Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 2123–2142

BIOORGANIC & MEDICINAL CHEMISTRY

Review

Designing Anticancer Drugs Via the Achilles Heel: Ceramide, Allylic Ketones, and Mitochondria

Norman S. Radin*

Mental Health Research Institute, University of Michigan, Ann Arbor, MI, USA

Received 29 July 2002; accepted 18 October 2002

Abstract—Published reports are reviewed as the basis of a proposal that an effective antineoplastic drug should contain several features: (a) resemblance to the natural lipid, ceramide; (b) an allylic alcohol and/or allylic ketone moiety; (c) a hydroxyl and/or a nitrogen atom near the allylic group; (d) conjugated double bonds as part of the allylic region. The drug should produce reactive oxygen species in tumor mitochondria, stimulate the generation of ceramide in the tumor, and condense with mitochondrial glutathione. It is pointed out that some antibiotics with these features are also active against cancer cells; perhaps anticancer drugs with these features will prove useful as antibiotics. Common problems in working with lipoidal substances are discussed.

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*Present address: 10150 Torre Avenue, #15 Cupertino, CA 95014-2129, USA. Tel.: +1-408-861-9487; fax: +1-408-446-4913; e-mail: gluconorm@aol.com

0968-0896/03/$ - see front matter
doi:10.1016/S0968-0896(02)00609-0

Introduction

Many published reports point to the common, naturally occurring sphingolipid, ceramide (Cer), as a prominent compound in the induction of tumor death via apoptosis. Yet Cer is normally converted enzymatically to other sphingolipids (sphingosine-1-phosphate and glucosphingolipids) that *stimulate* tumor growth, proliferation, angiogenesis, and resistance to attack by the patient's immune system. While the detailed mechanisms for all these effects are still under active study, it is becoming increasingly evident that the fate of a tumor—growth, proliferation, and metastasis versus tumor death and patient survival—is dependent on the ratios of the various sphingolipid enzyme activities and associated processes, such as lipid transport, availability of precursors, relevant cytokines, and levels of growth factors. Because of this multiplicity of pathways, the use of a single anticancer drug acting on just one pathway is rarely effective because the tumor's enzyme complex usually has time to adapt or mutate to increased activity in a different proliferative pathway. For example, a drug that stimulates Cer biosynthesis might fail to induce apoptosis simply because the cancer cell responds to the extra Cer by converting it to a proliferation-inducing metabolite. In a normal cell, the pathways are balanced according to the cell's current physiological role, but in a cancerous cell, a vital control factor has been tipped off the balance wheel. Table 1 lists some of the major actors in this apoptogenic scheme and Table 2 lists

Table 1. Factors leading to slow cell growth and apoptosis

	Enzyme	Reaction	Reaction modifiers
1	Sphingomyelinase (neutral pH optimum)	SM + H_2O→Cer + fatty acid	GSH, manumycin, scyphosphatin, ganglioside GM3, cholesterol-lowering agents inhibit. Arachidonic acid, etoposide, paclitaxel stimulate
2	Sphingomyelinase (acid pH optimum)	Same	Desipramine, 1,4-benzothiazine analogues inhibit.
3	Glucosylceramidase	GlcCer + H_2O→Cer + glucose	Inhibited by conduritol B epoxide, *N*-hexyl-glucosylsphingosine, Acidic phospholipids stimulate.
4	Cer synthase	Sphingosine + fatty acyl-CoA→Cer	Fumonisin B1, australifungin inhibit.
5	Fatty acyl sphingnine desaturase	*N*-Fatty acyl sphinganine + NAD→ Cer + NADH?	

Table 2. Factors leading to slow cell growth and apoptosis

	Enzyme	Reaction	Reaction modifiers
A	Sphingosine kinase	Sphingosine + ATP→sphingosine-1-phosphate	*N*,*N*-Dimethylsph, threo-sphinganine inhibit.
B	GlcCer synthase	Cer + UDP-glc→glucosylceramide	PDMP, PPMP, PPP, *N*-oleoylethanolamine inhibit.
C	SM synthase	Cer + lecithin→SM + diacyl glycerol (DAG)	DAG, lecithin-lowering agents, D609, *N*-(3-hydroxy-1-hydroxymethyl-3-phenyl-propyl)dodecanamide inhibit.
D	Ceramidase (acid pH optimum)	Cer + H_2O→sphingosine + fatty acid	*N*-oleoylethanolamine inhibits.
E	Ceramidase (neutral/basic pH optimum)	Cer + H_2O→sphingosine + fatty acid	D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol inhibits.
F	Ceramide kinase	Cer + APT→Cer-1-phosphate	
G	SM synthesis via phosphatidylethanolamine	Cer + PE→Cer-phosphoethanolamine; followed by *N*-methylation→SM	

major actors in the proliferative, anti-apoptotic scheme. Included in the tables are agents that allow a therapist to control the enzymes. More detailed lists of controlling agents[1,2] and reviews of sphingolipid metabolism and function[3–7] are available.

A vital control factor appears to be a complex set of oxido-reduction metabolites that control the levels of the different reactive oxygen species (ROS). Ceramide plays a role in this balance system, since it produces ROS that—in turn—can generate additional molecules of Cer.[2,8,9] Because of the existence of several such spiraling 'autocatalytic' reactions, the extra Cer may—in the uncontrolled cancer cell—be converted too rapidly to the proliferative sphingolipids, sphingosine phosphate and glucosphingolipids.[10] Glutathione (GSH), the major reducing agent in cells, normally reduces the ROS and thus blocks the spiraling generation of Cer. Moreover, because GSH inhibits neutral sphingomyelinase (SMase) (reaction **1**, Table 1), it slows formation of Cer from the major cellular sphingolipid, sphingomyelin (SM).[11] These two effects normally act to prevent large-scale generation of Cer and proliferation-stimulators formed from Cer. The effects also prevent cellular damage and mutation generation by ROS, thus help *prevent* the appearance of cancer. To some extent, precursors of GSH and other thiols act like GSH, as does oxidized GSH (GS-SG). It is significant that *exogenous* GSH also inhibits SMase, elevates SM level, and reduces the concentration of oxidized lipids.[12] Tumors are sometimes found to contain a high concentration of GSH, revealing one of the ways cancer cells avoid apoptosis despite their high rate of sphingolipid synthesis.[13]

The protective value of GSH may also be due to inhibition of the enzyme that desaturates dihydroCer (*N*-acyl sphinganine) to form Cer (reaction 5, Table 1). This enzyme is inhibited by dithiothreitol and by *N*-acetylcysteine, which is converted to GSH by cells.[14] As with other inhibitors of Cer synthesis, the net effect is avoidance of apoptosis by cancer cells. The relatively low pH typical of tumors may also protect them by the same mechanism, since the desaturase is very sensitive to pH.[14] Some tumors contain a low desaturase activity, suggesting the occurrence of a protective pro-proliferative mutation in cancer cells.[15]

In accord with these observations, researchers have found that drugs which undergo metabolic inactivation by reaction with GSH, or that stimulate the enzymes involving GSH use, or condense chemically with GSH can lead to tumor apoptosis. The same is true of enzyme reactions that lead to ROS production since they convert reduced sulfur compounds (GSH, GS-SG, methionine, cysteine) into compounds containing an S–O or S–NO link. Reduction of cellular GSH levels can also be achieved with an inhibitor of GSH synthesis, such as L-buthionine-(*S*,*R*)-sulfoximine. Its use leads to loss of GSH and accumulation of Cer. Used together with anticancer drugs, it enhances tumor apoptosis. These relationships explain why antioxidants tend to protect people against formation, growth, and mutation toward increasing malignancy of cancerous cells.

The spiraling reactions that produce elevated levels of Cer are a key to cancer chemotherapy, provided the accumulating Cer is not allowed to form proliferative sphingolipids. This means that glucosylation, forming glucosylceramide (GlcCer, reaction **B**, Table 2), and Cer hydrolysis (reactions **D** and **E**, forming sphingosine and, subsequently, sphingosine phosphate) must be blocked at the same time. Under these conditions, antioxidants and reduced sulfur metabolites are undesirable, since ROS synthesis is needed to promote Cer synthesis. Drugs that destroy reduced thiols (e.g., allylic ketones) are desirable since they allow ROS elevation or they themselves produce ROS.

Some of the anticancer drugs in current use have these effects, explaining (in part) why they are effective. However, many tumors develop the ability to synthesize proliferation-promoting metabolites and enzymes very rapidly, and thus are only initially sensitive to these drugs. If the tumor is not promptly killed by the chemotherapy, drug-resistant cancer clones already present in the tumor will survive and eventually become the dominant form. (Radiation administered simultaneously with chemotherapy can be helpful, since it generates additional ROS and Cer.[16]) Even if a tumor—at the time of diagnostic recognition—does not contain such clones, these mutant strains will eventually appear because of the increased mutation rate resulting from enhanced ROS generation. Some anticancer drugs produce ROS but are not effective enough to kill tumors, thus may eventually produce the opposite effect (cancer induction) due to the indiscriminate damage from the ROS. These considerations point to the need for poly-drug chemotherapy, to attack as many proliferative metabolites as possible simultaneously.

A lucky aspect of the above interactions is that *normal* cells appear to be relatively insensitive to manipulation of sphingolipid levels. While the number of published comparisons with cancer cells is small, it appears that the latter contain more sphingolipids and synthesize them somewhat more rapidly, and are thus likely to be more sensitive than normal tissues to drugs acting on sphingolipid enzymes. This is especially true for the more dangerous kinds of tumors, the ones that are resistant to current anticancer drugs due to appearance of mutations leading to the multi-drug resistance proteins. In some—perhaps all—cases, this resistance seems to be due to excessive synthesis of Cer and/or its product, GlcCer.[17,18] This simplest glucosphingolipid (GSL) is formed from Cer and UDP-glucose and is the precursor of hundreds of more complex GSLs. The first metabolic anabolite of GlcCer, galactosyl GlcCer, is also a stimulator of cell proliferation.[19,20] The GSLs containing sialic acids (the gangliosides) constitute a prominent group with myriad functions. Some gangliosides are responsible for apoptosis, or rapid cell growth, angiogenesis, and resistance to the antitumor immune response.[21–23] GSLs are also important players in cell–cell adhesion phenomena.

Increasingly, researchers are reporting that elevating the body's content of Cer and Cer precursors protects

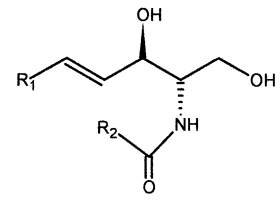
against cancer formation.[24,25] The antineoplastic drug, tamoxifen, is finding use in women as a cancer preventive agent; it may owe its effectiveness to its ability to slow the glucosylation of Cer.[26] This feature lends greater urgency to the need to develop new drugs that act like Cer or enhance Cer production.

The uptake of sphingolipids from blood or easily accessed tissues (skin, lungs) is probably significantly faster in cancer cells, because of their more rapid sphingolipid metabolism. Together with the relative insensitivity of normal cells, this makes sphingolipid-like drugs a promising group to investigate.

Other apoptogenic factors, not shown in Table 1, are the hydrolases that degrade the complex GSLs to GalGlcCer, then to GlcCer, which is hydrolyzed in reaction **3**. These enzymes are potentially useful targets for attempts at developing stimulators. The same considerations apply to the phosphatase that cleaves sphingosine-1-phosphate and the lyase that splits the phosphate into ethanolamine phosphate and a long-chain aldehyde. In many cell experiments in plastico, just one or two Cer-elevating agents has sufficed to slow or kill cancer cells. However, actual human tumors contain cancer clones with varying susceptibility and more than two drugs are needed.

From the names of some of the substances listed in Tables 1 and 2, one can see that they resemble Cer in structure. Many review articles show the structures of these compounds but only the natural metabolite, Cer, is shown in Figure 1. It should be noted that the double bond has the trans configuration, that the C-2 and C-3 atoms in the sphingosine chain are asymmetric, and that the valence angles of nitrogen are different from those seen in the common, ester-type glycerolipids. The C-2 and C-3 atoms have the D-erythro configuration. The length of the sphingoid chain varies, depending on the biological species. The most common chain is 18 carbon atoms long and chains containing more than one double bond and other substituents have been found. The length of the fatty acid moiety, typically C_{16}, C_{18}, $C_{22–25}$ in mammals, seems relatively unimportant although shorter chain lipids compete with the natural ones made by cells.

The three polar substituents at C-1, C-2, and C-3 are reminiscent of the triglycerides, so Cer can be thought of as resembling monoacylglycerol or monoalkylglycerol, or—disregarding the second hydroxyl group—as a diacylglycerol. The latter is a potent effector of protein kinase C, thus it is not surprising to learn that Cer also has potent effects on several enzymes, particularly those involving protein phosphates. Both Cer and diacylglycerol are galactosylated at the C-1 hydroxyl to form the glycolipids of the galactosyl series, seen mainly in the nervous system. Diacylglycerol is formed by the enzymatic phosphocholine exchange reaction between Cer and phosphatidylcholine, forming SM. This is a reversible reaction in which the product diglyceride inhibits the forward reaction quite well.



Ceramide $R_1 = CH_3(CH_2)_{12}$

$R_2 = CH_3(CH_2)_{15-25}$

Figure 1. Fatty acid sphingosine (ceramide).

PhytoCer is a ceramide in which the sphingoid base is 4-hydroxysphinganine (phytosphingosine). Although the name implies the presence of the Δ^4 double bond of sphingosine, it is simply 4-hydroxysphinganine. Originally thought to occur only in plants, phytosphingosine is now seen in animal tissues, but it has rarely been studied. Virtually every natural sphingolipid is formed from Cer and dihydroCer by addition to the C-1 hydroxyl group. Interesting enzymatically-active sphingolipids containing modifications of the sphingoid chain also occur in nature, especially in molds (e.g., fumonisins and australifungins). These sphingosine analogues inhibit Cer synthase (reaction **4**), thus tend to cause cancer and pose a significant public health hazard. The australifungins are of special interest to this article, since they contain the allylic ketone moiety in which a hydroxyl group is attached to the double bond, that is, the enolic form of a 1,3-diketone. Branched methyl groups on the sphingoid chain have significance, with *cis*-4-methyl sphingosine slowing the synthesis of 3-ketosphinganine (the first step in the biosynthesis of sphingolipids, the reaction between serine and palmitoyl-CoA).[27] The proposed mechanism of inhibition involved action of sphingosine kinase, forming the 1-phosphate ester, the actual inhibitor. These features deserve consideration in designing Cer-like drugs. This article explores the chemical nature of Cer that makes it produce ROS and apoptosis. The article also points to the importance—in any anticancer drug—of including an allylic alcohol or allylic ketone group in the drug's structure.

Significance of the Allylic Group in Ceramide

Numerous authors have compared Cer (*N*-acyl sphingosine) with its saturated version, dihydroCer. The latter is normally desaturated to form Cer (reaction **5**) and both compounds are converted to other sphingolipids: the free amines, sphingosine and sphinganine, Cer-1-phosphate, sphingomyelin, and the GSLs. The two kinds of ceramide are also formed by hydrolysis of the more complex sphingolipids and by acylation of the free amines (reaction **4**). *In virtually every study of the apoptotic effects of Cer, dihydroCer was found to be inactive*. Indeed, a wide variety of *other* physiological effects of Cer also could not be produced by dihydroCer. The latter, unlike Cer, did not produce ROS on incubation with cells or mitochondria although it is presumably eventually converted to Cer. The only chemical differ-

ence between the two sphingolipids is the presence of a double bond in the 4–5-position (Fig. 1). The hydroxyl group at C-3 is thus an allylic alcohol and it is reasonable to expect unusual reactivity for the OH.

Good evidence has been found for an ROS-forming process when Cer enters mitochondria[28–31] and it is not surprising that mitochondria contain two enzymes that hydrolyze Cer, thus protecting their cells against mutations and apoptosis. The two ceramidases have pH optima in the acid and in the neutral/alkaline range; the latter appears to be localized to the mitochondria.[32] Evidence for a significant amount of the acid enzyme in mitochondria has also been reported.[33] Many human prostate tumors contain a high level of acid ceramidase, which helps explain their ability to proliferate.[34] The importance of ceramidase was further demonstrated by experiments with an inhibitor of the neutral enzyme. This produced Cer accumulation in the tumor mitochondria and thus blocked the appearance of solid tumors in nude mice inoculated with human colon cancer cells.[35] The precursor of Cer in mitochondria is SM, formed in the mitochondria (reaction **C** or **G**). Some SM may also be transported from the cytosol.

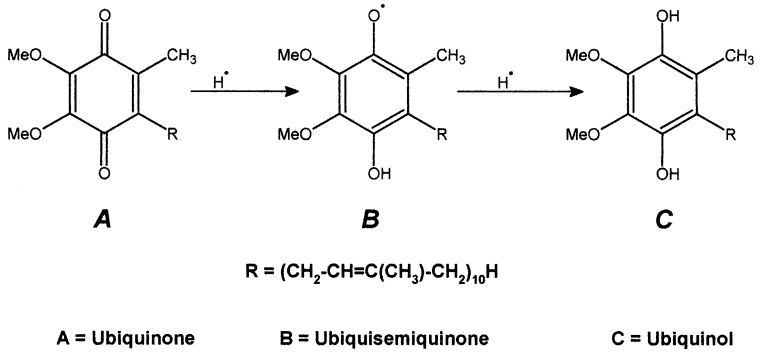
Studies of the process by which mitochondria generate H_2O_2 from Cer elicited the conclusion that the oxidant is generated at the ubiquinone site of the mitochondrial respiratory chain.[29,30] Tests with inhibitors of mitochondrial respiration indicated that there was a block at complex III, apparently interfering with ubiquinone function (Scheme 1). Ubiquinone (coenzyme Q = CoQ) exists in the mitochondrial electron transport system in three forms, as the quinone (**A**), the semiquinone (ubisemiquinone, a radical anion, **B**), and the diol (ubiquinol, **C**). CoQ and Cer are physically similar, having a long nonpolar side chain and low water-solubility. Antimycin A, like Cer, also blocks the operation of complex III. Using tumor necrosis factor-α as a producer of endogenous Cer, Corda et al. showed that antimycin—like Cer—produces H_2O_2.[36] This is apparently formed from the initial ROS, superoxide anion, by superoxide dismutase.

Antimycin A interferes with the ubisemiquinone transfer reaction and can itself produce apoptosis.[37] At lower doses, it simply competes with Cer's ability to generate apoptosis. Normally, electron flow from ubiquinol goes to cytochrome *c*, but when Cer blocks this flow, the cytochrome *c* dissociates and leaks out of the mitochondria, precipitating a complex series of 'death' reactions.

ROS release from mitochondria has also been produced by adding carbohydrate derivatives of ceramide (GSLs). The active GSLs examined so far are GlcCer, lactosylceramide (GalGlcCer), and gangliosides GD3 and GM1 (sialic acid-containing GSLs).[19,38] Sphingosine itself did not react and no one seems to have seen ROS formation by sphingomyelin, the major sphingolipid in cells. However the only GSL in the above group known to induce apoptosis in cultured cells is ganglioside GD3 (GalGlcCer attached to two sialic acid residues).[39] Using GD3 or a more stable analogue of GD3 as a chemotherapeutic agent in a cocktail of pro-Cer drugs might increase the cocktail's effectiveness.

Bhunia et al.[39] found that aortic smooth muscle cells, exposed to 50 μM [^{3}H]ganglioside GD3, readily took up the lipid but primarily on the surface. The lipid was labeled by catalytic reduction of the double bonds, so the sphingoid base was sphinganine. With *unlabeled* (primarily sphingosine-based) GD3 at 2.5–10 μM in a 10-min incubation, the cells generated superoxide anion and lost GSH (50% at 25–200 μM). GalGlcCer produced even more superoxide. Evidently, these glucosphingolipids penetrated the cells quite readily in this short incubation although the labeled GD3 did not. Cells thus treated exhibited elevated activity in NADPH/NADH oxidase and increased cell proliferation (incorporation of [^{3}H]thymidine into DNA). The Cer level was not determined.

It has been suggested that the block at complex III is the result of oxidation of the allylic alcohol moiety of Cer to an allylic ketone.[40] Allylic alcohols are noted for their reactivity and Cer is readily oxidized nonenzymatically at C-3 (not the C-1 hydroxyl) with a benzoquinone.[41] CoQ is also a benzoquinone and there may well be an electron transfer with Cer to form 3-ketoceramide or a free radical version of ketoceramide (Scheme 2). Allylic ketones can be expected to form assorted Michael condensation products by 1,4- or 1,2-addition. Such a condensation could explain the disappearance of GSH when Cer or a GSL is added to cells, but it is possible that other important thiols or amines in mitochondria also condense with the oxidized Cer. Thus Cer destroys



Scheme 1. Reactions of mitochondrial ubiquinone in complex III of the respiratory system.

GSH in two ways, by a condensation reaction with GSH and by oxidation of GSH with the ROS formed by Cer. This peculiar versatility of Cer may be partly due to the hydrogen bond formed by the two oxygen atoms at C-1 and C-3. Unfortunately no one seems to have searched for the formation of a ceramide-GSH (or other) adduct.

Another possibility is that Cer, after oxidation in the mitochondria, is also dehydrated at the C-1–C-2 position, forming a double bond that is conjugated with the 3-keto group, as well as the Δ^4 double bond (Scheme 2). It might be useful to synthesize a Cer with multiple double bonds on both sides of the hydroxyl group. An advantage of such an analogue is that it might not be converted to a proliferative sphingolipid. Conjugated isomers of the simple fatty acid, linoleic (especially combinations of *cis* and *trans* double bonds), have shown anticancer activity[42] and it is possible that such a combination in either long-chain moiety of a Cer would be especially useful. Perhaps the conjugated double bonds undergo allylic oxidation in vivo and thus act like Cer.

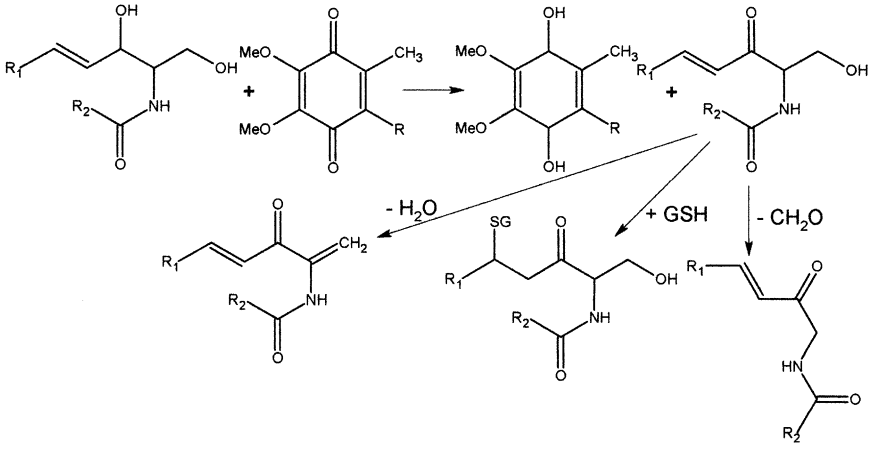
Another possible fate for the hypothesized ketoCer is a reverse Mannich condensation, by which the sphingolipid splits to form formaldehyde and 1-(*N*-fatty acylamino)-heptadec-3-ene-2-one (Scheme 2). This is an allylic ketone that should also condense with GSH. Formaldehyde is of course a reactive agent that might participate in apoptosis.

A close model of Cer is seen in 3-hydroxy-4-pentenoic acid, a simple allylic alcohol that is also capable of forming a hydrogen bond between oxygen atoms at C1 and C3.[43] Added to cells, it is oxidized in mitochondria to the allylic ketone, which then condenses with mitochondrial GSH. Apparently, no one has tested the substance for ROS and Cer formation. There is at present no reason to predict that this agent will react primarily with cancer cells. but it is possible that it may be useful as a moiety bound to a mitochondrion-seeking material, such as a Cer derivative.

The condensation with GSH can also occur enzymatically. A recent paper describes the action of GSH transferase in converting an anticancer allylic ketone into a GSH derivative.[44] Thus this enzyme also acts to lower the mitochondrial GSH level. As will be pointed out below, other anticancer drugs also generate ROS and deplete cells of their GSH.

If natural Cer is indeed oxidized to the ketoCer by ubiquinone, there may be some reversibility to the enzyme, allowing some conversion back to the original lipid. Adding $[^3H]_2O$ to the incubation mixture would introduce tritium into residual Cer. A similar test using [3-^{3}H]Cer should yield labeled water or H_2O_2.

Support for this ketoCer concept comes from analysis of the sphingoid bases in the ceramides of rat liver mitochondria, using chromatography and mass spectrometry.[45] Some ordinary Cer was found but there was 57% more *N*-acyl-ketosphinganine, a previously undetected kind of ceramide. This base was the only one seen in the GlcCer and GalGlcCer of the inner mitochondrial membranes, where the allylic alcohol group is presumably oxidized. These observations can be interpreted to mean that ketosphinganine (1-hydroxy-2-amino-3-keto-octadecane) is synthesized in mitochondria by the normal synthetic pathway and is then acylated to produce ketodihydroCer. This lipid does not contain an allylic alcohol group, thus should not react with the electron transport system or GSH. Apparently it is simply glucosylated to form 3-keto(dihydro)GlcCer, then the lactosyl GSL. The above proposed acylation has been shown to occur in mouse brain microsomes incubated with ketosphinganine[33] although it is generally believed that ketosphinganine is normally reduced to sphinganine *before* acylation. The ketodihydroCer, which is not allylic, is unreactive with GSH, and therefore accumulates in the mitochondria to a detectable extent. However the reaction of Cer with CoQ can be expected to keep the concentration of Cer low. Presumably the resultant ketone reacts too quickly with mitochondrial GSH to allow detectable accumulation.



Scheme 2. Hypothetical oxido-reduction reaction between ceramide and CoQ, with formation of ketoCer. This may condense with glutathione, or undergo dehydration, or a reverse Mannich reaction with formation of formaldehyde.

Surprisingly, the mitochondrial ceramides included a substantial portion of phytosphingosine as its sphingoid base.[45] This kind of ceramide, like ketodihydroCer, should be expected to accumulate in the mitochondria, since it lacks the allylic double bond.

A recent paper[46] presents the unexpected finding that *N*-acyl-phytosphingosine (phytoCer), exposed to SK-N-BE(2)C and N1E-115 cells for 24 h, induced apoptosis more effectively than Cer. The most effective fatty acid chain lengths were five and six carbons. Replacing the oxygen atom in the amide linkage with sulfur did not have much effect. (This is true of C_6-Cer, too.)[47] The authors looked for dehydration of the phytoCer to form Cer, but could not detect any. However, dehydration could have occurred with only a small fraction of the exogenous material in the mitochondria, where the C_6-Cer would be promptly attacked by the electron transfer system. This study did not examine the possibility that the phytoCer acted to influence the metabolism of endogenous Cer, possibly inhibiting its hydrolysis. This could be checked with a simple semi-quantitative evaluation of the cellular Cer content with a thin-layer chromatography plate. C_2-PhytoCer did not inhibit the growth of yeast cells.[47]

The above mitochondria-Cer-ROS hypothesis for apoptosis induction explains why some experiments with Cer formation using exogenous SMase failed to produce apoptosis. Apparently the Cer must enter into mitochondria during the time period used by the experimenter or be synthesized within mitochondria in order to start the death process. However there are other studies which indicate that exogenous SMase, as well as acid SMase,[48] can induce ROS, GSH depletion, and apoptosis. Perhaps certain cell types can transfer Cer rapidly from extra-mitochondrial sites to the mitochondria. Two mechanisms for transport of newly synthesized Cer normally bring the Cer to Golgi membranes, for conversion to GlcCer and SM.[49] Perhaps they also operate to bring Cer from lysosomes (which contain much acid SMase) to mitochondria. The mechanism for transport to the SM synthase site is inhibited by HPA-12, an aromatic analogue of Cer: N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl)-dodecanamide. Another factor to consider: some cell types may contain an especially active enzyme for converting Cer to GlcCer before it can reach the mitochondria.

Ceramide and its Analogues as Anticancer Drugs

Cer and its shorter chain homologues may well be practical anticancer drugs, provided they are not metabolized too quickly. They might, for example, be applied to skin cancers and precancerous lesions and bladder cancers in a simple lotion, or sprayed into lungs as a dispersion, for lung cancer. On ingestion, they can be expected to protect the intestine—and perhaps the entire body—against precancerous and cancerous lesions.[25] One would have to include inhibitors and stimulators of Cer metabolism to make sure that the Cer is not converted to proliferative sphingolipids.

On the basis of the many papers showing that cancer cells are killed by exogenous Cer, one would expect to see a host of new drugs—Cer or Cer derivatives—undergoing testing in tumor-bearing animals. Strangely, this has been done only rarely. Perhaps many of the currently active researchers working with Cer and apoptosis do not feel comfortable working with animals, preferring the neater, simpler, odor-free, faster-publishing cell culture dishes. Another problem is the high expense of preparing adequate amounts of sphingolipids, buying test animals, and caring for them. Perhaps the current rapid growth in funding for biochemical research will encourage a more realistic evaluation of putative cancer drugs. So many bioorganic papers end up with the sentence: 'This potent apoptogenic drug warrants further evaluation.' By whom?

A glucuronide derivative of Cer, added to the diet for only one week, was shown to undergo release of Cer in the intestine, partially protecting mice against the effects of a carcinogen, 1,2-dimethylhydrazine.[25] The derivative is hydrolyzed by an intestinal glucuronidase. Several papers have shown protective effects of other sphingolipids against colon cancer in animals; they are hydrolyzed to Cer, which may be the protective agent. A polyethylene glycol group attached to Cer in ester linkage would constitute a more water-soluble prodrug that might enter mitochondria and react like free Cer. Lecithin, often used as a dispersing agent for lipids, should be avoided since it reacts with Cer to produce SM (reaction **C**). (For the same reason, experiments that used phosphatidylcholine in cultures should be reevaluated.) However, a dispersion made with the aid of SM, if it penetrated intact to the tumor mitochondria, would be especially useful since the SMase in the tumor could generate additional Cer. Phosphatidylethanolamine also reacts with Cer to form Cer-phosphoethanolamine, which is then *N*-methylated to yield SM (reaction **G**).

Most studies of Cer in apoptosis have used cultured cancer cells with the short-chain fatty acid homologues of Cer, usually *N*-acetyl- or *N*-hexanoyl-sphingosine (C_2-Cer or C_6-Cer). This was done in the belief that natural Cer is too insoluble to enter cells. However some researchers have found that adding long-chain Cer dissolved in dodecane-EtOH gives excellent results.[50,51] (It is very likely that earlier unsuccessful results were simply the result of using coarse Cer suspensions that settled out at the bottom of the culture dishes.) The natural and short-chain ceramides should be compared in greater detail with tumors in animals. Like natural ceramides, the short-chain homologues are hydrolyzed to fatty acids and sphingosine, and also converted to short-chain GlcCer and SM.[52] The freed sphingosine undergoes acylation to form the natural long-chain ceramides. The short-chain Cer competes with long-chain Cer in the synthesis of complex sphingolipids, influencing cellular composition. In cultured kidney cancer cells, C_8-Cer and C_8-GlcCer led to large increases in natural Cer and GlcCer, and decreases in SM, DNA synthesis, and protein synthesis. Thus short-chain Cer or other sphingolipids can act as prodrugs and might prove effective in promoting apoptosis in vivo.

A set of such ceramides has been tested for ability to reverse the effect of fumonisin B1 on the growth of axons. Fumonisin is best known for its ability to block the acylation of sphingoid bases, thus preventing the synthesis of both dihydroCer and Cer. This Cer depletion slows the synthesis of the more complex sphingolipids and thus slows cell growth. The sphingoid bases accumulate, causing additional metabolic problems. Addition of exogenous Cer can overcome the growth inhibition. Van Overmeire et al.[53] found that neuronal axons whose growth rate was stimulated by basic fibroblast growth factor grew slowly when exposed to fumonisin, but the blockage by fumonisin was neutralized by C_2-Cer made from a 10-carbon sphingosine. Presumably the Cer homologue was converted to truncated GlcCer, which functioned adequately in the cells' growth process—for the duration of the experiment. This finding illustrates the peculiar bifunctional nature of Cer: under conditions where it is converted to proliferative sphingolipids, it can act as a growth stimulator.

It is interesting that the *N*-hexanoyl derivative of an ω-arylated five-carbon sphingosine also restored axonal growth of the fumonisin-inhibited neurons.[54] This implies that the cells were able to utilize the truncated Cer to make truncated SM and glucosphingolipids, but this would surely be toxic at a later stage. The phenyl group in this chain is conjugated with the allylic double bond, thus (in a longer incubation) might make the Cer very susceptible to oxidation and ROS production in mitochondria, constituting an effective anticancer drug.

The Bittman laboratory has synthesized some Cer isomers of potential value. The natural Δ^4 isomer, after assimilation into phospholipid/cholesterol vesicles, was found to bind to the Semliki Forest virus glycoprotein, while the Δ^5 isomer, 3-deoxyceramide, 3-methoxyceramide, and dihydroCer did not bind.[55] The acetylenic version of natural Cer was also able to bind to the protein. While binding to a virus seems unrelated to apoptosis, it does illustrate how precise the structure of active Cer must be and also raises the idea of testing acetylenic derivatives, in which the adjacent hydroxyl might be more rapidly oxidized in mitochondria. Another interesting possibility is a Cer containing a second trans double bond, conjugated with the Δ^4 double bond.[56] The conjugation may make the Cer more susceptible to mitochondrial oxidation, and the resultant ketone more likely to react with GSH, and so on.

Several fluoro derivatives of Cer have been tested. Molt and K-422 cancer cells were incubated 24 h with short-chain ceramides (30 μM) and evaluated for apoptotic cell content. Normal blood lymphocytes were used for comparison.[57] C_2-Cer was included as an example of a reliable apoptosis inducer, yielding 28–51% apoptotic cells. A similarly active inducer of apoptosis with K-422 cells was a truncated Cer made from hexanoic acid and a C_{12}-sphingosine containing fluorine instead of the C-3 OH. This raises the question for my hypothesis-how can an allylic fluorine atom be the equivalent of an allylic alcohol group? Analysis of the mode of apoptosis induction is called for. An interesting test might be to search for enzymatic replacement of the fluorine by an OH, or effects of agents known to block the effectiveness of Cer, or a possible accumulation of Cer due to ceramidase inhibition. Unfortunately, the fluorine compounds were quite toxic to normal cells too.

Macchia et al. have synthesized Cer analogues in which the polar end of the sphingoid base was replaced by a thiouracil or uracil ring.[58] Analogue I (Fig. 2) was more potent than C_2-Cer in inhibiting growth of human leukemia cells and even more effective with respect to apoptosis (DNA laddering and cytochrome *c* release). Treatment of mice implanted with human colon cancer cells for 5 weeks slowed tumor growth 43% without noticeable toxicity. The analogue's thiouracil ring might be considered to be an allylic ketone by disregarding any effects of the amide linkages in the ring, and thus might be expected to condense with GSH. However the drug's structure resembles that of 6-propylthiouracil (the Pr group is replaced by an Et and a long saturated chain is in the 5-position), and might act like this antithyroid agent.

Propylthiouracil produces elevated GSH levels in patients, which I would expect to stimulate tumor growth. The drug's mechanism of anticancer action needs more study.

The above analogue brings to mind a bicyclic anticancer drug consisting of uracil fused at the two N atoms with an eight-carbon conjugated enediyne, forming a second ring.[59] This drug was nontoxic to normal human cells but in cancer cells it slowed growth, depleted them of GSH, and synergistically augmented the effectiveness of doxorubicin (MI 3473) and ara-C (MI 2813), drugs frequently used in cancer therapy. The latter drugs produce Cer accumulation in tumors and it is possible that the enediyne does this too. The effectiveness of the enediyne was attributed not to the uracil portion but to the ability of thiols to displace the uracil and form a dithio ether with the C_8 chain. Presumably the same reaction could occur in cells with GSH but it is also possible that the three conjugated unsaturated bonds are readily oxidized to form an allylic alcohol.

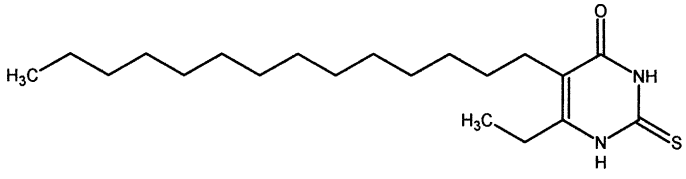
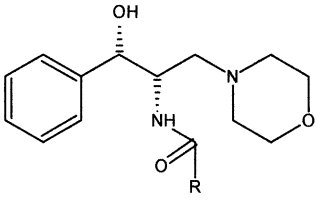


Figure 2. 4-Ethyl-5-tetradecyl-uracil.

A promising group of inhibitors and inducers of Cer accumulation and apoptosis is seen in the 'P-drugs,' analogues of Cer in which a phenyl group is attached to the C-3 carbon in place of the long alkenyl chain seen in sphingosine, and a small nitrogenous ring (morpholine or pyrrolidine) is attached to the C-1 carbon instead of the hydroxyl residue (Fig. 3).[60–64] The analogues differ from Cer also in inversion of the hydroxyl at C-3, making them D-*threo* isomers instead of D-*erythro*. (Several other studies comparing *threo*- and *erythro*-Cer analogues have noted higher apoptotic activity in the threo-isomers.) Over 200 papers have appeared on the effects of the P-drugs, which are known primarily as inhibitors of Cer glucosylation, forcing accumulation of Cer and SM. However inhibition of related enzymes has been found with D-PDMP, the best known of this group: (a) conversion of GlcCer to the galactosyl derivative, (b) conversion of GalGlcCer to the sialosyl derivative, ganglioside GM3, (c) conversion of GalGlcCer to the globoside GSLs, (d) SM synthase in malarial parasites, (e) the endoglycoceramidase that converts complex GSLs to Cer, and (f) the enzymes that synthesize and hydrolyze 1-acyl-C_2-Cer. The more recent variants of PDMP are said to be more specific.[39,62] It appears that many sphingolipid enzymes possess a similar site that is targeted by the Cer moiety of their substrates; PDMP and its analogus must bind to that site.

One of the inhibitors, D-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (PPPP), killed over 80 kinds of human cancer cells at ≈5 μM. Together with PDMP and the 3,4-ethylenedioxy derivative of PPPP,[65] it has proved remarkably nontoxic to mice, rats, and fish. At a low dosage, enough to slow GlcCer synthesis slightly, the P-drugs can overcome the toxic effects of accumulated GSLs in sphingolipidoses.[65,66] The drugs increase the incidence of apoptotic cell death when combined with other drugs that also elevate Cer concentration.

The C-3 hydroxyl group in the P-drugs, being adjacent to the phenyl ring, may be susceptible to oxidation in mitochondria and might thus produce apoptosis by the same route as Cer. However, PDMP labeled with [^{3}H] at C-3 produced very little labeled water in mice.[67] Cytochrome P450 oxidized much of the PDMP but the products were not identified and one cannot say whether they too were enzymatically active. However inhibitors of P450 augmented the half-life of the drug and its effects on sphingolipids. KetoPDMP (3-dehydro-PDMP) inactivated GlcCer synthase, apparently covalently, suggesting there is an accessible amine (lysine?) near the active site.[68]



PDMP R = $CH_3(CH_2)_8$
PPMP R = $CH_3(CH_2)_{12}$

Figure 3. Two P-drugs, *N*-decanoyl- and *N*-tetradecanoyl-derivatives of 095D-threo-1-phenyl-2-amino-3-morpholinyl-propanol-1.

A variant of the P-drugs, in which the phenyl ring was replaced by the aliphatic chain of sphingosine, was also an effective inhibitor of GlcCer synthase. but it did not force accumulation of Cer or demonstrate growth inhibition.[69] Perhaps it stimulates an enzyme that converts Cer to another metabolite.

Many Cer analogues, containing a chemically reactive region, have been synthesized but not evaluated biologically.[70,71] The authors pointed out that the analogues could be made to form 'tight' crystals that would probably dissolve slowly in the body, and thus might be especially useful as long-lasting therapeutic drugs. Another large series of Cer analogues was prepared and assayed for ability to inhibit the galactosidase acting on galactosylCer (the major glycolipid of axonal sheaths) and the galactosyltransferase that converts Cer to the glycolipid. GlcCer enzymes were also tested.[72–78] The amines used were either highly truncated sphinganine derivatives or variants of 1-phenyl-1,3-dihydroxy-2-propylamine, and the fatty acid was usually decanoate. A longer acyl chain variant resembling 1-desoxy-Cer, D-erythro-2-myristoylamino-1-phenyl-1-propanol (D-MAPP), was as effective as C_2-Cer in inhibiting HL-60 cell growth. It acted by inhibiting neutral/basic ceramidase (Table 2) and forcing mitochondria to accumulate natural Cer.[79]

An unusual ceramide made from dodecanoic acid and 4-amino-5-hydroxydecane resembles a short-chain dihydroCer but lacks the hydroxyl group at C-1; one could say there is an ethyl group there instead.[80] This amide produced cleavage of poly(ADP ribose) polymerase, a symptom of apoptosis, as well as death of Molt 4 cells (only 20% at 28 μM after 13 h). Under the same conditions the thio analogue of C_2-Cer (*N*-thioacetylsphingosine) produced 73% cell death. Since the 'ethylated' ceramide analogue did not contain an allylic alcohol group, its mechanism of action is obscure. No sign of elevated Cer levels could be detected. It is possible that the thiolated C_2-sphingosine was quickly oxidized by any ROS that might have formed. In general, adding a drug with ROS-absorbing properties seems counterproductive for cancer therapy but useful for preventing cancer.

Several studies showed that analogues of the sphingoid bases or Cer could be utilized as substrates, generating abnormal sphingolipids. For example, the diastereoisomer, L-*threo*-sphinganine, was acylated to form a dihydroCer which was then metabolized to form the abnormal L-*threo*-dihydroSM, but not L-threo-dihydroGlcCer.[81] Presumably a tumor that incorporated a significant amount of such an abnormal sphingolipid would ultimately be significantly damaged. Truncated Cer (*N*-hexanoyl) with a phenyl ring attached to the end of a C_5-sphingosine chain proved to form truncated

GlcCer in cultured neurons.[54] In a tumor this might be converted to more complex GSLs which might be toxic. It is interesting that glucosylation also occurred when the phenyl ring included a para-methyl, -pentyl, -fluoro, or -methoxy substituent. Even a bulky fluorescent substituent on the fatty acid moiety of Cer entered into the synthesis of more complex sphingolipids. This flexibility on the part of sphingolipid enzymes points to the possibility of making a prodrug consisting of Cer linked to a toxic material or an apoptosis-promoting material (perhaps a benzoquinone?).

Drugs in Current Chemotherapy or Proposed Use

Do anticancer drugs in current use produce apoptosis in the same way as Cer or simply act to elevate Cer levels in tumors? If a drug can penetrate mitochondria and interfere with ubiquinone metabolism, producing ROS, it could be reasonably considered to be a Cer agonist, starting the mitochondrial sequence of apoptotic steps. The fact that the ROS would destroy GSH and stimulate Cer synthesis from SM would mean only that the efficacy of the drug was enhanced by Cer-induced apoptosis. A major thesis of this paper is that the drug probably includes an allylic alcohol or allylic ketone (or quinone) moiety and is likely to condense with GSH after reaction in the mitochondria.

A drug that does not enter mitochondria or that directly affects a sphingolipid enzyme (causing an increased level of Cer) might not be a Cer agonist. However it should nevertheless be useful if the Cer produced outside the mitochondria could enter the mitochondria and block CoQ action. The drug should not lower cytosolic GSH too far, since that would slow the activation of caspases needed for apoptosis and would also be toxic to some enzymes. However some reaction with cytosolic GSH should be helpful, since this GSH is transported into the mitochondria to replenish mitochondrial GSH.[82]

Of course anticancer drugs differ from Cer and other drugs with regard to metabolism, ability to concentrate in specific tumors, and effects on other enzymes or binding sites. This is the basic problem of specificity and toxic reactions. The next section describes some anticancer drugs and their relationship to Cer generation (where known). To save space, some drug structures are referenced by compound number ('MI') in The Merck Index, 13th Edition.

Anthracyclines

These anticancer drugs are made by microorganisms and many variants have been synthesized in efforts to minimize cardiac toxicity and their tendency to induce multi-drug resistant tumor clones. They are tetracyclic structures in which one outer ring is aromatic, the adjacent ring is a quinone, the next ring is a hydroquinone, and the fourth ring is aliphatic. The hydroquinone hydroxyls can be considered allylic alcohols and are no doubt easily oxidized to a second quinone ring. Doxorubicin (MI 3473 = Dox = Adriamycin) may currently be the most used in the group. It generates Cer (by activating neutral SMase), H_2O_2, and apoptosis in cancer cells. Antioxidants and low O_2 levels reduce its effectiveness. In umbilical vein endothelial cells, Dox triggered Bcl-2 down-regulation, cytochrome *c* release from mitochondria, and the activation of caspases 9 and 3, suggesting the involvement of a mitochondrially instigated pathway of apoptosis.[83] Its effectiveness in human breast carcinoma cells was reduced by high levels of GSH peroxidase, which destroys ROS.[84] Buthionine sulfoximine enhanced the apoptotic action of Dox. These relationships are typical of Cer-induced apoptosis.

Cells overexpressing GlcCer synthase (which utilizes available Cer to form much GlcCer) were found to be markedly insensitive to Dox.[1] This illustrates the importance of blocking GSL synthesis in any therapeutic effort using Cer generating drugs. Prolonged treatment of patients with Dox leads to multi-drug resistance, the condition in which various chemotherapeutic drugs are excreted from cancer cells before they can achieve a therapeutic level. The excretion is usually due to high levels of the transporting proteins, MDR1 and/or MRP1, whose production may be induced by the high concentration of GlcCer.[1,2] Perhaps high production of Cer also induces the transporters. These and related findings explain why Dox effectiveness is enhanced by administration of a P-drug, which blocks GlcCer synthesis.

The ability of Dox to produce ROS and reduced GSH levels can be remarkably prolonged. After a series of low doses of Dox in rats, cardiac myocytes were isolated and found to produce above-normal amounts of ROS (increasing with time); they contained ~20% less GSH. These differences lasted at least 5 weeks after the drug injections had stopped, suggesting that the drug formed a long-lasting complex with a mitochondrial component that retained its ROS-generating activity.[85] It would be interesting to see if multiple Cer administrations, or doses of Cer-promoting drugs, produce similar long-term effects. Synthesis of a Dox–GSH adduct has been reported[86] and it is possible that the long-lasting complex is formed between Dox and a cysteine residue in a protein. The adduct, which was located in the cytoplasm, produced apoptosis via the mitochondria. Perhaps a little of the adduct is also formed in the mitochondria.

The prolonged nature of Dox-generated ROS may explain the belief that Dox is a weak carcinogen. Generation of ROS by any agent raises the risk of eventually creating a procancerous mutation in a susceptible pair of genes. This problem must affect any drug that elevates Cer levels, since Cer generates ROS. The conclusion to be drawn from this factor is that cancer therapy should be brief, utilizing as many drugs as possible simultaneously, then quenching the ROS by ingesting acetyl cysteine or other GSH promoter and extra antioxidants.

Docetaxel (MI 3431)

Docetaxel is an anticancer drug, related to paclitaxel (Taxol), with a complex structure that includes an allylic alcohol adjacent to a ketone group. The latter augments the conjugation activation of the alcohol group. The drug—at only 10 nM concentration!—produced growth inhibition of hepatoma cells, apoptosis, ROS, caspase activation, and DNA fragmentation.[87] These are typical Cer effects. However the drug-induced apoptosis was said to be independent of ROS formation, a discrepancy that needs more study.

Paclitaxel (MI 7052), a very close relative of docetaxel in which the allylic alcohol group is acetylated, behaved similarly. It has been shown to produce Cer and apoptosis in prostate cancer cells[88] and to block the drug-eliminating action of P-gp, the transport protein typically seen in multi-drug resistant cancer cells. The stimulation of Cer synthesis by paclitaxel is initially due to faster de novo synthesis from serine and palmitoyl-CoA, but the longer term effect is due to stimulation of SM hydrolysis. Protein kinase Cδ seems to be essential for Cer formation and the Cer itself promotes activation and transfer of the kinase into the mitochondria. Thus this constitutes another Cer self-augmentation spiral that tends to ultimately lead to apoptosis. From these findings, it seems likely that the neoplastic activity of the drug depends primarily on its ability to produce Cer. Perhaps allylic alcohols act by stimulating Cer elevation while allylic ketones act directly by interfering in the mitochondrial electron transport chain.

Many chemotherapeutic agents, such as taxol, docetaxel, and vinblastine, interact with microtubules and then induce apoptosis. Colchicine, which binds strongly to microtubules, stimulates GlcCer synthesis and possibly stimulates Cer synthesis too.[89] More precise control over cancer cell microtubules may enhance the action of antineoplastic drugs.

Tetracycline (MI 9271)

Tetracycline is an antibiotic that resembles doxorubicin to some extent, but the second ring contains only a single ketone group and the substituents on the four rings differ somewhat. One could think of part of the structure as being a 'benzoyl ethylene,' that is, an allylic ketone that is surrounded on both sides by conjugated double bonds. Also present on the fourth ring is another allylic ketone group. Thus it follows from my hypothesis that tetracycline should have anticancer activity. This has indeed been observed in several studies.[90,91] The tetracycline family of drugs tends to concentrate in bone and is therefore recommended for bone tumors, particularly metastases of breast cancer. Another beneficial feature is that tetracyclines inhibit matrix metalloproteinase, a highly active enzyme in invasive tumors. In general, they inhibit cancer cell growth and metastasis. Doxycycline (MI 3474) is another antibiotic, very similar to tetracycline, which produced a 70% reduction in tumor burden in mice carrying metastatic MDA-MB-231 breast cancer cells.[91] An even simpler tetracycline, CMT-3, reduced the growth of prostate cancer cells, produced ROS, caspase activation, and mitochondrial depolarization, and inhibited the invasive activity of the cells.[92] Given orally to rats carrying Dunning MAT LyLu prostate tumors, it inhibited tumor growth and metastasis significantly. Both variants of tetracycline retain the two allylic ketone groups, consistent with their anticancer activity.

Camptothecin (MI 1743)

This important antineoplastic drug can be viewed as a highly conjugated allylic ketone and allylic alcohol in which both oxygen atoms share the same double bond. A tertiary amine group is close to the ketone, a feature seen in other anticancer drugs. It has been reported to produce apoptosis by stimulating the de novo synthesis of Cer.[93] The authors point out that drug-treated cells exhibited marked changes in the morphology of the Golgi particles, where Cer is glucosylated to form GlcCer. This implies that depletion of cellular Cer by glucosylation plays an important part in protecting cancer cells, and the authors mention that treatment with PDMP (see P-drugs above) enhanced the apoptogenic effectiveness of camptothecin. That synergism has also been observed for taxol and vincristine.[94] The camptothecin analogue, CPT-11, which has the same allylic ketone group, also generated Cer and apoptosis in fibroblast 4B1 cells.[95] It was shown with human colonic tumor xenografts that the addition of SM enhanced the apoptogenic effect, presumably by increasing the availability of SM for the action of mitochondrial SMase.[96] This finding opens the possibility that SM, or a variant of SM capable of being hydrolyzed in mitochondria, could act as a prodrug to yield Cer or a Cer variant with superior apoptogenic efficacy.

Flavopiridol (MI 4122)

This simple flavonoid has apoptogenic and anticancer activity, and inhibits cyclin-dependent kinases.[97] Promising results have been obtained in patients. It is a substituted phenol that is fused to a substituted benzoflavone ring. The latter ring contains an allylic ketone conjugated on each side of the carbonyl group with two phenyl groups, so it can be considered highly conjugated and reactive. It could also be considered a benzoyl group attached to an ethylene group, thus resembling tetracycline. In U937 monoblastic leukemia cells it generated ROS and the typical apoptotic changes. These and other effects showed that it acted via the mitochondrial apoptosis pathway, like Cer.[98] Other reports of toxicity against cancer cells have appeared,[99] and normal human umbilical vein endothelial cells were found to undergo apoptosis.

Flavopiridol also inhibits phosphorylation of the receptor for epidermal growth factor, a substance prominent in some tumors. Cer has been found to inhibit EGF activity,[100] which may signify that flavopiridol acts by generating Cer. The Cer effect also points to another mechanism by which Cer blocks proliferation.

Baicalein (MI 944) has a similar allylic ketone structure, but fewer substituents. It is a plant component that is finding use in treating prostate cancer, as part of the herbal mixture PC-SPES. It produced apoptosis and cell cycle arrest in an assortment of cancer cells.[101]

Curcumin (MI 2703)

This yellow dye found in roots is a component of spice mixtures. It has been used in indigenous medicine and is now being promoted as a cancer preventive *and* treatment drug. It has also shown activity as an anti-inflammatory agent, antioxidant, and antibiotic—a truly versatile agent! It is a symmetrical molecule containing two allylic ketone sequences, each one conjugated with a substituted phenyl ring. The two carbonyl groups are separated by a methylene group. Thus it is an example of a benzalacetone (methyl styryl ketone) in which the phenyl ring has a guiacol configuration (3-methoxy-4-hydroxy-). The antimutagenic effect was attributed to the allylic ketone moieties, which are found also in other compounds with antimutagenic activity—flavonoids, coumarins, and dihydrofuranones. As may be expected, the substituents on the rings affected the potency of the compounds.

Curcumin has proved useful in slowing the appearance in rats of colonic aberrant crypt foci, precursors of colon cancer.[102] It inhibited proliferation of colon adenocarcinoma cell lines[103] and breast tumor cell lines.[104] The mechanism of curcumin's actions are not simple; it may inhibit the inducible nitric oxide synthase.[102] Compounds that alkylate thiol groups, such as allylic ketones, disrupt the complex between a chaperone, Keap1, and Nrf2, a member of the basic leucine zipper family of transcription factors.[105] The liberated Nrf2 then enters the nucleus, where it induces a host of 'phase 2' enzymes.[106] These enzymes control GSH-associated metabolism. Curcumin has been shown to induce the appearance of heme oxygenase,[107] a phase 2 enzyme. Possibly an important point of the study by Dinkova-Kostova et al.[105] is the finding that a hydroxyl group close to the allylic double bond greatly increased the induction power. Perhaps the hydroxyl on C-1 of Cer acts this way too.

Ciprofloxacin (MI 2337)

This antibiotic is a member of the quinolone family, containing two fused rings, one of which is a substituted benzene. The other contains the vital allylic ketone group close to a tertiary amine. Thus its predicted anticancer activity stems from the same conjugated 'benzoyl ethylene' group seen in tetracyclines. It inhibited growth of human bladder carcinomas cells, producing apoptosis.[108] Mitochondrial depolarization was observed, together with an alteration of mitochondrial Ca^{2+} within 5 min and Bcl-2 dependent subcellular redistribution of Bax to the mitochondrial membrane. Ciprofloxacin also produced swelling of isolated mitochondria, accompanied by cytochrome *c* release and caspase 3 activation. It also suppressed DNA synthesis in colon carcinoma cells, producing apoptosis.[109] It upregulated Bax and the activity of caspases 3, 8 and 9, while decreasing mitochondrial membrane potential. These are all typical of Cer anticancer effects. It is interesting that hundreds of papers describe the value of ciprofloxacin in cancer patients to minimize inbfections, while the drug may have incidentally helped fight their cancer.

Another quinolone antibacterial agent, cinoxacin (MI 2332), has a similar structure but apparently has not been tested for anticancer activity.

Δ^9-Tetrahydrocannabinol (MI 9283)

This famous mind-altering component of marijuana (THC) does not possesses an allylic alcohol group, so it might be expected to lack antineoplastic activity. Nevertheless significant activity has been reported in a series of papers from the laboratory of M. Guzman.[110,111] THC and other cannabinoids produced apoptosis of glioma cells and Cer accumulation. Inhibiting Cer synthesis de novo prevented the appearance of apoptosis, suggesting that the drug stimulated the synthesis of keto sphinganine. THC is rapidly oxidized by cytochrome P450 enzymes to 11-hydroxy-THC and 8-hydroxy-THC,[112] which are allylic alcohols and thus putative anticancer drugs. It should be possible to demonstrate the importance of this process by including an inhibitor of P450, such as piperonyl butoxide, which should neutralize the tumor inhibition.

THC has produced a distinct degree of protection in EL-4 tumor-bearing mice, as did the 'natural cannabinoid,' anandamide.[113] It is interesting that anandamide, *N*-arachidonoyl ethanolamine, is a truncated analogue of Cer. It resembles the inhibitor of ceramidase, *N*-oleoyl ethanolamine (Table 2), which promotes Cer accumulation in mitochondria and apoptosis. It should be noted that anandamide normally undergoes rapid hydrolysis to form free arachidonic acid, which is a stimulator of SMase and apoptosis. Arachidonic acid is also converted by enzymatic oxidation to 4-hydroxy-2-nonenal, which is both an allylic aldehyde and an allylic alcohol. This compound produces apoptosis, forms a conjugate with GSH, and lowers GSH levels in cells.[114] Consideration of these relationships points to a partial explanation of THC's benefits: by occupying the anandamide-binding site, it may force anandamide accumulation, hydrolysis, and activation of SMase by the liberated arachidonic acid. If this is significant, it may be useful to use THC together with arachidonic acid and an inhibitor of cyclooxygenase-2.

A synthetic THC agonist, WIN-55,212-2, produced similar anticancer effects.[115] This compound possesses an allylic ketone residue in which the double bond is part of an indole ring[116] so it too fits the allylic ketone-apoptosis hypothesis. Some other THC analogues that bind to the CB_2 receptor contain an allylic alcohol residue and may prove to have anticancer activity.[117] Cannabinoids also inhibit protein kinase B and stimulate ERK (extracellular signal regulated kinase). This too may involve Cer synthesis, as shown by the ability of

L-cycloserine, an inhibitor of the first step in sphingolipid synthesis, to block these effects.

Before the reader smiles again at THC's unexpected combination of effects—mind-alteration and tumor apoptosis—it should be noted that the psychological effects have been attributed to binding of THC to the CB_1 receptor while the receptor for THC and other apoptotic analogues in brain tumors is CB_2. Yet to be determined is the extent of occurrence of CB_2 in other tumors. Attempts have been made to synthesize THC analogues that bind primarily to CB_2 and thus avoid psychotropic effects. JWH-133, 1-deoxy-3-(1′,1′-dimethylbutyl)-Δ^8-THC, is a simplified version of Δ^8-THC that lacks the allylic alcohol residue but is rather specific for CB_2. At a dosage of only 50 μg/day in mice, it greatly slowed the growth of gliomas.[111] In cultured glioma cells, apoptosis and Cer accumulation were seen. No doubt this analogue also undergoes oxidation by P450 enzymes to allylic alcohols.

One of the effects of THC is the acceleration of glucose metabolism, which was attributed to acceleration of SM hydrolysis.[118] Rat astrocytes treated with THC showed faster glucose oxidation to CO_2 and incorporation into phospholipids and glycogen. Exogenous SM and C_8-Cer also increased glucose metabolism, suggesting that the apoptotic effect of THC was simply due to Cer production. SMase was found to accelerate the uptake of deoxyglucose by platelets and their basal glycolytic flux.[119] This may be a normal response of cells confronted with excess Cer: they absorb extra glucose in order to form UDP-glc and remove the Cer by glucosylation.

Gaucher disease patients, who hydrolyze GlcCer to Cer abnormally slowly (reaction **3**), were found to generate hepatic glucose 30% faster and exhibit 24% faster resting energy expenditure.[120,121] The plasma insulin activity was also abnormally high. Treating the patients with normal GlcCer glucosidase did not normalize these values, suggesting that they were not due to an intrinsic effect of the accumulated GlcCer.

It would seem wise to see if THC analogues elevate GlcCer levels, as well as Cer, and use the analogues together with an inhibitor of Cer glucosylation in order to maximize the increase in tumor Cer.

Raloxifene (MI 8190)

This drug is a SERM, a selective estrogen receptor modulator, in which a ketone group is attached to both a substituted phenyl ring and a benzothiene bicyclic structure. It is thus a highly conjugated allylic ketone that ought to show antineoplastic activity. It has been found to markedly reduce the incidence of breast cancer in women.[122,123] It also prevents or repairs osteoporosis, an interesting property which has been linked to GalGlcCer, the glycolipid formed from Cer by glucosylation and galactosylation. The P-drug, PDMP, inhibits the synthesis of this lipid and prevents the generation of osteoclasts.[124] Gaucher disease patients, who have high cell contents of GlcCer and GalGlcCer, suffer from a loss of bone, mainly in the long bones, apparently due to poor balance between osteoclasts and osteoblasts. Tumor necrosis factor-α stimulates the formation of Cer and, probably, GlcCer too; it protects osteoclasts against apoptosis.[125] Thus raloxifene may act like Cer in terms of bone protection as well as tumor apoptosis. Doxycycline, an antineoplastic agent with an allylic ketone group, increases the number of osteoblasts in the bones of treated mice.[91] Dihydroxy Vitamin D_3, an allylic alcohol, stimulates osteoblast growth[126] and has antineoplastic activity. While the connections to bone growth are complex, it is evident that sphingolipids are important controlling factors.

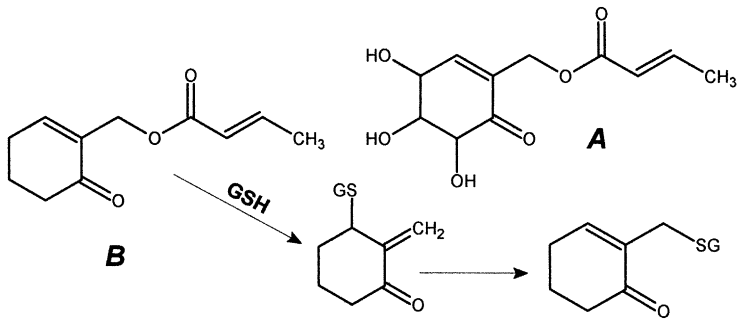
Mitoxantrone (MI 6238)

This antineoplastic drug is an analogue of anthraquinone, a tricyclic with a hydroquinone outer ring and two *p*-dialkylamino groups in the other outer ring. Thus it can be considered to be an allylic ketone and allylic alcohol that is easily oxidized to a diiminoquinone then, perhaps, to a hexaketo derivative. It was shown to react with NADPH and a rat liver enzyme, forming several kinds of ROS.[127] Reaction with DNA was reported by several researchers and Cer formation was also observed.[128] Phosphatidylcholine cleavage also occurred, with the appearance of diacylglycerol and phosphocholine. This loss of phosphocholine and appearance of diacylglycerol can be interpreted to mean that some of the accumulated Cer reacted with the phospholipid to form SM and diacylglcerol (reaction **C**).

1,25-Dihydroxy vitamin D_3 (MI 10079)

Vitamin D_3 is activated by two hydroxylation steps, forming 1,25-dihydroxy vitamin D_3 (DHD3). DHD3 is noted for its bone-building activity but has also shown considerable promise as an anticancer drug. It contains an allylic alcohol group in which the hydroxyl at C-1 is activated by a methene carbon atom that is itself conjugated to two double bonds. In MCF-7 breast cancer cells, DHD3 induced apoptosis, translocation of Bax to the mitochondria, generation of ROS, loss of the mitochondrial membrane potential, and release of cytochrome *c*.[129] It produced cell cycle arrest and apoptosis in a squamous cell carcinoma model, probably via involvement of Erk and Akt.[130] These effects were enhanced by including dexamethasone, itself an allylic ketone that is often used in cancer therapy. DHD3 stimulates neutral SMase, which leads to Cer increase.[131] Exogenous Cer can elicit the same effects as DHD3, which apparently acts by increasing cell levels of tumor necrosis factor, thus stimulating SMase to produce Cer.

A study with keratinocytes illustrates the complexity of interpreting biological reactions.[132] In these cells, DHD3 produced an elevated level of sphingosine-1-phosphate and growth *stimulation* instead of apoptosis. These cells may have a high level of ceramidase activity and/or sphingosine kinase activity, either of which



Scheme 3. Enzyme-assisted condensation of glutathione with crotonoyl 2-hydroxymethyl-2-cyclohexenone.

switches cells from apoptosis toward proliferation. This study exemplifies the importance of poly-drug control of Cer metabolism.

Use of DHD3 in cancer therapy is limited by its production of an imbalance in Ca^{2+} metabolism, so researchers have been designing analogues that retain the antiproliferation activity while minimizing hypercalcemia. Several such analogues, in which a second allylic (or acetylenic) alcohol is present, have shown good activity against colon cancer in mice[133] and reduction of tumor cell invasiveness.[134]

Illudins (MI 4923)

These mushroom-derived antibiotics have shown strong anticancer properties but their therapeutic index is low. They are tricyclics, one ring of which is the exotic cyclopropane. The middle ring contains an allylic ketone whose double bond is conjugated with another double bond. The third ring contains an allylic alcohol and a dimethyl group (illudin M). In illudin S, one of the methyl groups contains an OH group, which might act like the hydroxymethyl group in the crotonoyl ester described below. Thus the two structures suggest good antineoplastic activity. The allylic ketone residue can react with GSH, whereupon the cyclopropane ring opens to bind with a second nucleophile, such as DNA. This is an aromatization step that results in removal of a water molecule.[135] Thus the drug acts to kill cells by two routes: GSH removal and, perhaps, DNA or RNA damage. Irofulven (6-hydroxymethylacylfulvene, MGI 114, NSC 683863) is a semisynthetic derivative of illudin S that induces caspase-mediated apoptosis in pancreatic carcinoma cell lines.[136] It activates JNK1 and Erk1/2 but not p38. Irofulven has demonstrated activity against a broad range of solid tumors in both xenograft models and human trials. Human lung carcinoma cells and athymic mice bearing the human lung carcinoma MV522 xenograft responded well and good synergism was seen with thiotepa or mitomycin C (MI 6236).[137] The latter is an antineoplastic quinone (a diallylic diketone).

Glucocorticoids

Glucocorticoids are well-known inhibitors of cell growth and find frequent use in cancer therapy. They are allylic ketones, possessing a ketone oxygen at C-3 and a double bond at C4,5 and thus fit into the proposed rubric.

Dexamethasone (MI 2960) is a frequently used glucocorticoid-like drug possessing many properties, one of which is the induction of cancer cell apoptosis. It is a doubly allylic ketone resembling testosterone (MI 9255), with an extra double bond in the 1,2-position. It increased Cer levels in mouse thymocytes[138] and stimulated the conversion of Cer to GlcCer in renal cells,[139] suggesting that glucocorticoids stimulate synthesis of all the sphingolipids.

Testosterone and aldosterone, the mineralocorticoid, possess the same allylic ketone structure. Testosterone induces GlcCer synthase and, in the mouse kidney, markedly increases the concentrations of Cer, GlcCer, and the more complex GSLs.[140] This increased GSL concentration in kidneys explains why they grow faster in maturing male mice. It also explains why an inhibitor of Cer glucosylation (PDMP) causes kidneys to shrink.[141–143] Androgens produce elevated levels of H_2O_2 and can thus be expected to instigate the appearance of cancer in normal androgen-sensitive tissues. In tumors that have already appeared, androgens can be expected to slow tumor cell growth when Cer glucosylation is blocked with an inhibitor. Some benefit of testosterone has indeed been observed in patients whose prostate cancer cells have become insensitive to the growth-stimulating effects of testosterone, even without use of a glucosylation inhibitor.

Crotonyloxymethyl-trihydroxy-cyclohexenone

A *Streptomyces* product that has shown promising anticancer activity in plastico is an ester formed from crotonic acid and 2-hydroxymethyl-4,5,6-trihydroxy-2-cyclohexenone (Scheme 3A). The acid is attached to the hydroxymethyl group. Thus this compound can be considered to be an allylic ketone and also an allylic alcohol. A structural comparison of compounds related to the ester drew the conclusion that the antitumor activity was derived from the allylic ketone moiety.[144] A simpler version (Scheme 3B) in which the cluster of three hydroxyl groups is absent was found to be a potent antineoplastic drug. A study of this variant showed that it was readily converted by an enzyme, glutathionyl transferase, to a GSH adduct, while

releasing the crotonic acid residue, so that the product had a methylene double bond at C-2.[44] This unexpected product rearranged nonenzymatically, with the glutathione residue now attached to the methylene carbon; the allylic ketone system was still intact. Conceivably the product could add a second GSH group by 1,4-addition but the reaction was not detected in the short time examined. Condensation with model nucleic acids has been detected, which might be helpful if the derivative concentrates in the tumor.

The activation of the crotonoyl ester by an enzyme involving GSH is a novel pro-drug activation step that might be utilized for other anticancer drugs. It might be worth synthesizing a Cer analogue containing the entire crotonic acid ester (in which the crotonoyl group is part of the fatty acid or sphingoid base moiety).

Another potentially effective prodrug enzymatic activation approach is via *ipso*-substitution, a reaction which can convert *p*-substituted phenols to quinones or hydroquinones. For example, cytochrome P450 in rat liver microsomes—with its NADPH-reductase—can replace the halogen (even the fluoro!), nitro, nitrile, and hydroxymethyl derivatives of phenol with an OH.[145] Benzoquinone seems to be an intermediate, and it may react with cytosolic GSH instead of undergoing reduction. In the case of a *p*-methyl substituent (*p*-cresol), the product was an allylic alcohol/ketone (*p*-toluquinol = 1-hydroxy-1-methyl-4-keto-cyclohexadiene),[146] which may also react well with GSH.

Hazards of Research with Water-Insoluble Drugs and Sphingolipids

The literature on potential anticancer drugs is replete with errors of omission and commission, making it difficult to draw unequivocal conclusions about 'the' correct direction for future drug designs. Of course there are good reasons for errors of omission, such as a shortage of funds and facilities or collaborators with the necessary skills. Bioorganic researchers always hope that others will become interested in pursuing further study of their new drugs and thus demonstrate their practical value. However, including a little more sophistication in the first publication will probably increase the impact of the findings on other researchers. I urge editors and reviewers to keep these factors and common errors (see below) in mind when reviewing descriptions of new putative drugs.

(a) In vivo versus in plastico: some inactive drugs are hydroxylated in vivo, converting them to allylic alcohols which can then generate ROS. These processes take time, as much as three days, so short-term tests can be misleading. Even tests in plastico can involve multiple induction processes, thus evaluation of a new drug after a single, short incubation can be misleading—especially if the amounts of related metabolites are not also measured. All too often, researchers (and laymen who want to stop researchers from inflicting pain) forget that an intact human being is far different from a few cultured cells. If a drug has anticancer activity in plastico, it is worth doing a time study to see if there is a lag period, which might be due to a drug-activation process. If an inhibitor of P450 (piperonyl butoxide, cimetidine, etc.) prevents the anticancer effect, that is a good sign that the active drug has a modified structure.

This rule applies not only to putative antineoplastic drugs but also to modifying agents. A good example is BHT (2,6-di-*tert*-butyl-4-methylphenol), an antioxidant that is usually expected to block ROS generation and protect cells against cancer. However, it is attacked by a cytochrome P450 to form a derivative that produces apoptosis and condenses with GSH and cellular proteins.[147] A related example is seen with vitamin A (retinal), which has shown the ability to inhibit tumor growth. Retinoids of various types are being studied as antineoplastic agents, yet these substances do not contain an allylic alcohol or ketone group. Recently, a new metabolite of vitamin A alcohol (retinol), containing a conjugated allylic ketone structure (9-*cis*-4-oxo-13,14-dihydro retinoic acid), was discovered in mouse and human liver.[148] This structural feature may explain the anticancer activity of the retinoids. It also should remind us that an anticancer drug may be inactive in a cancer cell type that cannot transform the drug to the active form.

(b) When comparing related compounds for (say) apoptogenic activity, it should be remembered that the different compounds may exert their effect by different mechanisms. For instance, a Cer analogue might act simply to block an enzyme that normally destroys endogenous Cer.

(c) Testing a drug at only one concentration is risky since the chosen concentration may be above the point of maximum effectiveness. Comparisons of different drugs, all at the same molar concentration, will miss important differences if they differ with respect to the K_i or concentration yielding maximum effectiveness.

(d) Using radioactive precursors as a substitute for mass measurement is tantalizingly convenient but can be misleading since the amount of radioisotope in a product depends on the specific activity of each precursor, a factor that might be influenced by the test drug. The specific activity of each precursor may change with time so multiple time points are essential for clarity. It should be noted that sphingolipids labeled by oxidation at the C-3 position, then reduction with labeled borohydride, will yield partially racemized 3-hydroxyl groups. Not every investigator has separated the two forms before use. A similar ambiguity arises when researchers hydrogenate their sphingolipids catalytically with $^3H_2$, which converts the sphingosine moiety to sphinganine. Non-radioactive sphingolipid carrier added to dilute the radioactive lipid may act differently. It is often necessary to trace an author's previous publications to ascertain how the radioactivity was inserted.

(e) Almost all new drugs are evaluated with cancer cells, but it is important to compare them with very similar normal cells to see if there is reasonable discrimination

(i.e., check for the 'shoe polish effect'). Of course it is not always clear which normal cell is appropriate and it can be useful to use several different types.

(f) Many drugs that force Cer accumulation do so weakly, yet have the advantage of being quite nontoxic. They should be tested together with C_6-Cer or at least one active generator of Cer to see if there is useful synergy. Including an inhibitor of ceramidase is also informative. A popular inhibitor of Cer synthesis, fumonisin B1, is risky because of its many effects.

(g) Research results based on semisynthetic SM are questionable since almost all preparations were made by acylating partially racemized sphingosylphosphorylcholine.

(h) The use of lipoidal drugs always raises the problem: how does one add them to aqueous incubations? Many articles do not even mention the method used by the authors. Dispersing the drug in a detergent raises the hackles of researchers, for fear of damage to cell walls. Usually the drug is dissolved in EtOH or Me_2SO and a control incubation is included to show that the solvent, *alone*, has no noticeable effect. However there is the rarely considered possibility in such experiments—might the solvent affect the performance of the drug being tested? Me_2SO is a mild oxidant and reducing agent, and EtOH can be oxidized to the indiscriminate inhibitor, acetaldehyde. EtOH itself exerts many effects, attested to by the literature on alcoholism; one effect is induction of increased neutral SMase and apoptosis.[149] Thus ROS formed by the drug may react with the solvent. Comparing two different solvents in separate incubations might help validate the results.

In general, a mixture of solvents is more effective than a single solvent in preparing a concentrated lipid solution, so less solvent can be added to the culture dish. Isopropyl alcohol is usually a better and less toxic solvent than EtOH (and is uncontaminated with acetaldehyde), so it is preferable to the more common solvents. As mentioned above, dodecane is a good diluent for a polar solvent, and I recommend a mixture with isopropyl alcohol. It should be kept in mind that adding a drug in an organic solvent to a stock bottle of medium is risky because the diluted drug may well precipitate and/or adsorb onto the container walls. It is helpful to observe the diluted drug under a bright high-intensity light beam or centrifuge a sample of diluted drug in a glass tube and see if it is still in the supernatant medium.

Using a detergent or complexing agent (a cyclodextrin or bovine serum albumin) to add a lipid to the cell medium can extract lipid from the cell surface, particularly cholesterol. Such a lipid depletion will liberate surface SM and render it easier to hydrolyze. After such an experiment one should analyze the medium for cellular lipids.

It is also possible for a lipoidal drug, after addition to a culture dish, to adsorb onto the dish surface. This can be checked by analyzing the medium ± cells after a period of time to see if all the drug is present (or wash the dish with a good organic solvent and look for the drug in the wash). PDMP, the inhibitor of several sphingolipid enzymes, was found to adsorb onto the plastic dish.[64] Cells, when present, competed with the plastic for uptake. The adsorption was much greater when a more lipoidal homologue was used. This variable makes it difficult to compare different drugs in cell culture experiments.

(i) It is surely time for authors whose new drug produces apoptosis to analyze their cells for Cer, ROS, and GSH content. If an increase in Cer is found, it points to the need to assay other sphingolipids too.

(j, finally) Authors who describe shorter-chain or longer-chain lipids should refer to them as homologues, not analogues. A small but neglected rule.

Considerations in the 'Final' Design

After examining the above kinds of information—and disregarding thousands more publications—I propose the following:

1. The unusual 'Yin-Yang' nature of Cer (its ability to produce apoptosis vs the ability of its metabolites to produce proliferation) seems to place it at the crux of cancer control. Thus cancer therapy should control the level of Cer in tumors.

2. An effective drug should behave like Cer with regard to its mechanism for generating apoptosis. The mechanism seems to involve its ability to interfere with mitochondrial electron transport, specifically ubiquinone metabolism. The mitochondria may oxidize Cer's allylic alcohol to an allylic ketone, the main inhibitor. GSH may interfere with the inhibition so lowering GSH levels should be a prominent effect of the drug.

3. The existence of many antineoplastic drugs that contain an essential allylic alcohol or allylic ketone or quinone group suggests that they act like Cer. Some drugs that lack such a grouping are activated by an oxygenase that forms an allylic alcohol moiety. The other groupings in the drugs evidently function to bring them to a specific kind of tumor (a tumor with many binding sites normally used by a hormone, cytokine, enzyme, metabolite, etc.) A surprising number of drugs exhibiting *some* kind of therapeutic value also possess the allylic feature-one need only scan through the Merck Index to see this-and many of these drugs have been found to have anticancer activity. However, few have been examined for Cer or ROS production, reactivity with GSH, and apoptotic activity. Some of these drugs, normally used as antibiotics, may function by blocking the ability of microorganisms to bind to susceptible tissues. Many microorganisms bind to tissue GSLs, so it is possible that these antibiotics slow the synthesis of glucosphingolipids as they act on Cer metabolism. If this is correct, the converse may apply: other drugs that slow GSL synthesis may have antibiotic activity.

4. Part of the Cer structure also brings it to the tumor's mitochondria, either by direct transport or by internal

synthesis. This proposal is based on the high metabolic activity of sphingolipids in cancer cells, the major role of GSH in tumor survival and SM hydrolysis, and the high susceptibility of tumors to drugs that elevate Cer levels. Mitochondria contain critical enzymes that control Cer. Presumably the alkyl chains in the sphingoid base and fatty acid are significant targeting moieties, as well as the amide linkage and 'extra' hydroxyl at C-1. The fatty acyl group is attached very close to the two OH groups. It may be relevant that some of the allylic cancer drugs also contain an 'extra' oxygen atom attached to the allylic ethylene group, either as an OH or phenol ester (as in benzoflavones). It is possible to add an OH or oxirane group to the fatty acylamino chain of ceramides, at C-2 or C-3. 2-Hydroxytetracosanoic acid occurs normally in sphingolipids, especially in GalCer of myelin (the membrane wrapped around nerve axons). A tertiary amine group close to the allylic ketone seems to improve anticancer activity.

5. In order for a drug to enter mitochondria, the molecular weight should not be too high. It may be possible to enhance a drug's entry into mitochondria by attaching an appropriate 'targeting signal,' the kind of peptide sequence that brings proteins to mitochondria.[150]

6. Conjugation of the allylic double bond or the allylic ketone with several double bonds or aromatic rings seems to help apoptogenic activity. This may sensitize the OH group to oxidation in the mitochondria.

7. The polyunsaturated fatty acids, like linoleic and arachidonic, normally react with oxygen to form allylic hydroperoxides and allylic alcohol groups along the chain. Although these compounds ought to exert anticancer action and generate Cer, they seem to stimulate cell proliferation and counteract the apoptogenic action of Cer. For example, oxidized lipoprotein can stimulate GSL synthesis in aortic endothelial cells.[20] Perhaps they do stimulate Cer synthesis but also its conversion to GSLs. These long-chain linear allylic alcohols may block apoptosis by competing with the oxidation of Cer in the mitochondria. Thus an antineoplastic drug should probably not contain the methylene-interrupted double bond sequence (and polyunsaturated fatty foods should be avoided). This kind of fatty acid hydroxylation is blocked by NSAIDS, such as celecoxib, which help prevent appearance of cancer cells and, in functioning tumors, produce apoptosis. Conversely, the toxic effects of NSAIDS might be blocked by Cer.

8. Many anticancer drugs are designed to counter some specific abnormality in the level of a tumor component, such as an enzyme (normal or mutated). While this has obviously produced some benefits for patients, I think the abnormality is simply a reflection of the high mutation rate in tumors. The mutation is observed because of its survival value to the tumor, such as a mutation that keeps the GSH level high and thus blocks Cer formation from SM. An example of this is the finding that nearly all prostate cancer cells lack the enzyme, pi-class glutathione *S*-transferase. This enzyme, which combines GSH with various drugs, normally acts to lower GSH levels and thus produce enough Cer from SM to slow cell proliferation.[151,152] I urge researchers trying this approach to analyze the tumor's sphingolipids before and after the treatment.

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Norman Radin was born 7/20/20 in New York City. BA from Columbia College 1941, PhD in Biochemistry, Columbia University, 1949. Asst./Assoc. Prof., Northwestern Univ. Medical School 1952–1960. University of Michigan: Research Biochemist, Mental Health Research Inst., 1960–1991; Director of Training, 1960–1978; Prof. Neurochem. in Psychiatry, 1984–1991; Prof. Emeritus, 1992–present. Member of Physiological Chemistry Study Section, NIH, 1965–1969; Mental Retardation Research and Training Committee, Natl. Inst. Child Health and Human Development, 1970–1974. Executive Editor, *Analytical Biochemistry,* 1980–1985. NIH Sen. Jacob Javits Neuroscience Investigative Awardee, 1984–1991. Research interests: development of radioisotope methods (Hyamine hydroxide, synthesis of labeled metabolites), lipid analysis methods (chromatography, enzyme assays), large scale sphingolipid isolation procedures, characterization of sphingolipid metabolic pathways, design of inhibitors of these pathways (PDMP, PPMP, PPPP), chemotherapy via alternative enzyme pathways and 'metabolic balance control' (urea cycle, Gaucher disease, sphingolipid disorders, cancer).