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A review of ceramide analogs as potential anticancer agents

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Summary

Ceramide serves as a central mediator in sphingolipid metabolism and signaling pathways, regulating many fundamental cellular responses. It is referred to as a “tumor suppressor lipid”, since it powerfully potentiates signaling events which drive apoptosis, cell cycle arrest, and autophagic responses. In the typical cancer cell, ceramide levels and signaling are usually suppressed by over-expression of ceramide-metabolizing enzymes or down-regulation of ceramide-generating enzymes. However, chemotherapeutic drugs as well as radiotherapy increase intracellular ceramide levels while exogenously treating cancer cells with short-chain ceramides leads to anti-cancer effects. All evidence currently points to the fact that the up-regulation of ceramide level is a promising anti-cancer target. In this review, we exhibited a full scroll of anti-cancer ceramide analogs as down-stream receptor agonists and ceramide metabolizing enzyme inhibitors.

Keywords

Ceramide; Sphingosine; Sphingolipid signaling pathway; Ceramidase; Glucosylceramide synthase; Anticancer agents; Enzyme inhibitors

Anti-cancer targets in the ceramide signaling pathway

Ceramide signaling pathway

There are two major pathways known to trigger the generation of ceramide. In the sphingomyelinase pathway, hydrolysis of plasma membrane sphingomyelin or lysosomal sphingomyelin by sphingomyelinases produces ceramide and phosphorylcholine. Exposure of cells to stress factors, such as pro-inflammatory cytokines, oxidative and nitrosative stress, UV- and γ-irradiation, or chemotherapeutic agents has been shown to rapidly activate sphingomyelinase activity, resulting in increased ceramide formation [1].

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The *de novo* synthesis of ceramide begins with the condensation of palmitate and serine by serine palmitoyltransferase to yield 3-keto-dihydrosphingosine, which is, in turn, reduced to dihydrosphingosine, and acylated by dihydroceramide synthase to produce dihydroceramide. Finally, desaturation of dihydroceramide by dihydroceramide desaturase generates ceramide. The *de novo* synthesis of ceramide occurs in the endoplasmic reticulum (ER), and the newly formed ceramide does not enter cytosol immediately. On the contrary, it is subsequently transported to the Golgi apparatus by either vesicular trafficking or the ceramide transfer protein CERT. Once in the Golgi, ceramide is converted to sphingomyelin (SM) by sphingomyelin synthase on the luminal side of the Golgi, or to glucosylceramide (GluCer) by glucosylceramide synthase on the cytosolic surface of the Golgi, and in turn to the complex glycosphingolipids [2]. The *de novo* formation of ceramide can be induced by several factors including tumor necrosis factor-α, hypoxia, and certain chemotherapeutic agents [3, 4].

Ceramide can also be synthesized by re-utilizing free sphingosine that is derived from the degradation of glycosphingolipids and other complex sphingolipids. This recycling of sphingosine is termed the salvage pathway.

Ceramide and apoptosis

Whether through endogenous elevation of ceramide level or exogenous treatment with cell-permeable short-chain ceramides, a series of biological effects, denoted as apoptosis, cell cycle arrest, differentiation, and autophagy, can be triggered.

In the past two decades, numerous studies have focused on the ceramide-induced apoptosis pathway in the fields of physiology, biochemistry, pathophysiology, and pharmacology. A classic mitochondria-dependent apoptosis can be triggered by endogenous and exogenous ceramide signaling. An immediate downstream target of ceramide is known to be CAPPs (ceramide-activated Ser-Thr protein phosphatases), such as PP2A (protein phosphatase 2A). The effect of ceramide on CAPPs leads to inactivation of an anti-apoptotic kinase, AKT (serine/threonine-specific protein kinase or protein kinase B), through protein dephosphorylation [5]. Activation of PKCζ by ceramide has been implicated in the regulation of membrane potential, inhibition of AKT, and pro-apoptotic functions [6, 7]. JNK and p38 can also be activated by ceramide through apoptosis signal-regulating kinase 1 (ASK1). JNK and p38 increase the level of BAX, a pro-apoptotic protein in mitochondrial pathway, while p38 also contributes to the inactivation of AKT. Thus, the downgrading of AKT by several signals (PP2A, PKCζ, and p38) decreases the phosphorylation of BCL-2. At the same time, PP2A's catalytic subunit (PP2Ac) also inhibits BCL-2 phosphorylation, leading to increased p53/BCL-2 binding. Finally, downgraded BCL-2 levels and a suppressed BCL-2/BAX ratio results in intrinsic apoptosis [8]. Cathepsin D is considered as a specific target for lysosomally generated ceramide, and is involved in both mitochondria-dependent and independent apoptosis pathways [9]. Therefore, ceramide analogs can act on the ceramide downstream targets, imitating ceramide-like apoptosis.

The permeability of mitochondria is the critical factor inducing the release of apoptotic proteins, such as cytochrome *c*, in turn leading to apoptosis. Ceramide can directly induce mitochondrial outer membrane permeabilization (MOMP), which is a key event in apoptotic

signaling, through the formation of ceramide channels. This MOMP process is concomitant with and promoted by pro-apoptotic BAX translocation to mitochondria [10, 11]. This is why a group of positively charged ceramide analogs was developed to target ceramide-induced apoptosis [12, 13].

Ceramide metabolism and cancer

In vitro assays have shown that a short-chain ceramide molecule, C_6-Cer, modestly activates the catalytic subunit of PP2A (PP2Ac), an immediate ceramide downstream target. In contrast, 4–5 double-bond saturated analog, C_6-dhCer, inhibits PP2Ac. Further studies demonstrated the strict structural requirements for interaction of ceramide with PP2A (an amide group, a primary hydroxyl group, and a secondary hydroxyl group) [14]. However, many ceramide analogs that do not activate or even inhibit PP2A, do exhibit apoptotic activity in cancer cells (like D-*e*-MAPP and 3-keto-C_6-ceramide) [15, 16]. Thus, other agents inducing apoptosis in ceramide signaling pathways are expected to exist.

Multiple enzymes are directly involved in regulating intracellular ceramide concentrations. These include ceramide-generating enzymes, such as ceramide synthase, cerebrosidase, sphingomyelinase, and ceramide-metabolizing enzymes, such as ceramidase, glucosylceramide synthase, sphingomyelin synthase, and ceramide kinase. Inhibition of glycosphingolipid biosynthesis or ceramide degradation gives rise to an elevation of endogenous ceramide level. Among these enzymes ceramidase (CDase) and glucosylceramide synthase (GCS) play important roles in regulating ceramide levels.

Ceramidase—Ceramidases hydrolyze the amide bond of ceramide producing sphingosine (Sph) and a fatty acid. According to their optimum activity pH, ceramidases are classified into acid [17], neutral [18, 19], and alkaline ceramidases [20]. They modulate the intracellular ceramide levels, especially regulating the lysosomal pool of ceramide (by acid CDase). A large amount of evidence supports the notion that CDase inhibitors serve as potential ceramide-increasing agents useful for cancer chemotherapy. Ceramide analogs such as B13 [21, 22] and AD2646 [23] can antagonize ceramidases, exhibiting interesting activities in cancer chemotherapy and have been widely used in research on ceramide signaling pathways.

Glucosylceramide synthase—Glucosylceramide synthase (GCS), also called UDP-glucose ceramide glucosyltransferase, is another important ceramide metabolizing enzyme. GCS, located on the cytosolic surface of the Golgi apparatus, catalyzes the conversion of ceramide into glucosylceramide [24]. Generally, up-regulation of GCS levels prevents the accumulation of a ceramide pool, which reduces ceramide-induced apoptosis in response to certain cytotoxic drugs [25–27]. On the other hand, evidence also shows a close relationship between GCS and P-glycoprotein (P-gp, an important multidrug-resistance protein). Overexpression of P-gp in resistant cancer cells is concomitant with high GCS expression [28, 29]. GCS inhibitor treatment in multidrug-resistant cancer cells down-regulates the expression of MDR1 (P-gp-encoding gene) [30]. Furthermore, drug-resistant cancer cells exposed to GCS inhibitors become sensitive to anticancer agents [30–32]. These results strengthen the notion that inhibition of GCS is a promising therapeutic strategy for

combating multidrug-resistance, thus, promoting the design of ceramide analogs as GCS inhibitors.

Ceramide kinase and ceramide-1-phospate—Phosphorylation of ceramide by the ceramide kinase (CerK) produces Ceramide-1-phospate (C1P) which plays an opposing physiological role compared to ceramide. C1P is a potent inhibitor of apoptosis, which blocks apoptosis through inhibition of acid sphingomyelinase and serine palmitoyltransferase in macrophages [33, 34]. C1P also stimulates cell proliferation through activation of the PI3- kinase/PKB, JNK and ERK1/2 pathways [35, 36]. Therefore, blocking ceramide kinase to decrease the generation of C1P is the third anti-cancer target of ceramide analogs as enzyme inhibitors.

Sphingosine kinase (SK), sphingosine-1-phospate (S1P), and S1P receptors

Sphingosine, the metabolite of ceramide generated by ceramidase, can be phosphorylated by sphingosine kinase (SK) to yield sphingosine-1-phospate (S1P), another important signaling molecule in the ceramide pathway. Sphingosine-1-phosphate (S1P) is implicated in many critical cellular processes. However, unlike ceramide, it promotes cell survival, proliferation, and migration, as well as angiogenesis and allergic responses [2].

S1P mediates cell survival through different pathways, such as an intracellular signaling mechanism (for example, regulation of BCL-2 family members) and an S1P receptor-dependent mechanism, referred to as “inside-out” signaling. In the former pathway, S1P can negatively mediate apoptosis by up-regulating the expression of anti-apoptotic proteins such as BCL-2 [37], while down-regulating the pro-apoptotic proteins BAX [37, 38]. It was also observed that exogenous S1P blocks the translocation of BAX to the mitochondria, which enhances the stability of mitochondrial membranes [39]. Furthermore, up-regulated expression of BCL-2 family members can increase SK1 (sphingosine kinase 1) levels, which in turn generates more S1P from sphingosine, resulting in S1P signal amplification [40, 41].

In the S1P receptor-dependent mechanism, S1P is secreted from the inside to the outside by the members of the ATP-binding cassette (ABC) family transporters [42], and binds to and signals through S1P receptors in an autocrine manner. To date, five S1P receptors have been identified, which are specific G protein-coupled receptors (GPCRs), designated as $S1P_{1–5}$. Through these receptors, S1P regulates different cellular processes. The functions of S1P receptors are under investigation. Some results support that $S1P_1$ and $S1P_3$ mediate cell proliferation [43], migration [44, 45], invasion [46, 47], and angiogenesis [48].

S1P is interconvertible with ceramide, which is a negative mediator of apoptosis. It has been postulated that the ratio between S1P and ceramide determines cell fate. Targeting the conversion of ceramide to sphingosine 1-phosphate is a novel strategy for cancer therapy. Since phosphorylation of sphingosine by sphingosine kinase (SK) is the sole known source of S1P, SK inhibitors are promising cancer chemotherapeutics.

As described above, two main approaches to promote anti-cancer activity in the ceramide-sphingosine-S1P axis have been identified as 1) use of an exogenous supplement of ceramide to promote apoptosis, and 2) inhibition of ceramide metabolizing enzymes to

regulate the ceramide/S1P rheostat. Since both of these can be achieved by employing ceramide analogs (mimics or derivatives), these types of compounds have attracted a lot of attention in the past few years. Herein, we will review each step to discovery and development of anti-cancer ceramide analogs.

Development of ceramide analogs

Structural characteristics of ceramide

Ceramide molecules contain a sphingoid long-chain base (sphingosine) backbone, linked to a fatty acid molecule through an amide bond. (Figure 2) In earlier studies, the importance of the 4,5-trans-double bond was emphasized, and treated as the mark of sphingosine backbone. This conclusion was based on the notion that targeting ceramide-downstream effectors is the sole purpose to design ceramide analogs. With the development of ceramide-metabolizing enzyme inhibitors, many non-4,5-double bond agents such as S18 [49], PDMP [50], and FTY720 were designed [51]. To date, the 2-amino-1,3-propanediol or 2-amino-propanol moieties are considered as common characteristics of ceramide analogs. These structural backbones are marked in red throughout this review.

In order to increase water-solubility, the long-chain fatty acid of native ceramide structure is often replaced by short-chain fatty acids (acetic, hexanoic and octanoic acids) to form C_2-, C_6-, and C_8-ceramides (C_2-, C_6-, and C_8-Cer). Exposure of cells to short-chain ceramides has lead to ceramide-like anti-cancer activity in many preclinical studies [5, 52–55]. Thus, in most studies short-chain ceramides, but not the naturally occurring ceramides, have been used as research tools.

Steorespecific apoptotic activity of short-chain ceramides

Since two chiral centers exist in the ceramide molecule, the natural D-*erythro*-ceramide has three stereoisomers, denoted as L-*erythro*-ceramide, D-*threo*-ceramide, and L-*threo*-ceramide. To investigate the relationship between stereochemistry and activity, four C_2-Cer isomers and four C_2-dihydroceramide (C_2-dhCer) isomers were synthesized and evaluated in leukemia cells [56]. (Figure 3) The four isomers of C_2-Cer were active in inhibition of cell growth and induction of apoptosis with modest differences in potency (ranking as follows: L-*threo*-C_2-Cer > D-*erythro*-C_2-Cer = L-*erythro*-C_2-Cer > D-*threo*-C_2-Cer). This observation suggested that the non-natural stereoisomer L-*threo*-C_2-Cer was more pro-apoptotic than the natural stereoisomer D-*erythro*-C_2-Cer. On the other hand, with C_2-dihydroceramide (C_2-dhCer) only the *threo* compounds were active in these assays, whereas the *erythro* compounds were completely inactive. Thus, lack of the 4,5-double bond lead to inactivity in *erythro*-C_2-dhCer, but not in *threo*-C_2-dhCer. This systematic study provided a basic view of the relationship between ceramide stereoisomers and pro-apoptotic activities. Stereospecific activity is a common feature for ceramide analogs, suggesting that the targets of ceramide analogs also own specific spatial configurations. A large number of ceramide analog studies involve the investigation of stereostructure-activity relationships [14, 57–62].

Discovery and development of B13 (inducing apoptosis and inhibiting ceramidase)

In 1992, a group of phenyl modified ceramide analogs (L-MAPPD, D-NMAPPD, D-e-MAPP), was reported [57]. (Figure 4) The idea of this design approach might have been derived from a previous modification of GCS inhibitors (discussed below) [63]. In the original paper, this group of analogs showed ceramide-like anti-proliferation activity in human myelocytic leukemia HL-60 cells. Very interestingly, this activity had stereospecific requirements. The L-isomer L-*e*-MAPP was found to be inactive, while D*e*-MAPP was soon reported to be a neutral ceramidase inhibitor *in vitro* and induced a concentration-and time-dependent growth suppression accompanied by an arrest in the G_0/G_1 phase of the cell cycle [15]. In that paper, the explanation provided for the inactivity of L-*e*-MAPP was that L-*e*-MAPP is metabolized by alkaline ceramidase to an extent similar to that seen with C_{16}-ceramide. However, years later, D-NMAPPD (later named as B13, Figure 4) was found to be an acid ceramidase inhibitor, and induce apoptosis in several cancer cell lines [21, 22, 64]. Thus, D-*e*-MAPP and D-NMAPPD (B13) not only up-regulated ceramide levels in cultured cells but also potently induced apoptosis [1].

Almost at the same time, ceramide's amide group proved to be not required for apoptosis, and the replacement of the carbonyl group by methylene group substantially decreased the time required for cells to die, with maximum DNA fragmentation occurring at 6 h as opposed to the 18 h required by D-*erythro*-C_8-Cer [58]. The most potent compound (D-*threo*-C_8-ceramine) in this group is shown in Figure 4. According to the structural features of B13 (D-NMAPPD) and D-*threo*-C_8-ceramine, a group of ceramide analogs, including AD2646 (its hydrochloric salt was called LCL-204 by another group, Figure 4) AD2672 and AD2687, was created [65]. When these compounds were applied to HL-60 cells, they inhibited the biosynthesis of sphingomyelin (SM) and glycosphingolipids and induced apoptosis. AD2687 (trimethyl sphingosine analog) induced cell death at lower concentrations. An investigation of mechanisms involved found that these compounds were able to kill leukemic cells through distinct pathways implicating caspase activation and mitochondrial events [23, 66].

Structural optimization of B13-like ceramides has been ongoing. The newest B13-like ceramide analogs are LCL-464 [67, 68] and KPB-27 [69], which showed potent inhibition of ceramidase as well as ceramide-like anti-cancer activities.

Glucosylceramide synthase (GCS) inhibitors (PDMP family)

The investigation of glucosylceramide synthase Inhibitors was initiated prior to that of ceramidase inhibitors. The original purpose of these studies was to treat Gaucher's Disease, a genetic lack of adequate β-glucosidase activity [63]. The precursors of this family of compounds are N-decanoyl-2-amino-3-hydroxypropiophenone (RV-49, a 3-ketone-4-phenyl-ceramide) and N-acyl-norephedrine (a 1-dehydroxy-4-phenyl-ceramide). (Figure 5) Using RV-49 and N-acyl-norephedrine, 2-decanoylaminopropiophenone was yielded with a higher inhibition potency for glucosylceramide synthase than the lead compounds. Introducing a morphorlinyl group into the 1-position produced a non-competitive GCS inhibitor, RV-378 (Figure 5). It inactivated the enzyme, probably by covalent reaction with the enzyme's active site. Reduction of RV-378 at 3-ketone formed the more potent analog

RV-583 that is a competitive GCS inhibitor [63, 70]. When discovered, RV-583 was originally a mixture of four stereoisomers. After determination of each isomer, RV-583 got a new name PDMP (Figure 5). It was found that only the D-*threo*-PDMP (1S,2R) is active against GCS [71]. Shortly after, it was observed that D-*threo*-PDMP caused growth inhibition and ceramide accumulation in cultured rabbit skin fibroblasts [72]. Replacement of the morphorlinyl group of RV-583 by a pyrrolidinyl group produced a pair of *erythro*- and *threo*- isomers (BML-129 and BML-130). Both of these compounds showed very effective growth inhibition of several kinds of cancer cells [73]. This was the first time that GCS inhibitors were linked to anti-cancer activities. Moreover, only *threo*-isomer BML-130 inhibited GCS in MDCK cell homogenates, while *erythro*-isomer BML-129 did not. Then, PPMP, PPPP, and 4′-hydroxy-P4 were prepared based on the structural modification of D-*threo*-PDMP and BML-129/130 [50, 74, 75], leading to the discovery of the most potent GCS inhibitor, 4′-hydroxy-P4, at the end of the 20th century. In 2006, a group of PDMP-family analogs were reported by Hillaert et al. Among these, compounds 16c and 18 (Figure 5) were derived from PPPP with the replacement of backbone phenyl group, and showed a comparable inhibitory potency as PDMP [76]. The newest PDMP-family member is CCG-20358 (Figure 5), with a bulky N-acyl group to increase the rigidity of the whole molecule. This compound (CCG-20358) inhibited GCS in the nM range, and in an *in vivo* assay in mice led to a dose-dependent decrease in brain glucosylceramide contents by intraperitoneal injection [77].

Derivatives of sphingosine as sphingosine kinase (SK) inhibitors

DHS and DMS—As a sphingosine derivative, D,L-*threo*-dihydrosphingosine (DHS or safingol, Figure 6) was the first identified sphingosine kinase (SK) inhibitor. DHS was shown to be a competitive inhibitor for SK1, and a substrate for SK2 [61]. Shortly after, N,N-dimethylsphingosine (DMS) was proved to be more potent than DHS [78], and served as a competitive inhibitor for both SK1 and SK2 [79, 80]. DMS induces apoptosis in both human epidermoid carcinoma KB-3-1 and its multidrug-resistant (MDR) subclone KB-C2 cells *in vitro* [81]. Human colonic carcinoma cell lines HT29, HRTI8, MKN74, and COLO205 were shown to be more susceptible to apoptosis upon addition of DMS, with potency comparable to C_2-Cer [82]. DHS and DMS have since been intensively used in studies involving ceramide metabolism. However, neither DHS nor DMS were a specific sphingosine kinase inhibitor, since they also inhibited protein kinase C [83] and ceramide kinase [84].

FTY720 (Fingolimod)—When the long-chain hydrophobic group of sphingosine was shifted from the 3-position to the 2-position, a new group of sphingosine derivatives, 2-substituted 2-amino-1,3-propanediols, was developed. Among these analogs, FTY720 (2-amino-2-(2-(4-octylphenyl)ethyl)-1,3-propanediol hydrochloride, Figure 6) has been the most investigated. This compound is known for its immunosuppressive properties. However, FTY720 is also able to induce growth arrest and apoptosis of various cancer cell lines, including human prostate cancer cells [51], bladder cancer cells [85], renal cancer cells [86], pancreatic cancer cells [87], breast cancer cells, colon cancer cells [88], and brain tumor stem cells [89]. The *in vivo* assays showed that FTY720 inhibited tumor growth and metastasis [90], and reduced tumor vascularization and angiogenesis [91]. The pro-apoptotic

effect of FTY720 was shown to be associated with mitochondria-dependent activation [51, 92] as well as inhibition of SK1 [93]. It is very interesting that phosphorylation of FTY720 by SK2 produces a biologically active molecule, FTY720-phosphate (FTY720-P) [94, 95] that could bind to and activate four of the five S1P receptors, $S1P_{1,3–5}$ [96]. Although FTY720-P is an $S1P_1$ receptor agonist, it leads to endocytosis and proteasomal degradation of the $S1P_1$ receptor in T lymphocytes [97], thereby preventing their egress from lymph nodes [97]. It is unclear whether the anti- tumor activity of FTY720 is partly from down-regulating $S1P_1$ receptor. However, the structural optimization of FTY720 is in progress [98].

ROME—Since FTY720 is a substrate of SK2, it was O-mono-methylated, forming (S) and (R)-FTY720-OMe (ROME) to avoid metabolism by SK2 (Figure 6). The inhibitory activity of ROME was stereospecific. (R)-FTY720-OMe specifically inhibited SK2, and not SK1, while (S)-FTY720-OMe failed to inhibit SK2. Prolonged treatment of HEK 293 cells with (R)-FTY720-OMe induced a reduction in SK2 expression and inhibited DNA synthesis in HEK 293 cells [60]. Treatment of MCF-7 cells with ROME prevented actin enrichment into lamellipodia in response to S1P, suggesting that metastasis could be inhibited. As a SK2-selective inhibitor, (R)-FTY720 methyl ether increased sphingosine and decreased S1P levels, but had no effect on ceramide levels and did not induce apoptosis in LNCaP cells [99].

KRP-203—Recently, a FTY720-like immunosuppressant, KRP-203 (Figure 6) has been reported to be a $S1P_1$ receptor-selective agonist in contrast to FTY720 [100]. Both of the compounds had a similar high affinity for the $S1P_1$ receptor with an ED_{50} in the nM range. However, for $S1P_3$ the FTY720-P displayed an ED_{50} of 1.74 nM whereas KRP-203-P had an ED_{50} of>1 mM [101]. KRP-203 proved to not only regulate T-cell responses but also those of B cell. The anticancer activity of KRP-203 is under investigation.

S16, S18, and T18—In contrast to FTY720, modification of 2-amino-1,3-propanediol on the amino group produced amidated 2-amino-1,3-propanediol derivatives (S16, B16, S18, and T18, Figure, 6). S16 was the first compound of this group. Incubation of neuroblastoma cells with C_{16}-serinol (S16) increased the concentration of endogenous ceramide by 50–80% and caused apoptosis in rapidly dividing low-density cells but not in confluent cells. Further studies indicated that apoptosis in neuroblastoma cells induced by C_{16}-serinol was at least partially mediated by activation of PKCζ, an important regulator in ceramide-induced apoptosis pathway (shown in Figure 1) [102]. Structural optimization of S16 created a new generation 2-amino-1,3-propanediol, S18 (N-(2-hydroxy-1-(hydroxymethyl)ethyl)-oleoylamide, Figure 6), which induced apoptosis in a variety of neuronal and non-neuronal cancer lines. S18 triggered pro-apoptotic signaling pathways without prior elevation of endogenous ceramide, suggesting that it probably replaced ceramide to activate downstream effectors [49]. However, unfortunately the SK inhibition activity of this group has not been investigated.

Other SK inhibitors, such as SKI I,II,V [103], B-5354 [104], F-12509A, and S-15183a,b [104] do not belong to the ceramide structure system, and they are not discussed here.

Multifarious modifications in the recent decade

Over the past ten years, there have been a variety of approaches to the design of new ceramide analogs. According to the modification sites, these design approaches can be classified into 1) sphingosine backbone modification, 2) N-terminal side chain modification, and 3) mixed structural modification.

It is known that the sphingosine backbone is more important than the N-terminal side-chain for the pro-apoptotic activity of ceramide. The purpose of backbone optimization is to discover a better pharmacophore than 3-hydroxy-4-ene (the main active moiety of ceramide), which can more effectively activate ceramide downstream effectors, inducing apoptosis. On the basis of the reported results, both the 3-hydroxy and the 4,5- double bond are replaceable. A relatively rigid structure with a conjugated system (such as a conjugated diene, an enone, or an aromatic ring) in the sphingosine backbone improves the pro-apoptotic activities [16, 64, 105]. The following table provides information on a number of ceramide analogs and their activities.

Unlike the backbone modification, the N-terminal side-chain modification focuses on the metabolic properties of ceramide, such as water/lipid solubility [118]. Modifying the N-terminal amide group could avoid ceramide analog metabolism by ceramidase [59, 119] or inhibit ceramidase metabolizing endogenous ceramide [120]. A major success of N-terminal side-chain modification was the discovery of positively charged ceramide analogs (LCL124, LCL29, and LCL30, etc) [118]. These analogs own a pyridinium functional group, which dramatically increases the water-solubility of the whole molecule. Furthermore these positively charged ceramide analogs can target negatively charged intracellular compartments, and accumulate mainly in mitochondria-, and nuclei-enriched fractions, in turn increasing mitochondrial membrane permeability and triggering the mitochondrial apoptosis cascade. These compounds are displayed in table 2 with other N-terminal side-chain modified ceramides.

The mixed modification on both chains of ceramide has been reported only in the past couple of years. However, the limited information available points to the fact that 1-position modification highly correlates with the inhibition of sphingosine kinase (SK) and glycosylceramide synthase (GCS).

Future Perspective

To date, thousands of potential anti-cancer ceramide analogs have been synthesized and evaluated aiming at the diverse targets in the ceramide signaling pathway. Since ceramide is in the center of sphingolipid metabolism, its analogs are intrinsically involved in multiple mechanisms of action. Sometimes, multiple mechanisms point in the same direction, and we can obtain multi-action anticancer agents. However, most often, selective agents are more desirable. As in the case of any other anti-cancer agents, improving the selectivity of the agent for a certain target over other targets, for a certain organ over other organs, and for tumor cells over normal cells, are the best approaches to develop new ceramide analogs. Thus, mechanism investigation is as important as activity screening for these chemicals and a multi-evaluation system around ceramide-related targets is desired. In recent years, the

crystal structures of some ceramide targets, such as sphingosine kinase [132], neutral ceramidase [133], and protein phosphatase 2A (PP2A) [134] have been published. These developments prompted a change in the approach to the design of ceramide analogs from ligand-based optimization to ligand/protein-based optimization, and accelerated the development of ceramide-based anti-cancer agents.

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Executive Summary

Anti-cancer target system surrounding ceramide

- Activating CAPPs (ceramide-activated Ser-Thr protein phosphatases)
- Inhibiting ceramidase, glycosylceramide synthase, and ceramide kinase
- Inhibiting sphingosine kinase and sphingosine-1-phospate receptor

Structure-activity relationship of anti-cancer ceramide analogs

- Stereospecific activity is a common feature for ceramide analogs
- Sphingosine backbone modification by rigid groups increasing apoptotic activity
- 1-Position modification leading to the inhibition of glucosylceramide synthase
- Introducing positively charged groups increases mitochondrial membrane permeability and triggers the mitochondrial apoptosis cascade

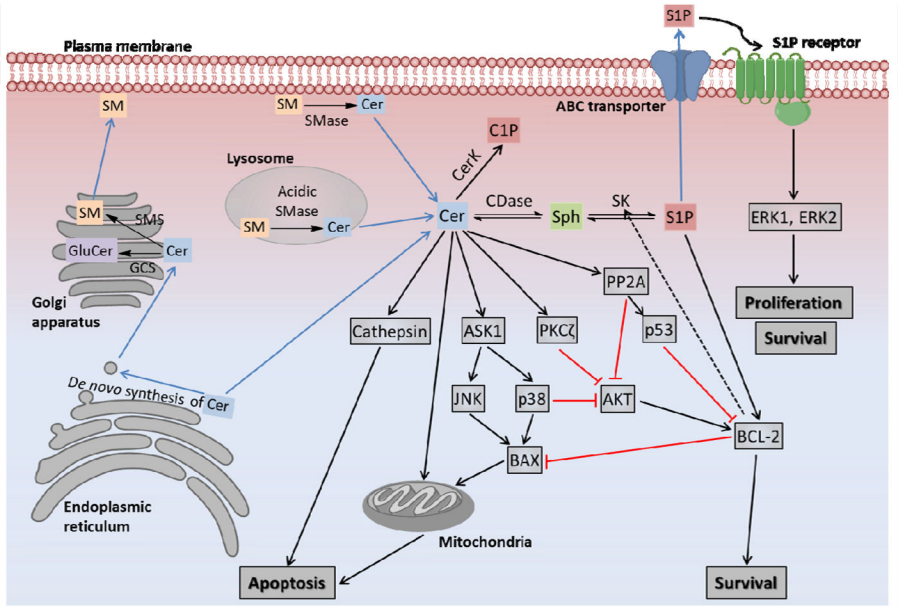


Figure 1.

Ceramide metabolism and signaling pathways and their anti-cancer targets. Cer, ceramide; CDase, ceramidase; Sph, sphingosine; SK, sphingosine kinase; S1P, sphingosine-1-phosphate; CerK, ceramide kinase; C1P, ceramide-1-phosphate; SMS, sphingomyelin synthase; SM, sphingomyelin; GCS, glucosylceramide synthase; GluCer, glucosylceramide; SMase, sphingomyelinase; ASK1, Apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; BAX, BCL-2–associated X protein; PP2A, protein phosphatase 2A; AKT, serine/threonine-specific protein kinase; BCL-2, B-cell lymphoma 2; ERK, extracellular-signal-regulated kinase; ABC transporter, ATP-binding cassette transporter.

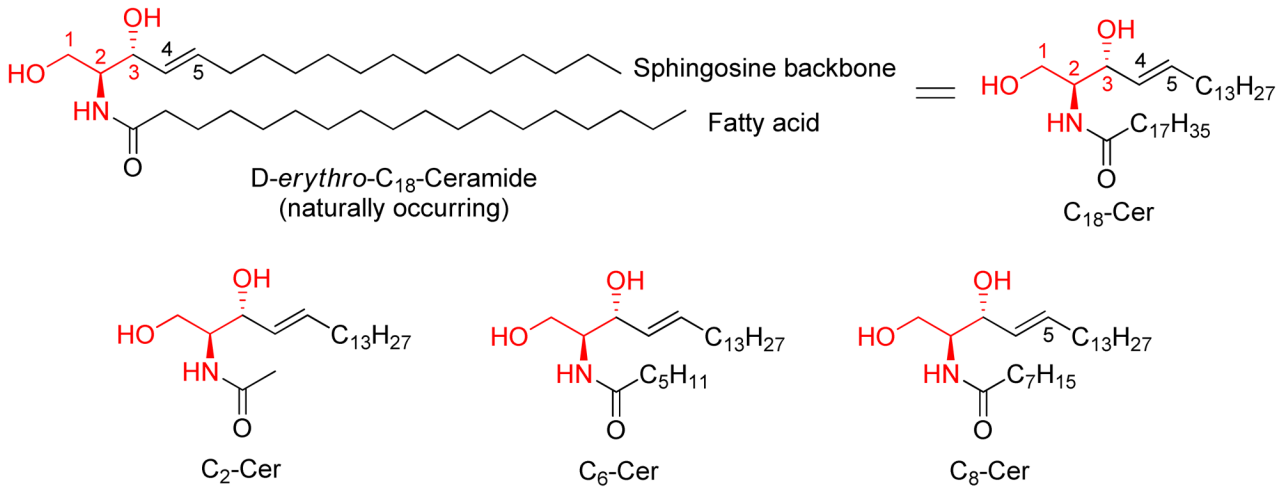


Figure 2.

Structures of the naturally occurring ceramide, D-*erythro*-C_{18}-ceramide (C_{18}-Cer), and synthetic short-chain ceramides, C_2-Cer, C_6-Cer, and C_8-Cer

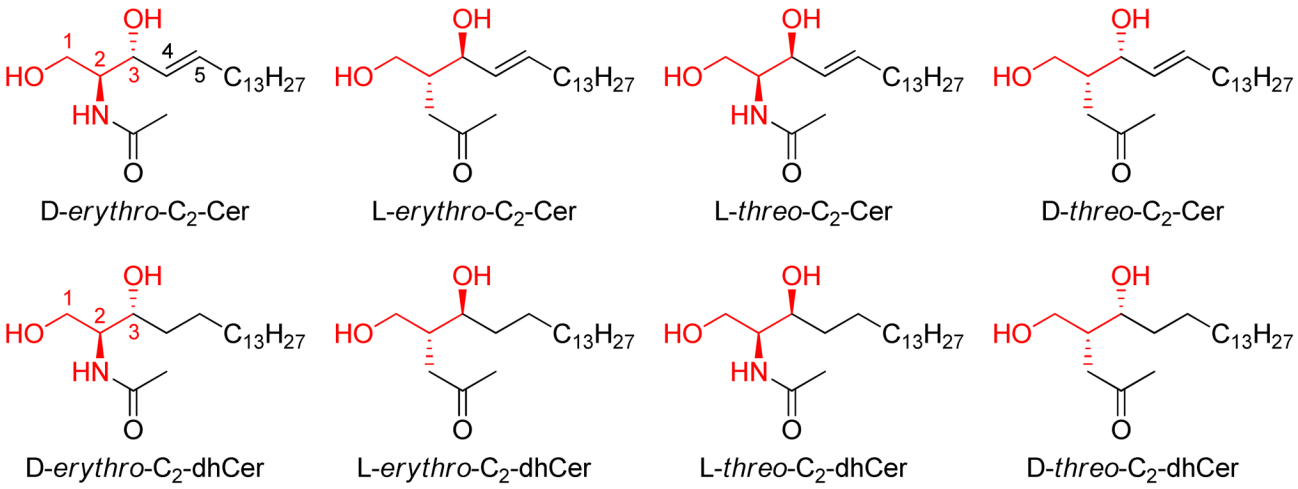


Figure 3.

Structures of the four stereoisomers of C_2-Cer and the four stereoisomers of C_2-dhCer studied by [56]

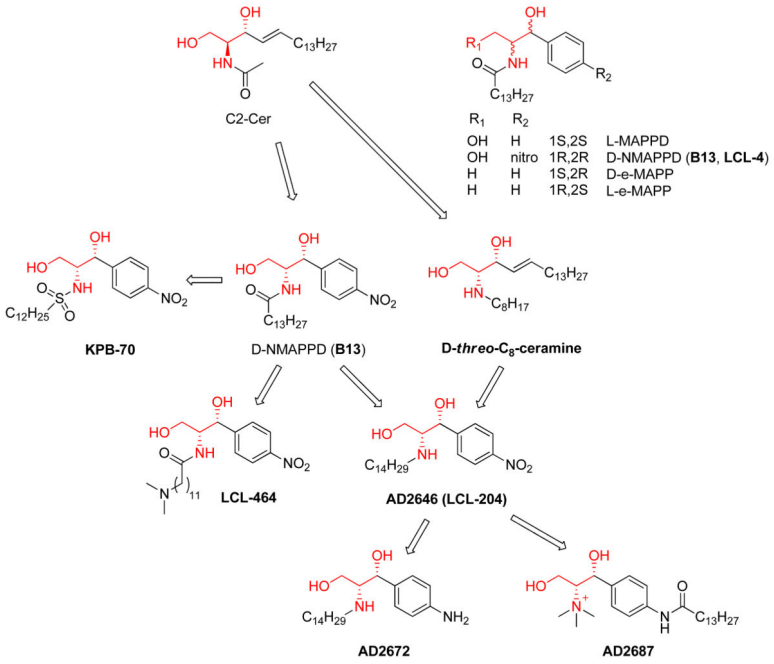


Figure 4.
The B13-family of ceramide analogs

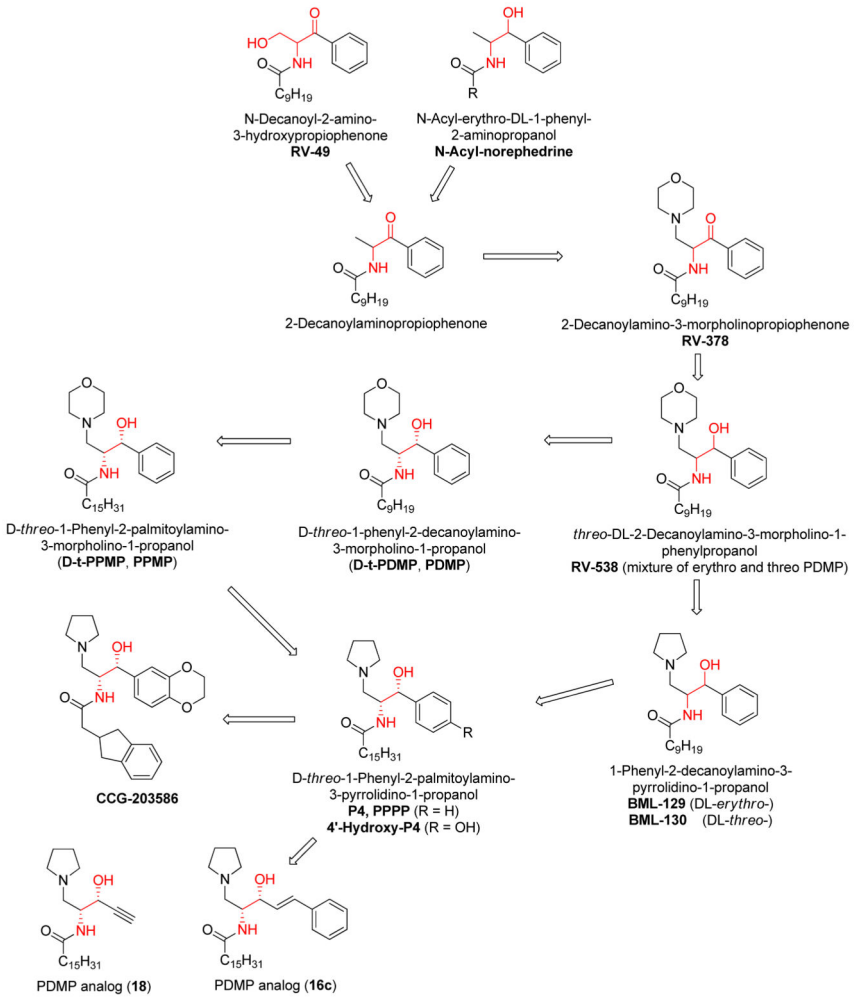


Figure 5.
Evolution of PDMP-family ceramide analogs (targeting glucosylceramide synthase, GCS)

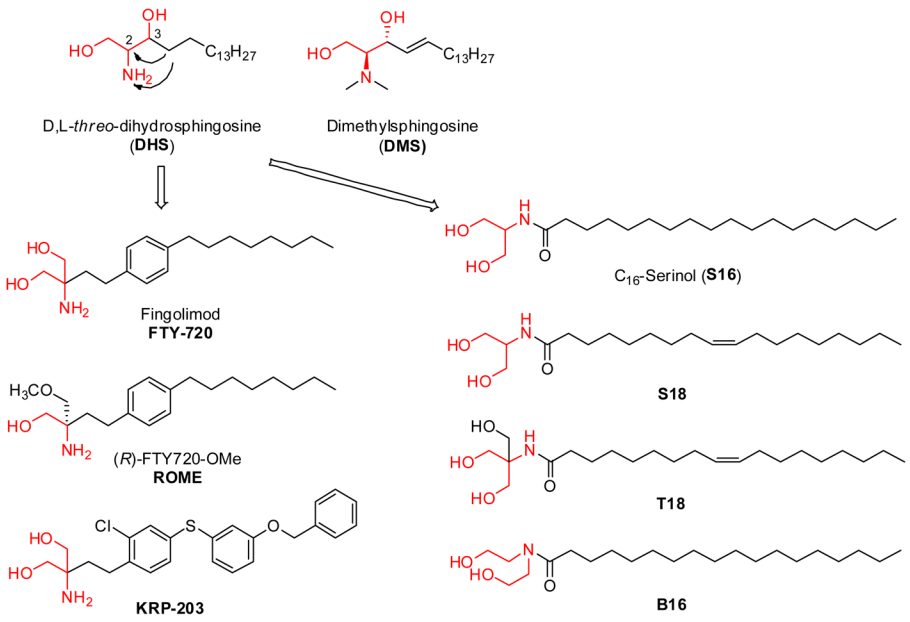
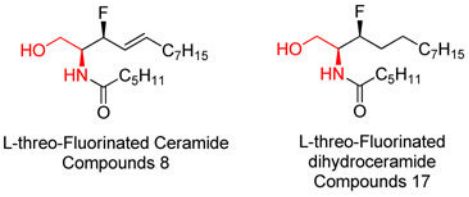
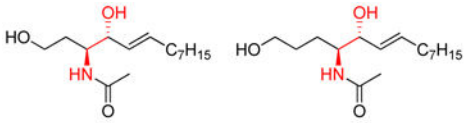
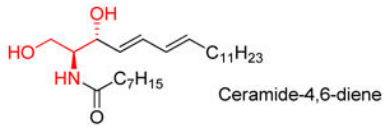
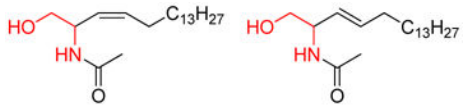
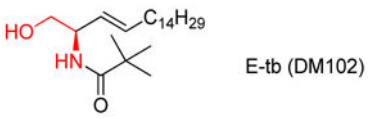
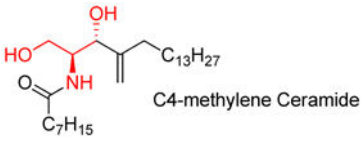


Figure 6.
Sphingosine-derivative sphingosine kinase inhibitors

Table 1

Ceramide backbone modification, structures, and activities

Name and structure	Activity	Reference
 L-threo-Fluorinated Ceramide Compounds 8 L-threo-Fluorinated dihydroceramide Compounds 17	• Compared to C_2-Cer, higher apoptogenic activity in human lymphoma cell lines, and a lower apoptogenic activity in T-lymphoblastoid cells. • Apoptogenic activities in peripheral blood lymphocytes are compatible with that of C_2-Cer.	[106]
 C2-Cer-1 C2-homo-ceramide C2-Cer-2 C2-bishomo-ceramide	• Low but considerable potency in comparison with C_2-Cer • Induction of apoptotic cell death in HL-60 human leukemia cells.	[107, 108]
 Ceramide-4,6-diene	• Induction of apoptosis by 8 h via the mitochondrial pathway • Higher potency than C8-Cer in TNFα-resistant (IC_{50} of 11.3 versus 32.9 μM) and TNFα-sensitive (IC_{50} of 13.7 versus 37.7 μM) MCF-7 cells. • More potent than C8-Cer in inducing cell death in MDA-MB-231 and NCI/ADR-RES breast cancer cell lines (IC_{50} of 3.7 versus 11.3 μM, and 24.1 versus 86.9 μM, respectively).	[105]
 RZ-2 (2R) SZ-2 (2S) RE-2 (2R) SE-2 (2S)	• Cis-isomers were the most potent than trans-isomers. • Cis-isomers possessing high and comparable apoptotic activities compared with C_2-Cer • Induction of apoptosis examined in HL-60 cells	[109]
 E-tb (DM102)	• Inhibition of acid CDase in AC10X cell lysates and intact AC10X cell (IC_{50}: 15 μM), no influence on neutral CDase • Inducing cell death on A549 cells with LD_{50} values nearly 40 μM • Inducing concentration-dependent cell cycle arrest at G(1) and 20–25% apoptosis/late apoptosis/necrosis after 24h incubation at 50 μM	[110]
 C4-methylene Ceramide	• Higher anti-proliferative activity on mouse embryonic fibroblast (MEF) cells than C_8-Cer	[111]

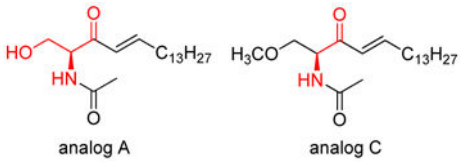
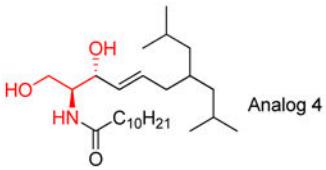
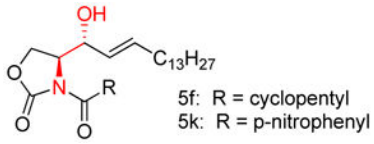
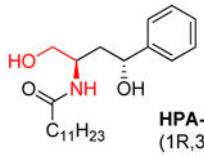
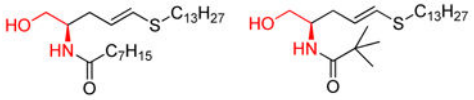
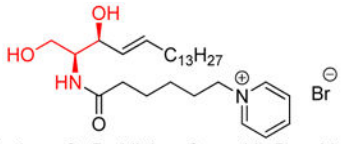
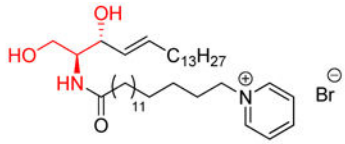
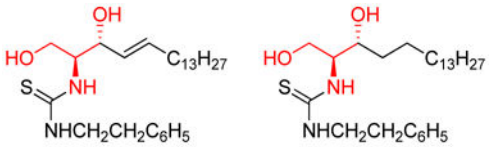
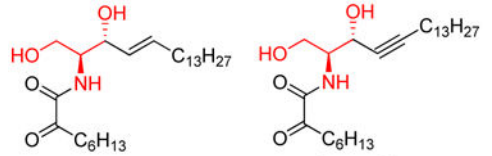
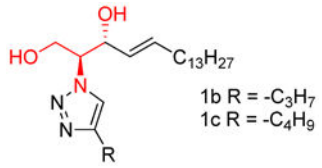
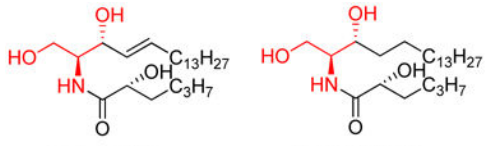
Name and structure	Activity	Reference
 analog A analog C	• Induction of mitochondrial apoptosis in HL-60 cell • More potent than C_2-Cer	[16]
 Analog 4	• Significantly high potent than C_2-Cer in inducting cell death in several cell lines, including MCF-7, MDA-MB-231, SKBR-3, and SupT1 (IC50: 12.30, 22.36, 10.15, and 15,45 μM, respectively) • The induction of apoptosis by Analog 4 in terms of Annexin V binding and DiOC6 labeling was superior to that achieved with C_2-Cer	[112]
 5f: R = cyclopentyl 5k: R = p-nitrophenyl	• Induction of cell death and DNA fragmentation in HL-60 cell • More potent than C_2-Cer	[113]
 **HPA-12** (1R,3R)	• Inhibiting ceramide trafficking from the ER to the site of SM synthesis, but not affecting ER-to-Golgi trafficking of protein	[114–116]
 **RBM2-1B** **RBM2-1D**	• Reducing cell viability in A549 (IC_{50}, ~ 20 μM) and HCT116 cells (IC_{50}, ~ 30 μM) • Inducing apoptotic cell death in HCT116 cells • Increase of dhCer, dhSM, Glu-dhCer levels, decreasing GluCer level, no obviously influence on Cer level in HCT116 cells	[117]

Table 2

Ceramide N-terminal side-chain modification, structures, and activities

Name and structure	Activity	Reference
 L-*threo*-C_6-Pyridinium Ceramide Bromide	• Inhibiting the growth of various human head and neck squamous cell carcinoma (HNSCC) cell lines at low IC_{50} concentration • Targeting negatively charged intracellular compartments, and accumulated mainly in mitochondria-, and nuclei-enriched fractions upon treatment of human UM-SCC-22A cells • increasing inner membrane permeability and triggered release of mitochondrial cytochrome c	[12, 118, 121]
 D-*erythro*-C_{16}-Pyridinium Ceramide Bromide	• Positively charged ceramide analog • highly cytotoxic to several cancer cell lines	[13, 122, 123]
 PEITC-So PEITC-Sa	• Cytotoxicity and chemomodulatory in the HL-60 • Inhibition of conventional and novel PKC isoforms	[124]
 α-ketoamides Compound 12 α-ketoamides Compound 14	• Inhibition of acid CDase in cultured Farber fibroblasts	[120]
 1b R = -C_3H_7 1c R = -C_4H_9	• Induction of cell death in human colon carcinoma HCT-116, human bronchioalveolar carcinoma NCI-H358, human chronic myelogenous leukemia K-562 • 1b and 1c (IC_{50}, ~12 μM) more potent than C_2-Cer (IC50, 24,5, 19.6, 35.1 μM in HCT-116, NCI-H358 and K-562, respectively) • DNA fragmentation in K-562 cells was observed	[119]
 (2'R)-2'-OHCers (2'R)-2'-OHdhCers	• Suppressing cell growth in MCF-7 cells • (2′R)-isomers (IC_{50}, 3–8 μM) were more active than the (2′S)-isomers and (IC_{50}, 8–12 μM) and C_6-Cer (IC_{50}, 12 μM)	[59]

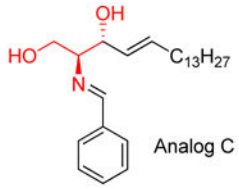
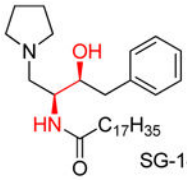
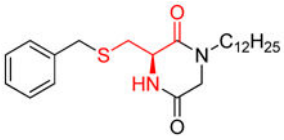
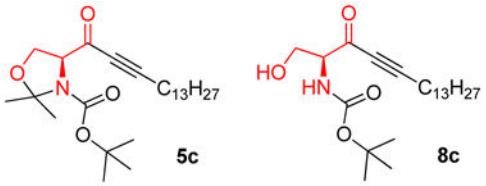
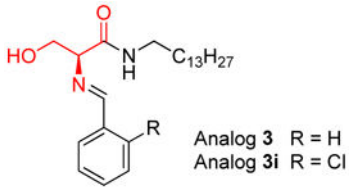
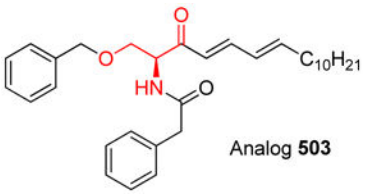
Name and structure	Activity	Reference
 Analog C	• Induction of cell death in MCF-7 (IC_{50}, 9.0 μM), MCF-7TN-R (IC_{50}, 3.6 μM), and MDA-MB-231 (IC_{50}, 4.8 μM)	[125]

Table 3

Other modifications on ceramide, structures, and activities

Name and structure	Activity	Reference
 SG-14	• Exhibiting selective inhibition of SK2, do not affecting SK1 and PKC	[126]
 diketopiperazine backbone	• Inducing apoptosis in human prostate cancer PC3 cells through mitochondrial pathway • Decreasing the phospho-Akt and phospho-Bad levels • Decreasing Bcl-2 expression slightly with increasing Bax/Bcl-2 ratio	[127]
 **5c** **8c**	5c • Selective inhibition SK1 over SK2 and PKC 8C • Inhibition of both SK1 and SK2 at the same level	[128]
 Analog **3** R = H Analog **3i** R = Cl	• Compatible pro-apoptotic activity with C_8-Cer • Breast cancer apoptosis was examined in MCF-7 cells • Dramatically increasing Sph and S1P levels in resistant cancer cells	[129–131]
 Analog **503**	• Inhibition of cell viability in several sensitive and resistant cell lines (IC_{50}, ~5 μM) • Inhibiting GCS in cultured cells	Unpublished data from authors' lab