REVIEW ARTICLE

Killing tumours by ceramide-induced apoptosis: a critique of available drugs

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Over 1000 research papers have described the production of programmed cell death (apoptosis) by interventions that elevate the cell content of ceramide (Cer). Other interventions, which lower cellular Cer, have been found to interfere with apoptosis induced by other agents. Some studies have shown that slowing the formation of proliferation-stimulating sphingolipids also induces apoptosis. These relationships are due to the two different aspects of Cer: Cer itself produces apoptosis, but metabolic conversion of Cer into either sphingosine 1-phosphate or glucosphingolipids leads to cell proliferation. The balance between these two aspects is missing in cancer cells, and yet intervention by stimulating or blocking only one or two of the pathways in Cer metabolism is very likely to fail. This results from two properties of cancer cells: their high mutation rate and the preferential survival of the most malignant cells. Tumours treated with only one or two drugs that elevate Cer can adjust the uncontrolled processes to either maintain or to ‘aggravate’ the

excessive growth, angiogenesis and metastasis characteristics of tumours. These treatments might simply elevate the production of growth factors, receptors and other substances that reduce the effectiveness of Cer. Tumour cells that do not adapt in this way undergo apoptosis, leaving the adapted cells free to grow and, ultimately, to ‘subdue’ their host. Thus it is important to kill every type of cancer cell present in the tumour rapidly and simultaneously, using as many different agents to control as many pathways as possible. To aid this approach, this article catalogues many of the drugs that act on different aspects of Cer metabolism. The techniques described here may lead to the development of practical chemotherapy for cancer and other diseases of excess proliferation.

Key words: glutathione depletion, glucosphingolipid-induced proliferation, multidrug resistance, poly-drug chemotherapy, reactive oxygen species, sphingolipid.

INTRODUCTION

Readers unfamiliar with sphingolipid metabolism should consult some of the 600+ reviews available (e.g. [1–8]). The reviews

typically describe only a few metabolic steps [namely, the hydrolysis of sphingomyelin (SM) to form ceramide (Cer) and the *de novo* synthesis of Cer from serine and palmitoyl-CoA], but all the steps in Cer metabolism are important in cancer. The mechanisms by which Cer produces apoptosis are understood too incompletely to warrant discussion here, but they appear to involve the allylic alcohol group in Cer at C-3, since dihydroCer (a precursor of Cer) has rarely shown biological effects. Oxidation of the C-3 hydroxy group in mitochondria, generating reactive oxygen species (ROS), has been proposed as a major step [9]. In addition, Cer controls protein phosphorylation and calcium levels, possibly by formation of an intermediate cyclic phosphate ester between the C-1 and C-3 hydroxy groups. These properties appear to explain the multitude of Cer effects. This review describes eight approaches to elevating tumour Cer levels, which are outlined in Table 1. The Table should help the reader follow the many cross-references to various processes and drugs that are impossible to avoid.

Table 1 Eight approaches to use in multidrug cancer chemotherapy

Therapeutic approach	Available drugs
Use exogenous Cer or analogue	C ₂ -Cer, Cer glucuronide, pegylated Cer, various analogues
Stimulate <i>de novo</i> synthesis of Cer	Palmitic acid, serine, THC analogues, gemcitabine, vitamin C, valspodar, retinoic acid analogues, camptothecin, etoposide, paclitaxel, fludarabine
Stimulate SM hydrolysis	Deplete glutathione by synthesis inhibition, thiol oxidation, competitive utilization, or Michael condensation. Oxidized LDL, arachidonic acid elevation, ketoconazole, irinotecan, doxorubicin, dihydroxyvitamin D ₃ , mitoxanthrone, dexamethasone, arabinofuranosylcytosine
Stimulate GlcCer hydrolysis	Acidic phospholipids, vitamin A, saposin C analogues, CPZ
Inhibit GlcCer synthesis	P-drugs (PDMP, PPMP, PPPP, ethylenedioxy-P4), tamoxifen, mifepristone, <i>N</i> -butyl deoxynojirimycin, anti-androgens
Inhibit Cer hydrolysis	<i>N</i> -Oleylethanolamine, <i>D</i> -MAPP
Inhibit SM synthesis from Cer	Deplete tumour of PtdCho
Elevate Cer in tumour by radiation	Synergizes with above drugs

1. USE OF EXOGENOUS Cer

Treating cancer cells *in vitro* with exogenous Cer (the structure of which is given in Figure 1) almost always produces apoptosis. Naturally occurring Cers are very insoluble in water, so most tests have utilized the more easily dispersed short-chain Cer, *N*-

Abbreviations used: Cer, ceramide; C₂-Cer, *N*-acetylsphingosine; COX-2, cyclo-oxygenase-2; CPZ, chlorpromazine; DAG, diacylglycerol; GlcCer, glucosylceramide; GSL, glucosphingolipid; LDL, low-density lipoprotein; *D*-MAPP, *D*-erythro-2-tetradecanoylamino-1-phenyl-1-propanol; MDR, multidrug resistance; NSAIDs, non-steroidal anti-inflammatory drugs; P4 (or PPPP), *D*-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; PDMP, *D*-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PPMP, *D*,*L*-threo-(1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol); PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; ROS, reactive oxygen species; S1P, sphingosine 1-phosphate; SM(ase), sphingomyelin(ase); SPT, serine palmitoyltransferase; THC, tetrahydrocannabinol; UDP-Glc, UDP-glucose.

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Figure 1 Structure of Cer

acetyl sphingosine (C_2 -Cer), or C_6 -Cer or C_8 -Cer. The Cer is usually dissolved and added in ethanol or DMSO, and probably forms a fine suspension on dilution with cell medium. These Cers inhibit cell growth and have been shown to compete metabolically with natural Cers and their metabolites, forming free sphingosine, short-chain SM and short-chain glucosphingolipids (GSLs) [10,11]. Some researchers use natural Cer, typically sphingosine bound to a C_{16-26} fatty acid. The lipid is added in a solution of ethanol and dodecane, which apparently yields a cell-penetrating fine dispersion [12]. *In vivo*, easily accessed tumours (e.g. skin) may respond to dispersions or ointments containing Cer. The use of lecithin as a dispersing agent should be avoided, because it may promote conversion of the Cer into SM by phosphocholine exchange (see section 7 below).

(a) Cer 1-glucuronide, hydrolysed to Cer in the intestine after ingestion, was found to reduce the carcinogenic activity of 1,2-dimethylhydrazine, with significant reductions in colonic cell proliferation and the appearance of aberrant crypt foci [13]. If the liberated Cer is absorbed into the bloodstream, the glucuronide (or a similar polar derivative) may be of more general value. Dietary sphingolipids (typically ≈ 0.3 g/day) are also hydrolysed to some extent in the digestive tract, and may constitute natural anti-carcinogens [14].

(b) Cer can be solubilized by attaching poly(ethylene glycol) at a suitable site. Attaching poly(ethylene glycol) chains at the C-1 hydroxy group via a succinic acid 'spacer' has been shown to greatly increase the circulation lifetimes of liposomes formed from SM, cholesterol and vincristine (an anti-cancer drug that promotes Cer formation in cancer cells) [15]. It is very likely that pegylated Cer can penetrate tumours and undergo hydrolysis, liberating the Cer. A more effective liposome might be created by using Cer instead of cholesterol, enabling all four components to elevate tumour Cer levels. Cholesterol should be avoided, since it binds tightly to SM and hinders its hydrolysis (see section 3 below).

(c) Variant analogues of the basic Cer structure ought to be tested, especially ones with a lower melting point, which might penetrate tumours more readily. Apoptosis-promoting analogues have been described, and favourable structural features were proposed for further syntheses [16]. Intensification of the allylic hydroxy group, using conjugated double bonds instead of a single one, can be expected to yield increased apoptogenic activity. Cer containing an extra allylic alcohol group (at C-6) has been prepared [17]; this too may be a potent analogue of Cer.

2. STIMULATING Cer SYNTHESIS *DE NOVO*

The first step, a condensation between serine and palmitoyl-CoA by serine palmitoyltransferase (SPT), appears to be the rate-limiting step in Cer synthesis. This is followed by reduction of the product to form sphinganine, which is then acylated and desaturated (Figure 2).

(a) Cer is made from palmitate (primarily) and a second fatty acid, and therefore its synthesis can be accelerated by adding

palmitic acid to cell media [18–20]. In a growth medium for astrocytes, it gradually produced apoptosis, an effect that could be blocked by inclusion of L-cycloserine (SPT inhibitor) or fumonisins B1 (sphingol acylation inhibitor) [21]. Thus one can conclude that the apoptosis was not due to a toxic action of the fatty acid, such as detergency. Exposure of rat pancreatic islets to 0.5 mM palmitic acid for 4 days resulted in a 400–800% increase in β -cell DNA fragmentation that was attributed to accelerated Cer synthesis [22]. In accordance with these studies, exogenous palmitate enhanced [14 C]serine incorporation into Cer. A drug that protects palmitate from mitochondrial oxidation, by blocking the formation of carnitine palmitate, enhanced the apoptogenic action of palmitate.

Carnitine, needed for fatty acid oxidation in mitochondria, inhibited Cer synthesis in the murine haematopoietic cell lines LyD9 and WEHI-231 [18]. Presumably it created a shortage of Cer's fatty acid precursors. This inhibition could be minimized by adding an inhibitor of carnitine palmitoyltransferase I (etomoxir), which enhanced the apoptogenic effect of palmitate. Carnitine blocked apoptosis of skeletal muscle cells, consistent with its protective action against the myopathy seen in congestive heart failure [23]. (The latter effect may be a complication of Cer-elevation chemotherapy, like the limitation on doxorubicin chemotherapy.) Carnitine also interferes with the generation of Cer from SM [24]. These factors suggest that dietary carnitine supplements should be avoided during cancer therapy and, perhaps, as a cancer-preventative measure.

(b) Rapid fatty acid synthesis in various tumours has been noted, supporting the evidence for rapid sphingolipid synthesis in cancer cells. In LNCaP prostate cancer cells, it was found that fatty acid synthase and related enzymes are elevated by androgens [25]. Testosterone also elevated glucosylceramide (GlcCer) synthase and lowered GlcCer glucosidase in mouse kidneys [26], so it is possible that the hormone stimulates all the steps involved in elevating Cer and GSL levels. Thus anti-androgen therapy may act to reduce Cer levels. If appropriate inhibitors of the Cer-converting enzymes are included in the anti-neoplastic cocktail, supplemental androgens might be helpful in treating tumours of androgen-sensitive tissues.

It has been suggested that administration of androgens might slow the growth of androgen-independent prostate cancers [27], and experiments with such cells have shown that this works [28,29]. Thus it follows that anti-androgen therapy is appropriate during the initial treatment, and then, when androgen-insensitive cancer clones have appeared, androgen supplementation should be started. This is close to the intermittent androgen-deprivation technique, which has been effective in blocking the appearance of resistant prostate cancer in patients [30].

(c) The K_m for serine in the SPT condensation step is relatively high, so eating serine might stimulate Cer synthesis. It might also enhance synthesis of phosphatidylserine (PtdSer), which stimulates Cer formation from GlcCer and SM.

(d) Tetrahydrocannabinol (THC) has produced Cer elevation and apoptosis in cancer cells by stimulating SPT [31,32]. Synthetic analogues of THC showed diminished psychological effects, but more anti-cancer activity [33,34]. These seemed to act via binding to the CB₂ (cannabinoid type-2) receptor, rather than CB₁. Activity against lymphoblastic cancer in mice was demonstrated with THC [35]. Anandamide, a naturally occurring 'mammalian cannabinoid', also produced tumour apoptosis in mice, presumably because it acts like THC to stimulate SPT [35].

(d) The *de novo* Cer-synthesis pathway may also account for the anti-neoplastic effectiveness of gemcitabine. Treatment of A549 cells with 1 μ M gemcitabine trebled Cer levels, and also increased 3-fold the incorporation of [3 H]palmitate into Cer. The

Figure 2 Cer synthesis *de novo*

pathway was confirmed by pulse-labelling experiments and inhibition with 50 nM myriocin, a fungal sphingosine analogue that inhibits SPT [36].

(e) Vitamin C enhanced Cer synthesis in keratinocytes cultured for 9 days [37]. GlcCer was also elevated, possibly because of the increased availability of its lipoidal precursor. The specific activity of Cer synthase (the acylation step) was increased markedly, but SPT and GlcCer synthase activities were unchanged. The acylation reaction was assayed with cell homogenates, [¹⁴C]-lignoceric acid, reduced CoA, NADPH and NADH, a system in which it is assumed that the availability of sphingols is not rate-limiting. Studies of vitamin C effects in human cancer or cancer cells have given variable results which, I believe, are due to the importance of ascorbate concentration: like Cer, it can stimulate or inhibit growth. Ascorbate can promote or inhibit the production of ROS, a vital factor in the connection between Cer and cancer growth [9,38]. The vitamin attacks GSH, and so it can be expected to stimulate SM conversion into Cer (see section 3a).

(f) A drug acting against multidrug-resistant tumours, PSC 833 (valsopodar), was found to produce a considerable increase in cellular Cer in the multidrug resistance (MDR) cell line, KB-V-1 [39]. The increase was seen as early as 15 min, continuing at least for 24 h, with apoptosis and loss of cells. Fumonisin B1, a fungal sphingosine analogue that inhibits sphingol acylation, blocked the increase, suggesting that the extra Cer originated from *de novo* synthesis, rather than from hydrolysis of complex sphingolipids. However, experiments based on fumonisin require further substantiation, since the toxin produces elevated levels of sphingols and stimulates DNA synthesis [40], despite statements in the literature asserting that it only blocks sphinganine acylation. The free sphingols themselves interfere with sphingolipid metabolism, and can be phosphorylated to yield the counter-apoptogenic sphingosine 1-phosphate (S1P). (These reactions may explain why fumonisin contamination of food can lead to cancer.) Testing the effect of myriocin or of chloroalanine, a serine analogue that blocks SPT, would help confirm the involvement of the *de novo* pathway.

(g) Retinoic acid, another elevator of Cer levels and producer of apoptosis, was reported to act by stimulating the first step in

sphingol synthesis in PCC7-Mz1 stem cells that differentiate into brain cells [41]. Because some anti-cancer activity was observed, there has been a search for more effective retinoids. One derivative, fenretinide [*N*-(4-hydroxyphenyl)retinamide], also induced an increased Cer level and apoptosis [42], and has shown promising effects in cancer patients [43–45]. However, it was found to elevate GlcCer and SM levels in cultured cells [46], evidently because of the higher Cer concentration, so an inhibitor of GlcCer synthase ought to be included in the therapeutic cocktail. (SM accumulation should be harmless if a stimulator of SM hydrolysis is also included.) Fenretinide seems to stimulate directly the acylation of sphinganine, as well as the serine–palmitate condensation [41,47].

Exposure of six human lymphoblastic leukaemia cell lines to fenretinide led to Cer accumulation and apoptogenic cell death, but non-malignant lymphocytes were little affected [48]. This is a typical difference between normal and cancer cells with respect to sphingolipid metabolism. ROS appeared together with the Cer. Like Cer, the drug generates ROS in mitochondria, acting at some point in the electron-transfer chain, probably involving coenzyme Q [38,49]. ROS have the ability to stimulate Cer synthesis, and so the process forms an expanding spiral of Cer generation, ROS synthesis and mitochondrial damage, leading to apoptosis [9,50].

Since ROS stimulate neutral SM hydrolase, it is likely that a closer look at the changes produced by stimulators of the *de novo* route will show that some of the Cer is formed from SM (section 3). It is important that inclusion of an inhibitor of Cer glucosylation, PPMP [D,L-threo-(1-phenyl-2-hexadecanoyl-amino-3-morpholino-1-propanol)], increased Cer levels further, and increased fenretinide cytotoxicity in four of the cell lines [48].

(h) Camptothecin, an important anti-neoplastic agent, induced rapid production of Cer and apoptosis of mitogen-activated peripheral lymphocytes [51]. Camptothecin also induced apoptosis in retinal pigment epithelial cells, synergistically with CD95 (also a Cer promoter) [52]. It also induced accumulation of p53 and the p53 response protein, p21, as well as the pro-apoptotic BAX protein. Elevation of Cer in prostate cancer cells was attributed to faster *de novo* synthesis, since fumonisin almost

completely blocked Cer formation, and the specific activities of acid and neutral sphingomyelinases (SMases) decreased [53]. However, the fumonisin did not affect the apoptogenic action of camptothecin: a puzzling finding.

The importance of Cer accumulation in the action of camptothecin (and that of its more stable analogue, homocamptothecin) was shown in colon HT29 cells. Including glucosylation inhibitors PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) and PPMP in the cell medium markedly enhanced apoptosis by the drugs [54]. These inhibitors not only forced Cer accumulation, but also blocked formation of pro-proliferative GSLs (see section 5a).

(i) Etoposide, an anti-cancer drug that inhibits topoisomerase II and breaks DNA strands, also elevated Cer levels, lowered SM levels, and killed C6 glioma cells by apoptosis [55]. The effects were attributed to stimulation of SMase activity on the basis of the finding that adding GSH or *N*-acetylcysteine (a precursor of GSH) inhibited the increase in Cer and apoptosis (see section 3i). However, etoposide also inhibited SPT, slowing the incorporation of [¹⁴C]serine into Cer. In addition, PDMP increased the effect of etoposide on Cer accumulation and apoptosis.

In a different study, the Cer accumulation in Molt-4 leukaemia cells due to etoposide was also attributed to SPT stimulation [56]. Direct enzymic assay of SPT showed that the drug rapidly elevated its activity. (Users of labelled palmitate should note that it is incorporated into Cer by two processes: the initial condensation, then acylation. Thus the specific radioactivity of the resultant Cer depends on the radioactivity in the precursor pool used for acylation, as well as SPT action.)

The above two effects of etoposide were noted also in prostate cancer cells [57]. The early stimulation of SPT and, later, stimulation of SMase are probably due to the ability of Cer (formed by any process) to generate more Cer via any of several spiralling 'autocatalytic' processes [38].

(j) Paclitaxel (Taxol), a well-known anti-cancer drug, produced Cer and acted synergistically with exogenous Cer to produce apoptosis in a leukaemia T cell line, Jurkat [57]. Apparently, it behaves like etoposide, stimulating SPT at first, and then SMase later [58]. Protein kinase C δ was found to play an important role in Cer accumulation.

(k) Another anti-neoplastic drug, fludarabine, produced accumulation of Cer, a concomitant loss of SM in leukaemia cells, and apoptosis in WSU and JVM-2 leukaemic B cells [59]. *N*-Acetylcysteine, a precursor of GSH, elevated cellular GSH levels and partially blocked the production of Cer and apoptosis, supporting further the role of SMase in the process. However, pre-treating the cells with fumonisin B1 prevented fludarabine-induced Cer generation and apoptosis, suggesting that the drug also stimulated the *de novo* synthesis of Cer. This is like the effects of etoposide (see section 2i above). Perhaps an increase in *de novo* Cer synthesis elevates SM, which induces an elevated level of SMase.

3. SPEEDING THE FORMATION OF Cer FROM SM

This reaction has gained the most attention from students of Cer-induced apoptosis (Figure 3). Several SMases have been characterized, with differing pH optima, solubility and need for cations.

(a) Neutral, Mg²⁺-stimulated SMase has an exciting characteristic: GSH inhibits it, so any factor that lowers the GSH level increases the rate of Cer formation from SM [60]. Antioxidants or GSH precursors (*N*-acetylcysteine) tend to elevate the GSH concentration and prevent apoptosis, while inhibitors of GSH synthesis (buthionine sulfoximine) promote apoptosis. ROS (H₂O₂, nitric oxide, etc.) and ROS-producing substances

destroy GSH and speed Cer formation and apoptosis. Some anti-cancer drugs react with GSH via a Michael condensation reaction or produce ROS, and may therefore exert their beneficial effects via these mechanisms. The ROS can attack many cell constituents, including proteins and fatty acids. Most cellular sulphur compounds form S-O and S-N links, although some researchers believe that the oxidation stops at the disulphide level (GSSG).

Some drug-detoxifying enzymes also consume GSH, thereby speeding SM hydrolysis. GSH levels can be readily lowered to some extent by eating acetaminophen, a popular inflammation remedy. This condenses with GSH to form a thioether, and is then excreted. It is known to be an inducer of oxidative stress, DNA fragmentation and apoptosis. A substantial amount can be taken for a while, without too much liver damage.

γ -Glutamylcysteine synthase (the enzyme involved in GSH synthesis) and GSH occur at high levels in many tumours, giving them the ability to resist Cer-elevating agents. It would appear to be useful to slow GSH synthesis either with available inhibitors or by limiting the patient's content of glutamic acid with large doses of 4-phenylbutyric acid [61]. Thus, in general, the use of many kinds of drugs tends to deplete tumours of their GSH and promote apoptosis by Cer formation.

(b) Oxidized low-density lipoprotein (LDL) has been found to stimulate neutral SMase in smooth-muscle cells [62]. The LDL stimulated not only mitogen-activated protein kinases and Cer production, but also other sphingolipid enzymes, including alkaline and acid ceramidase and sphingosine kinase. The resultant increase in SIP may explain why proliferation, instead of apoptosis, was seen. In a similar study with human macrophages, the Cer increase was also accompanied by increased neutral and acidic SMase [63]. Oxidized LDL stimulated the formation of lactosyl-Cer from GlcCer in smooth-muscle cells [64]. This GSL, like the lipoprotein, exerts a proliferative action on the endothelial cells within arteries, thus narrowing arterial passageways. Although these findings support the general belief that one should routinely eat antioxidants to prevent atherosclerosis and cancer, it might be more effective simply to eat an inhibitor of Cer glucosylation and rely on ROS-induced Cer synthesis as a preventive agent.

(c) An unusually simple drug, Betathine (β -alanyl cysteamine disulphide), has been producing good effects in patients with myeloma, melanoma and breast cancer. It probably oxidizes GSH via the typical thiol/disulphide-exchange reaction, to form a mixed disulphide with β -alanyl cysteamine, thus depleting cells of their GSH and stimulating hydrolysis of SM. Betathine increased the surface expression of tumour necrosis factor α (TNF α) in T-cells and monocytes, which is significant, since TNF α stimulates Cer synthesis from SM.

(d) Arachidonic acid stimulates SMase, partially explaining the observation that depleting HL-60 cells of their arachidonic acid rendered them resistant to apoptosis [65,66]. The free fatty acid is liberated from glycerol esters and phospholipids by several kinds of enzymes, so stimulation of these enzymes may help speed Cer formation from SM. Increasing the activity of phospholipase A₂, which liberates arachidonate, has been shown to increase Cer and produce apoptosis [38].

Hydrogen peroxide was found to stimulate arachidonate release by phospholipase A₂ [36]. Thus the enzyme may react to peroxide formation from Cer or other ROS sources by speeding additional Cer synthesis from SM. This enzyme may be the acyltransferase that converts C₂-Cer into the 1-*O*-arachidonoyl ester, utilizing phosphatidylcholine (PtdCho) or phosphatidylethanolamine as the arachidonate source [67]. This enzyme also hydrolyses the Cer arachidonate ester, forming free arachidonate,

Figure 3 Cer synthesis from SM

and thus acts like a phospholipase in which C₂-Cer plays the role of a catalyst. The enzyme is inhibited by PDMP, but its physiological significance is still unclear.

(e) Stimulation of SMase by arachidonate may account for a part of the mechanism by which non-steroidal anti-inflammatory drugs (NSAIDs) and cyclo-oxygenase-2 (COX-2) inhibitors inhibit the development of colorectal tumours [68]. By blocking conversion of arachidonate into tumour-proliferating prostaglandins, etc., the NSAIDs and COX-2 inhibitors may act to elevate the arachidonate concentration to speed SM hydrolysis. The COX-2 inhibitor, celecoxib, is itself able to induce apoptosis in prostate carcinoma cells (both androgen-sensitive and -insensitive cells) [69]. COX-2 is present in both cancer-cell lines, whereas normal prostate epithelial cells contain almost no COX-2 and are insensitive to the apoptotic action of celecoxib. Inhibitors of either COX-1 or COX-2 were found to produce Cer accumulation and growth retardation [70]. This picture is complicated by recent findings that the apoptogenic action of COX-2 inhibitors is not related to their efficiency as inhibitors [71], indicating a second mode of action.

(f) Ketoconazole, an anti-fungal agent, inhibits 5-lipoxygenase (which produces the leukotrienes from arachidonate), and thus also acts to elevate arachidonic acid levels [72]. It has shown apoptotic activity and is sometimes used in prostate-cancer chemotherapy [73]. In the latter case, ketoconazole is particularly useful, since it is a strong inhibitor of cytochrome CYP3A4, and thus inhibits the formation of androgens (see section 2b). It could be useful to see whether it really induces elevation of the arachidonate level and Cer accretion *in vivo*. Since Cer also suppresses CYP3A4 (probably via nitric oxide elevation), ketoconazole's effect on the enzyme may be due to Cer elevation.

(g) Exogenous SM itself is an obvious (but only recently appreciated) 'prodrug' that can use tumour SMase to elevate Cer, if it can be delivered to the tumour. The use of dietary SM was mentioned above in section 1a. The efficacy of two anti-cancer drugs, 5-fluorouracil and irinotecan, for the treatment of human colonic tumour xenografts in mice has been shown to be enhanced by the use of 10 mg/day exogenous SM [74]. (Irinotecan is a derivative of camptothecin, both of which produce apoptosis in tumours by producing Cer [75].) These authors point out that

some tumours contain little SM, which may limit procedures that stimulate SM hydrolysis. Perhaps such tumours divert much of their newly formed Cer to GSL synthesis rather than to SM synthesis, and thus protect themselves against the apoptogenic effect of Cer. This is particularly the case for cells that overexpress GlcCer synthase [76]. Use of SM made from shorter-chain fatty acids might improve the efficacy of this approach, presumably speeding transportation to the tumour.

(h) Doxorubicin, an important anti-cancer drug, apparently acts by producing ROS and Cer, release of mitochondrial cytochrome *c*, and apoptosis [77–79]. There is a concomitant loss of SM and condensation of the drug with GSH. Thus it appears that the source of the extra Cer is SM.

Prolonged use of doxorubicin in patients often results in the development of MDR. This effect can also be produced in cultured cells by adding the drug at low-toxicity concentrations over a series of cell passages to eliminate ordinary, drug-sensitive cells, leaving MDR cells. These clones are now resistant to several anti-neoplastic drugs, as well as to exogenous Cer. In the case of MCF-7 breast cancer cells, the MDR cells exhibited a high enzymic capacity to convert Cer into GlcCer and contained a high level of GlcCer and other GSLs [77,78]. These and other observations suggest that exposure to doxorubicin produces rapid Cer synthesis, which causes death of ordinary cancer cells, but the Cer is rapidly glucosylated to form GlcCer and more complex GSLs in MDR cells. Not only does this induction protect the MDR cells from apoptosis, but the accumulated GSLs stimulate tumour growth. Thus doxorubicin treatment is very likely to produce therapeutic effects in patients initially but, later, to stimulate the proliferation of surviving cancer cells. Adding a blocker of Cer glucosylation to MDR cells neutralized their drug rejection, illustrating the value of such an inhibitor in poly-drug chemotherapy (see section 6).

(i) Vitamin D₃ is converted in the liver and kidney into an allylic alcohol, dihydroxyvitamin D₃, which stimulates Cer synthesis from SMase [80]. Various studies have shown that it produces Cer elevation and typical apoptogenic changes in cells. High doses produce toxic effects on Ca²⁺ metabolism, so current research is aimed towards developing more therapeutically specific analogues [81–83].

(j) Mitoxantrone, an important anti-neoplastic drug, induced rapid hydrolysis of SM in leukaemic cancer cells, with release of Cer and PtdCho and loss of SM [84]. The Cer levels decreased soon after, and the SM content gradually recovered, suggesting that the Cer was rapidly converted back into SM by exchange with PtdCho. Pretreating the cells with D609 (tricyclodecan-9-yl xanthogenate), an inhibitor of PtdCho phospholipase, greatly increased the amount of Cer formation while preventing the appearance of diacylglycerol (DAG). This effect, and results from another similar report [85], suggest that D609 also blocks the synthesis of SM from PtdCho and Cer (see section 6c).

(k) Dexamethasone, a glucocorticoid-like drug that is often used in chemotherapy, stimulates the hydrolysis of SM by acid SMase, producing apoptosis [86]. However, it also stimulates GlcCer synthesis [87], which tends to counteract the apoptogenic action of Cer. Thus dexamethasone probably should be used only with a GlcCer synthase inhibitor. Perhaps the elevated glucose resulting from glucocorticoid use speeds Cer glucosylation (see section 5).

(l) 1- β -D-arabinofuranosylcytosine ('Ara-C'), a useful anti-neoplastic drug, acts to elevate Cer formation from SM by neutral SMase [88]. The drug rapidly activated Lyn protein tyrosine kinase in U937 cells, translocating it to the cell-surface rafts that contain SMase and SM [89]. This stimulates Cer synthesis and apoptosis. Daunorubicin, a cancer drug resembling doxorubicin, acted similarly.

4. ACCELERATING GlcCer HYDROLYSIS

This section is focused on the following reaction:

(a) This method of increasing Cer has received little attention to date. GlcCer glucosidase is available for use in Gaucher disease patients, lowering their GlcCer content and reducing the symptoms of GlcCer excess. Using the enzyme for chemotherapy would be expensive, but it probably would be needed only for a short time. Acidic phospholipids, such as bis(monoacylglycerol)-phosphate and PtdSer, stimulate the glucosidase and should help to raise Cer levels. The latter phospholipid produced decreased GlcCer levels in the liver when injected into mice [90]. It is available as a dietary supplement, apparently surviving digestive hydrolysis. A more metabolically stable analogue of an acidic phospholipid might be better.

(b) Robert Glew and colleagues [91] found that GlcCer β -glucosidase reacts much faster if retinol (vitamin A) or pentanol is present as the glucose acceptor. The resultant glucoside is readily hydrolysed by cytosolic glucosidase (a different enzyme), and thus offers a method for accelerating Cer formation. The method has not been tested *in vivo*. It is interesting that retinol, which is metabolized to an allylic ketone, has shown some promise in cancer therapy, and so this approach may have been tried unintentionally [16].

(c) GlcCer glucosidase requires a small, heat-stable protein for activation: saposin C. This protein's active region is in its C-terminal end [92], and it may be possible to synthesize a peptide-like fragment that is sufficiently stable to use *in vivo* to block GSL accumulation.

(d) Chlorpromazine (CPZ), the anti-psychotic drug, may stimulate or induce glucocerebrosidase. Injected into mice at 10 mg/kg, it produced a 25% increase in liver Cer within 6 h [93]. In similar mice, a covalent inhibitor of Cer glucosylation (2-decanoylamino-3-morpholinopropiophenone) produced a 69% increase in Cer and a 20% decrease in GlcCer within 5 h. Use of both drugs together enhanced the loss in GlcCer. Perhaps CPZ

actually inhibits GlcCer synthesis, and the disappearance of GlcCer is simply due to the normal catabolic turnover of the GSLs. Injected into mice, 60 mg/kg CPZ was found to induce a 15-fold increase in apoptosis of T-lymphocytes in the thymus [94]. The significance of these observations is shown by the finding that CPZ and trifluoperazine induced apoptosis, abnormal cell cycling and expression of p53 in Chinese hamster V79 cells [95]. CPZ also induced apoptosis in lymphoblasts stimulated with interleukin-2. CPZ is a stimulator of the serine-base-exchange enzyme that is responsible for PtdSer synthesis (see section 4a above). Unfortunately, CPZ exerts many biological effects, so a search for more specific analogues might be productive.

Under this section heading, it should be noted that stimulating the hydrolysis of another proliferation stimulator, S1P, might be useful too. The phosphohydrolase acting on S1P yields sphingosine, which is readily acylated to produce Cer [96]. The authors cite interesting evidence, pointing to S1P as a controller of Cer levels. They suggest that free sphingosine (which is formed primarily by hydrolysis of sphingolipids) may be more rapidly acylated after release from S1P than by the direct re-acylation pathway. Thus chemotherapy with the free sphingol might benefit from development of a hydrolase stimulator.

5. BLOCKING GlcCer SYNTHESIS

This route, seemingly unknown to many researchers, has received remarkably little consideration in the apoptosis literature, but it is important because cells can escape apoptosis and accelerate proliferation by converting Cer into GlcCer (Figure 4) and the more complex GSLs. In several studies using glucosyltransferase inhibition, Cer accumulation was reduced or absent, because much of the Cer was diverted to SM synthesis [97,98]. This increase in the SM level can be converted into a Cer increase by stimulating SM hydrolysis (see section 3).

(a) The most studied inhibitor of GlcCer synthesis *in vitro* is PDMP [99–101]. This analogue of Cer or GlcCer is named as '*N*-[2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl decanamide]' by Chemical Abstracts. Much higher inhibitory activity has been obtained with the longer-chain homologue, PPMP, and new structural variants, *D-threo*-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP; also known as P4) and ethylenedioxy-P4 [102]. PDMP, injected into mice carrying Ehrlich ascites cell carcinoma, led to one-third of the mice being completely cured, and greatly prolonged life-spans in the other mice [101]. Most of the cured mice were immune to a second challenge with the cancer cells, indicating that PDMP did not interfere with the immune system. It is likely that the drug aided the development of antibodies by blocking the release of gangliosides, acidic GSLs which are toxic to the dendritic cells needed for an immunological response [103].

Later studies showed that PDMP also inhibits the glycosyltransferases forming several simple GSLs, Cer glycanase (a hydrolase that releases Cer from the higher GSLs), and Cer:lecithin transacylase. Generally, low concentrations *in vitro* of DL-P4 (0.2 μ M) strongly inhibited GlcCer synthesis, whereas higher concentrations (> 1 μ M) also produced Cer accumulation, a slowing of proliferation, and strong apoptosis. Perhaps the inhibitor acts at two different sites in sphingolipid metabolism.

It is curious that the 'P-drugs' (PDMP, PPMP, PPPP, etc.) appear to be strikingly non-toxic in fish embryos and in young mice and rats. Mice injected twice daily for 8 weeks with ethylenedioxy-P4 showed considerable loss of GSLs, but no noticeable toxic symptoms [102]. Injected into 'Fabry disease' mice (lacking α -galactosidase A), the inhibitor lowered tissue

Figure 4 Cer conversion into GlcCer

levels of the accumulated GSL, globotriaosyl-Cer, indicating its probable value in treating the heritable glucosphingolipidoses [104,105].

Normal cells appear somewhat less dependent on GSL synthesis than cancer cells. Perhaps normal cells make much more sphingolipid than is normally needed, possibly an indication of rapid turnover and careful homeostasis. MDR cancer cells contain even higher GlcCer synthase activity, and thus are more sensitive to the proliferation-slowing effects of PDMP [77,106].

Important effects of the P-drugs have been noted in cancer cells *in vitro* [106,107]. Mouse myeloma cells exposed to 0.4 μ M P4 suffered from apoptosis, as well as a large increase in the H-2 Class II differentiation antigens, loss of CD44 (an adhesion protein correlating with metastasis), and an increased surface expression of myeloma-specific immunoglobulin, the antigenic 'paraprotein' characteristic of myeloma [108]. Depletion of GSLs in Lewis lung carcinoma cells with PDMP for 6 days reduced the ability of the cells to metastasize to mouse lungs over a 3-week period by 70 % [109]. Growing the depleted cells for an additional day in the absence of PDMP partially restored the GSL content, and completely restored the ability to metastasize. A group of gangliosides plays a special role in promoting metastasis [110].

The specific activity of GSLs in promoting tumour growth is seen by their ability to augment growth-factor activity. In a study with cultured fibroblasts, 1 μ M P4 blocked GSL synthesis without affecting Cer levels [97]. The GSL-depleted cells now resisted the proliferative effects of epidermal, fibroblast and platelet-derived growth factors, as well as that of insulin-like growth factor-1. On the other hand, non-depleted cells showed enhanced proliferative responses if they were pre-incubated with gangliosides, suggesting that these GSLs are needed for cell proliferation. Perhaps the latter stimulatory effects were the result of activation of endogenous growth factors.

Other studies showed a need for GSLs for growth-factor activity, but increasing the Cer level blocked the effect. Pre-treatment of rheumatoid synovial cells with C₂-Cer completely inhibited cell-cycle progression resulting from platelet-derived growth factor [111]. Several anti-apoptotic kinases were also inhibited. It is clear from many studies of this type that the ratio between Cer and GSL concentrations is an important consideration, not simply the absolute concentrations. The same has been observed for Cer and SIP concentrations [112].

Surveyed in approx. 80 kinds of human cancer cells, P4 was an effective growth inhibitor over a similar range of concentrations, leading to Cer accumulation. This is what one would expect of a drug acting at the 'crux' of cancer, i.e. the central aspect of the disease. Also suggestive of a crucial role is the fact that the amino

acid sequence of GlcCer synthases in a wide range of organisms has shown remarkably little evolutionary change [113]. In addition, the expression of the synthase gene in macrophages from elderly people is only approximately one-fifth the activity of that in middle-aged individuals [114]. This slowed activity is in agreement with the generally slower growth of malignant tumours in old people.

(b) The anti-neoplastic drug, tamoxifen, may be effective, in part, by directly inhibiting GlcCer synthase, as well as by acting as an anti-oestrogen [115]. Blocking Cer glucosylation was particularly useful in MDR cancer cells, where exogenous Cer did not produce apoptosis but the combination of Cer and tamoxifen did [77]. Tamoxifen resembles PDMP also in its ability to inhibit Cer glycanase (see section 5a) [116]. Recent data suggest that tamoxifen is useful to prevent breast cancer, acting perhaps by lowering chronically tissue GSL concentrations, allowing the immune system to kill pre-cancerous lesions. Newer drugs, such as raloxifene (a pure anti-oestrogen), might have similar activity against GlcCer synthase.

Fenretinide's effectiveness in blocking carcinogen action in rats (see section 2g) was enhanced by tamoxifen [44]. This synergy was also seen in breast cancer cells and in some colorectal cell lines. Tamoxifen binds to some oestrogen receptors, behaving like natural oestrogen at those sites, thus suggesting that the sites influence sphingolipid metabolism. Oestradiol was found to lower GlcCer synthase and to increase GlcCer glucosidase in mouse kidneys [117], so any oestradiol agonist may be expected to force Cer accumulation too. The GlcCer-depleting action of tamoxifen was seen in MDR breast-cancer cells, and direct inhibition of the synthase was demonstrated in melanoma subcellular particles. Like PDMP, tamoxifen can produce an elevated level of DAG and lower the activity of protein kinase C [118].

Both tamoxifen and PPMP sensitized doxorubicin-resistant (i.e. MDR) cancer cells to the apoptotic action of doxorubicin by slowing Cer glucosylation [119]. Tamoxifen's anti-cancer activity can thus be attributed, at least in part, to its inhibition of GlcCer synthesis and, presumably, to accumulation of Cer. Two other drugs active against MDR, cyclosporin A and verapamil (a Ca²⁺-channel blocker), also lowered GlcCer content in MDR cells.

(c) RU486 (mifepristone), the abortion-inducing drug, inhibits Cer glucosylation, produces Cer accumulation and induces apoptosis [120]. It has also shown promise in cancer therapy, perhaps due to its ability to slow GlcCer synthesis. This explains its ability to reverse MDR (see section 3h). Mifepristone also inactivates cytochrome CYP3A4, binding to the enzyme while displacing iron porphyrin from the active site [121]. Mifepristone

is a conjugated allylic ketone, and thus may condense with a thiol group in the enzyme, as well as with GSH.

Since the drug produces abortions, one might consider whether this property is due in part to blockage of GSL synthesis in the fetus or associated tissues. If so, glucosylation inhibitors, such as the P-drugs, might augment mifepristone's abortifacient activity.

(d) A chronically low blood sugar level may slow the glucosylation of Cer and its glycosylation products. This expectation is supported by the findings that animals on a low-calorie diet develop cancer much later in life and live much longer than *ad libitum* eaters. Moreover, tumours depend greatly on the available glucose. The glucose concentration in blood affects the concentration of cellular UDP-glucose (UDP-Glc); thus we would like to know whether the concentration of UDP-Glc in tumours also reflects the blood-glucose level. Also, is the concentration of UDP-Glc saturating for GlcCer synthase? If, as found in rat kidneys [122], the concentration is below the saturating level, then the blood concentration of glucose is rate-limiting. Thus it may be possible to slow GSL biosynthesis and raise tumour Cer levels simply by minimizing the patient's carbohydrate intake or by slowing glucose absorption. Glucosamine, widely eaten to prevent joint pain, competes with glucose for UTP (after N-acetylation), thus making glucose less readily available. Glucosamine has been found to produce apoptosis in R28 cells, but sphingolipids were not measured.

Mouse cortical tubule cells exhibited the corresponding opposite effect, with growth in a high-glucose medium stimulating GlcCer synthesis and cell proliferation and simultaneously lowering the Cer level [123]. These effects were prevented by adding a P-drug, PDMP, to the medium. PDMP blocked the fast glucosylation of endogenous Cer (see section 5 above) and the proliferative action of GSLs, forcing the cells to accumulate Cer. These findings support the premise that excessive glucose acts to stimulate growth by speeding the glucosylation of Cer.

(e) N-Butyl deoxynojirimycin also inhibits GlcCer synthesis, producing considerable depletion of GSLs in various mouse organs. It was tolerated for over 1 year in patients with Gaucher disease, a genetic disorder affecting the β -glucosidase that normally degrades GlcCer. Toxic effects were seen, mainly a tendency to produce peripheral neuropathy, which might be the result of depleting neural gangliosides. A new isomer of the drug is more specific and may prove superior in human use.

(f) Anti-androgen chemotherapy (bicalutamide + leuprolide acetate) may elevate Cer by reducing the induction of GlcCer synthase resulting from androgens in sensitive tissues. The stimulation of kidney growth by testosterone has long been known, and assay of mouse kidneys for GlcCer synthase showed that the exogenous hormone acted to increase the activity of the enzyme in both males and females [117]. In addition, GlcCer glucosidase, the enzyme that converts GlcCer into Cer, had a decreased activity, thereby augmenting the increase in GSL levels and growth rate. Male mouse kidneys show high GSL levels, reflecting the normal action of androgens and suggesting that the synthesis of GSL precursors (sphingols and Cer) is also enhanced. Other androgen-sensitive organs, such as the prostate gland and lungs, presumably respond similarly. Testosterone and some other androgens stimulate breast-cancer-cell proliferation via binding to oestrogen receptor α [124], and so there is the possibility that anti-androgen therapy would be useful for breast cancer too. The use of anti-androgens or orchiectomy is standard treatment for prostate cancer, and its success may be due in part to slowing GSL synthesis and producing Cer accumulation.

Leydig cells, which specialize in androgen synthesis in response to chorionic gonadotropin, demonstrated a feedback control of testosterone by Cer [125]. Several treatments that elevate cellular

Cer content [short-chain Cer, D-MAPP (D-erythro-2-tetradecanoylamino-1-phenyl-1-propanol), SMase, tumour necrosis factor and interleukin 1] all inhibited the response to the gonadotropin. Thus Cer elevation in such cells exerts the desirable goal of lowering testosterone production and, with that, inhibiting GSL production.

The use of oestrogens in prostate cancer therapy is supported by the observation that oestradiol depressed GlcCer synthase levels in mouse kidney [117]. The oestrogenic properties of a herbal preparation (PC-SPES) may account for its activity against prostate cancer [126].

Gaucher disease patients, who accumulate GlcCer, tend to develop cancers of the B-cell type, emphasizing the importance of slowing GlcCer synthesis [105].

6. BLOCKING CERAMIDASE ACTION PRODUCES Cer ACCUMULATION

This section focuses on the following reaction:

(a) N-Oleoyl ethanolamine, a greatly truncated form of Cer, inhibits acid ceramidase. It induces Cer accumulation, mitochondrial permeability, cytochrome *c* release and apoptosis. Acid ceramidase occurs at a relatively high concentration in mitochondria, which seems to be the major, or primary, sub-cellular organelle in the production of apoptosis [127]. This suggests the importance of Cer hydrolysis in promoting cancer cell growth, since the enzyme prevents excessive accumulation of Cer and may contribute sphingols to the synthesis of sphingol phosphates. The inhibitor was reported to also directly stimulate neutral SMase in WEHI-231 cells, elevating Cer and ROS production in yet another way [128].

Another study showed that the amide inhibited GlcCer synthase when incubated with neuroepithelioma cells [129]. Glucosylation of a short-chain Cer, hexanoyl sphingosine, was not greatly inhibited, but the glucosylation of natural, long-chain Cers was considerably slowed. This is a reminder that the specificity of an inhibitor is often investigated inadequately, and that short-chain and normal-chain Cers are very different. At any rate, all three enzyme effects act to produce elevated Cer levels, and the compound deserves serious consideration for chemotherapeutic cocktails.

Acid ceramidase also occurs in lysosomes, which probably degrade most of the cellular Cer produced during catabolism of sphingolipids.

(b) Neutral/alkaline ceramidase is inhibited by a Cer analogue, D-MAPP [130]. This ceramidase may also be important, as shown by the inhibitor's ability to produce growth inhibition and accumulation of Cer in cultured cells. It is significant that the enzyme occurs primarily in mitochondria [131], where Cer may exert a major part of its apoptotic action. A related inhibitor, B13 (*p*-nitro-D-MAPP), was even more effective, acting to prevent the growth *in vivo* of two different aggressive colon-cancer-cell lines. It was noted that human colon tumours contained less than half the normal concentration of Cer. This is a typical characteristic of cancer cells: they mutate to reduce the concentration of Cer by various means.

An important role for the ceramidases is promoting synthesis of sphingol phosphates, which tend to prevent apoptosis and produce proliferation [112]. Sphingol kinase acts on sphinganine produced by *de novo* synthesis, and on both sphinganine and sphingosine produced from Cer (although the kinase is named 'sphingosine kinase'). Inhibitors for the kinase, *N,N*-dimethyl sphingosine and *threo*-sphinganine (safingol), should be

included in therapeutic cocktails. The latter is acylated, forming an abnormal Cer, which is highly cytotoxic, especially in the presence of fenretinide and P4 [46].

(c) The diversion of Cer by the synthesis of Cer 1-phosphate should be mentioned here, although the extent of its roles is obscure. The ester is formed by a kinase [132,133]. Its formation can be reduced by D609 [134], which also blocks the hydrolysis of lecithin and SM. Cer 1-phosphate appears to stimulate proliferation, and the effect can be reduced with the mitogen-activated protein kinase/extracellular-signal-related kinase kinase ('MEK') blocker, PD98059 [135]. This subject warrants further study.

7. PROTECTING Cer FROM CONVERSION INTO SM

(a) Cer is normally converted into SM by transfer of phosphocholine from lecithin (PtdCho), leaving DAG (Figure 5). A second route, little studied, is the similar exchange with phosphatidylethanolamine, yielding Cer 1-phosphoethanolamine and DAG. This route requires two transmethylation steps to produce SM. The phosphocholine exchange is reversible and can be slowed by reducing the tumour's content of the two glycerophospholipids.

Exogenous lecithin (which presumably accelerates SM synthesis) can prevent apoptosis caused by the Cer-elevating substances, P4 and oleoylethanolamine, C_2 -Cer or SMase [136]. A further indication of the importance of lecithin is the observation that cells depleted of lecithin tend to undergo apoptosis. The ability of tumours to protect themselves against Cer-induced apoptosis is illustrated in prostate tumours, in which there is increased (total) choline, and in metastatic tumours, which contain much lecithin. SV40 ('simian virus 40')-transformed human lung fibroblasts had much higher SM synthase activity

and ability to convert exogenous C_2 -Cer into SM [137]. Another example of apoptosis prevention was seen with neurons in primary culture, which underwent apoptosis when exposed to the Cer-elevating agents C_2 -Cer, SMase or N-oleoylethanolamine (see section 6a), but which were unharmed when PtdCho was included in the medium [136]. An unexpected finding was that exogenous Cer inhibited lecithin synthesis, apparently by product/precursor-feedback control (SM versus lecithin). One of the anti-apoptogenic effects of GlcCer is its ability to stimulate the biosynthesis of lecithin [138], so we see another balance problem between Cer and GlcCer. This is another reason to include inhibitors of GlcCer synthase in therapeutic cocktails.

(b) DAG, the by-product of SM synthesis, is a strong inhibitor of the synthase *in vitro* [139], possibly because it acts as a substrate to reverse the action of the enzyme, forming PtdCho and Cer. PDMP and P4 were found to produce marked increases in both Cer and DAG in kidney cancer cells [140]. The DAG presumably originated from the accelerated synthesis of SM, resulting from the blockade of GlcCer synthesis. A more stable analogue of DAG might be clinically useful.

(c) Phospholipase C lowers PtdCho levels, forming DAG and phosphocholine. The DAG can be expected to inhibit conversion of Cer into SM and to elevate Cer levels. If the DAG is hydrolysed to free fatty acids, they may act as Cer precursors and elevate Cer levels by this route too. The inhibitor of SM synthase and PtdCho phospholipase C, D609, was found to elevate Cer levels and enhance the Cer-increasing ability of daunorubicin to produce apoptosis [84,137]. Although D609 has been found to exert effects on additional processes, it might be useful as a component of a poly-drug attack.

(d) Hexadecylphosphocholine, a PtdCho analogue having value as an anti-neoplastic drug [141], may slow SM synthesis from Cer and lecithin. At a concentration of 25 μ M, it induced

Figure 5 Cer conversion into SM

an increased Cer level, lowered PtdCho and SM, and produced apoptosis. The ester is too toxic for use in patients, but a newer ester, erucylphosphocholine, may be more useful.

(e) Tamoxifen, which is finding increasing use as an anti-cancer agent (see section 5b), also lowers PtdCho levels by stimulating phospholipases C and D [118]. This effect is synergistic with its ability to slow Cer conversion to GlcCer.

8. Cer ELEVATION BY RADIATION

UVB radiation induced an increase in the mRNA for the serine-palmitate condensation (SPT), resulting in a 50 % increase in enzyme activity [142]. Presumably this leads to increased Cer production and, if the radiation is sufficient, to cell death. The tendency of UV light to eventually produce skin cancer might be due to excessive increases in the proliferative sphingolipids. UVB also induced activation of neutral and acidic SMases, thus increasing Cer by a second method [143].

In another study, UVA radiation increased the level of Cer in keratinocytes; however, so did a source of activated oxygen [144]. Both increases were reduced by vitamin E, providing further evidence for a direct effect of ROS. Presumably the ROS destroyed part of the cellular GSH, releasing Cer from SM by SMase activation. Although the authors found no change in the levels of neutral and acid SMase by assay in dilute cell extracts, the assays could not reflect the GSH effect, because the GSH was diluted out by the reaction medium. From a therapeutic viewpoint, the application of UV light to non-surface tumours does not seem easy, but it might help with skin cancer provided the increased Cer is forced to accumulate.

γ -Radiation of Fas-sensitive Jurkat cells also produced mitochondrial elevation of Cer, as well as of phosphatidylglycerol [145]. The source of the Cer was not determined. Another study with X-ray radiation indicated that the apoptosis of the damaged cells was the result of inducing elevated Cer synthase activity [146]. Fumonisin B1 protected the radiation-damaged cells from apoptotic destruction, suggesting that the *de novo* synthesis of Cer was involved. However, this observation might also mean that the fumonisin elevated the sphingosine phosphate level, which also blocks apoptosis.

A later study concluded that the Cer increase was due to SMase action. The apoptotic damage could be blocked by injecting basic fibroblast growth factor into irradiated mice. This growth factor elevates GlcCer synthase activity, thus preventing Cer's apoptotic action.

Synergy between radiation and a Cer-elevating drug was reported for human prostate LNCaP cells [147] and for Jurkat cells [148]. The latter, exposed only to γ -rays, did not show appreciable damage or a Cer increase until 24 or 48 h. Treatment of unexposed cells with three separate sphingolipid inhibitors for 2 days (DL-PDMP, D-MAPP or imipramine) also produced no distinct Cer accumulation, but a combination of all three produced marked increases. Together with radiation, the combined drugs yielded greater increases in total and mitochondrial Cer, apoptosis and ROS, and a great loss of GSH. This study illustrates how subtoxic doses of four different modalities work synergistically to kill cancer cells.

Radiation therapy may generate Cer by another route, since it produces free radicals that oxidize GSH and thus activate neutral SMase. This pathway may be more important in slow-growing cancers, where Cer synthase may not be much more active than in normal cells, or where ceramidase is unusually active.

In patients, radiation of the prostate gland, combined with slowing of GlcCer synthesis by androgen ablation, has recently been shown to be superior to either treatment alone. Not only is

the tumour forced to accumulate Cer, but the Cer also shrinks the gland, enabling the use of decreasing radiation dosages.

Radiation from ^{60}Co activated nuclear SMase, forming Cer and producing apoptotic changes in the DNA of radiosensitive erythromyeloblastic cells [149]. Unexpectedly, the non-nuclear cell materials (lysates and organelles) did not respond to radiation; thus there seems to be an important interaction between the different cellular organelles in the reaction. It will be interesting to see what part of the cells is needed for the nuclear changes.

Another complication in the effects of radiation was found in TSU-Pr1 prostate cancer cells, where a rapid loss of half the sphingosine kinase activity was observed [150]. This decrease explains why free sphingosine appeared. Apoptosis did not appear until Cer appeared. The role of the free sphingol is still unclear, but free sphingosine is rapidly acylated to form Cer in several cell types. An important observation in this study is that the effectiveness of radiation was enhanced by substances that elevated cellular Cer. This was shown also with PDMP. The important conclusion from the above reports is that radiation of tumours can be more useful if accompanied by drugs that elevate Cer.

9. ASSEMBLING THE PIECES OF DATA

The complexity of Cer metabolism has inevitably led to reports that seem to contradict each other. Too few analyses for individual sphingolipids have been made to allow a firm interpretation of the relevant interactions. Many experiments have been run for only a few hours, but the gradual upward spiralling of Cer levels calls for longer times, such as 24 to 48 h. The sphingolipid composition of cultured cells changes with cell density, probably because sphingolipids shed from cells influence other cells. Many studies have used preliminary labelling with radioactive precursors as a substitute for actual mass measurements, opening the results to questions of validity. Weak familiarity of many investigators with lipid technology has yielded many dubious reports [16]. Nevertheless, despite the multiplicity of Cer effects on 'cell proliferation versus apoptosis', it seems likely that the anti-cancer agents described in this review act, more or less intensely, on all kinds of cancer cells.

Tumours differ with regard to the activities of the individual enzymes and controlling factors, so one cannot rely on the belief that only the major factors should be controlled: there are often clones present in patients' tumours that show additional differences. If the dominant enzyme in a given cell type is the rate-controlling factor, slowing it with a drug will simply shift the rate control to a different enzyme, necessitating use of a second drug. This means that many anti-cancer agents have to be utilized in unison to assure a profound elevation of Cer and tumour death. The therapist has the promising (but daunting) choice of at least one drug from each of the above eight approaches. Poly-drug therapy can be expected to lower the dosages normally used with the individual drugs, and thus to minimize toxic side-effects. A wonderful side-benefit of this approach is that blocking Cer glucosylation will allow the patient's immune system to attack all the failing cancer cells [151], and thereby finish off the problem.

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