some cells treated with PPMP, ceramide increases (146) as a result of blocking glycosylation. The mode by which agents, such as tamoxifen, cyclosporine A, mifepristone, and verapamil, elevate ceramide is poorly understood. Although these drugs block the formation of glucosylceramide from ceramide (79,80,126,130,131), direct interaction with GCS has not been demonstrated. These agents, therefore, cannot be considered to be glycosylation inhibitors; instead they might induce apoptosis by direct binding to target proteins of ceramide. However, work with 4-HPR suggests direct targeting of the enzymes of de novo ceramide synthesis (83,145). Pre-exposure of intact neuroblastoma cells to 4-HPR elicits time- and dose-dependent increases in serine palmitoyltransferase and ceramide synthase, as measured in in vitro assays with microsomes (our unpublished data).
The role of GCS in regulating cellular response to chemotherapy has also been demonstrated by introducing GCS antisense complementary DNA (cDNA) into doxorubicin-resistant cancer cells (Fig. 4). Transfecting drug-resistant MCF-7/AdR cells with GCS antisense cDNA decreases cellular glycosylation, effectively reversing drug resistance (147,148). Reverse transcription-coupled polymerase chain reaction, western blot, and in vitro assays showed that MCF-7/AdR/asGCS, the cell line generated, has decreased GCS mRNA, GCS protein, and GCS enzymatic activity and is 28-fold more sensitive to doxorubicin than the parent MCF-7/AdR cell line. Exposure to doxorubicin causes both time- and dose-dependent increases in ceramide levels, caspase-3 activity, and cell death in MCF-7/AdR/asGCS cells transfected with GCS antisense when compared with MCF-7/AdR parent cells. These findings demonstrate that transfection of GCS antisense restores sensitivity to anthracyclines and resumption of ceramide/caspase apoptotic signaling. A reverse scenario also has been demonstrated by transfecting drug-sensitive MCF-7 breast cancer cells with GCS sense cDNA (Fig. 4), conferring chemotherapy resistance (21,22).

**CONCLUSIONS**

Cancer cells develop multiple, and often overlapping, mechanisms that allow them to become resistant to chemotherapeutic agents. The dysfunctional metabolism of ceramide is another one of these inherent or acquired mechanisms that contribute to cellular drug resistance. Numerous studies have helped define the ceramide signaling pathways that contribute to cell death. Studies also indicate that alterations in these cell death signaling pathways may contribute to resistance to standard chemotherapeutic agents in several in vitro cancer models, including breast, prostate, and squamous cell cancers. Investigators have demonstrated the efficacy of targeting ceramide synthesis or degradation pharmacologically to enhance the cytotoxic effects of several clinically relevant drugs. In addition, knocking out the GCS enzyme (which converts ceramide to an inactive metabolite) by transfection of cells with GCS antisense cDNA has been shown to completely reverse breast cancer cell resistance to anthracyclines (148). Targeting ceramide metabolic and cell death signaling pathways is an attractive clinical treatment strategy for overcoming drug resistance and continues to be studied actively. A Rapid Access to Preventive Intervention Development grant recently has been issued by the Developmental Therapeutics Program, National Cancer Institute, National Institutes of Health, Bethesda, MD, to support drug-directed toxicology of 4-HPR and safingol, agents that increase ceramide and enhance chemotherapeutic cytotoxicity in tumor cell lines in vitro (145).

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NOTES

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