Pharmacological Modulation of Sphingolipids and Role in Disease and Cancer Cell Biology

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Abstract: Sphingolipids comprise a family of bioactive lipids that exert antagonizing roles in diverse cellular functions such as cell proliferation, growth arrest or apoptosis. Synthesized in the ER/Golgi, sphingolipids are subsequently distributed to different compartments, most predominantly in the plasma membrane, where they integrate signaling platforms. In addition to its precursor role in the synthesis of complex glycosphingolipids, ceramide has been identified as a cell death effector and its generation increases in response to apoptotic stimuli including stress, radiation, chemotherapy, and death ligands. In contrast, sphingosine-1-phosphate (S1P) has been mainly characterized as an antiapoptotic sphingolipid mediating cell proliferation and survival. Thus, the relative balance between ceramide and SIP has important implications in disease pathogenesis, and therefore the pharmacological modulation of enzymes involved in regulation of the ceramide to SIP ratio could constitute a novel therapeutic approach for the treatment of human diseases and cancer.

Key Words: Ceramide, sphingosine-1-phosphate, mitochondria, apoptosis, necrosis, cancer therapy.

INTRODUCTION

The early conception of sphingolipids, as simple constituents of biological membranes has been challenged in the last years by the constant flow of data showing the participation of sphingolipids in the control of different cellular mechanisms, including cell proliferation, cell differentiation, apoptotic cell death, cell contraction, retraction, and migration [1-4]. Several sphingolipids, such as ceramide or GD3 ganglioside, have been causally involved in the activation of cell death pathways [5-7], while others, such as sphingosine 1-phosphate (S1P), promote cell proliferation and survival [8, 9]. This surprising capacity of structurally related sphingolipids to regulate opposing cell processes has sparked a great interest in the exploitation of these divergent properties in the biomedical arena [3, 10]. By acting on a single pathway, sphingolipid regulating drugs may have key potential benefits as they can simultaneously potentiate cell death and reduce pro-survival mechanisms as it would be desirable in cancer therapy while, in other instances in which excessive cell death causes organ dysfunction, the goal in their use will be to decrease cell death and activate survival pathways. Therefore, the knowledge of the enzymes involved in sphingolipid metabolism and their regulation during drug therapy, stress or inflammation may facilitate the design of strategies for the treatment of diverse pathologies. The genetic and biological progress on the characterization of the sphingolipid metabolic pathways during the last decade is a paradigm of how basic science may impact on biomedical research by providing the practical tools to confront diverse medical settings.

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SPHINGOLIPID METABOLISM AND TRAFFICKING

Ceramide synthesis occurs mainly in the endoplasmic reticulum (ER) from where is transported to the Golgi for its transformation in sphingomyelin, globotriaosyl ceramide or gangliosides (Fig. **1**). Ceramide synthesis begins with the condensation of L-serine and palmitoyl-CoA to form 3-ketosphinganine [11], the rate-limiting step in ceramide biosynthesis, by the pyridoxal phosphate-dependent enzyme serine palmitoyl transferase (SPT). 3-ketosphinganine is then reduced to the sphingoid base sphinganine by the 3-ketosphinganine reductase, which is then acylated by ceramide synthase (CS) to generate dihydroceramide. The action of dihydroceramide desaturase introduces a *trans*-4,5 double bond converting dihydroceramide into ceramide (Fig. **2**). As inferred, the *de novo* ceramide biosynthesis requires the coordinate action of SPT and CS to generate ceramide. In addition to the *de novo* synthesis, ceramide can arise from hydrolysis of sphingomyelin-engaging sphingomyelinases (SMases). Several SMases have been characterized of which two are of relevance in signaling. The membrane-bound neutral SMase (NSMase), with an optimum pH of approximately 7.5 and an acidic SMase (ASMase), with an optimum pH of about 4.8, further subclassified into an endosomal/lysosomal ASMase and a secretory Zn^{2+}-dependent SMase [5, 12].

Once generated, ceramide may be converted into a variety of substances (Fig. **3**). Deacylation by ceramidases (CDase) yields sphingosine, which may be then phosphorylated by sphingosine kinase (SK) to S1P, a powerful sphingolipid endowed with survival properties [1, 3, 9, 10]. Two distinct SKs have been cloned so far that perform different cellular functions. In addition, ceramide may be phosphorylated by ceramide kinase generating ceramide-1-phosphate, which similar to S1P, exhibit mitogenic and antiapoptotic properties [13].

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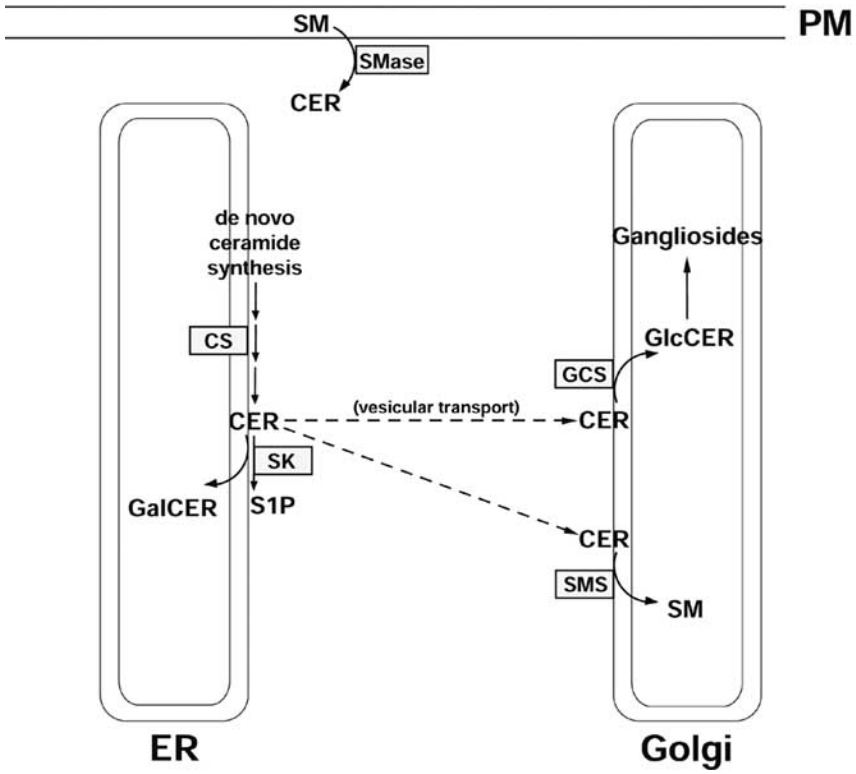

Fig. (1). Ceramide (CER) is synthesized in the endoplasmic reticulum (ER) and is the main source of many sphingolipids. Among them, galactosylceramide (GalCER) is generated in the ER, while glucosylceramide (GlcCER), the precursor of gangliosides, and sphingomyelin (SM) are produced in the Golgi through the action of glucosylceramide synthase (GCS) and sphingomyelin synthase (SMS), respectively. Ceramide may also be formed in the plasma membrane (PM) by the activation of specific sphingomyelinases (SMase). Cellular trafficking of ceramide and other sphingolipids are a topic of great interest whose full understanding will help the design of new strategies to control cell death regulation and disease pathogenesis.

Ceramide may also be transferred to the Golgi compartment through vesicle-dependent and –independent mechanisms [14, 15]. Recently, a new insight in this process has been provided with the identification of a novel cytoplasmic protein named CERT that mediates the ATP-dependent transport of ceramide from the endoplasmic reticulum to the Golgi in a vesicle-independent manner [16]. CERT contains a phosphatidylinositol-4-monophosphate-binding (PtdIns4P) domain and a putative domain for lipid transfer, which is also present in StAR and MLN64, two cholesterol binding proteins [14]. Indeed, the C-terminal START domain of CERT is responsible for its ability to specifically target ceramide extracted from phospholipid bilayers to the Golgi. CERT-mediated transport of ceramide from the ER to the Golgi mediates SM synthesis, in a process regulated by oxysterol binding protein OSBP and vesicle-associated membrane protein–associated protein, VAP [17].

Ceramide in the Golgi apparatus may be converted to SM by transfer of phosphorylcholine from phosphatidylcholine *via* sphingomyelin synthase. Alternatively, specialized glycosyltransferases transfer a glucose residue in a β-glycosidic linkage to the C1-hydroxyl of ceramide to produce glucosylceramide in the Golgi (Fig. **1**), while the addition of galactose to form galactosylceramide occurs in the ER [6, 15, 18]. Most of the glycosphingolipids (GSLs) arise from the glucosylation rather than galactosylation of ceramide, a step catalyzed on the cytosolic surface of the Golgi by the rate-limiting enzyme glucosylceramide synthase (GCS). Glucosylceramide (GlcCer) is then transferred to the lumenal leaflet of the Golgi, where it is modified by the addition of a galactose moiety to produce lactosylceramide from which most gangliosides derive by the action of specific glycolipid-glycosyltransferases. For instance, sequential addition of one, two, or three sialic acids to lactosylceramide results in the formation of GM3, GD3, and GT3, respectively. Since ceramide provides the carbon backbone of GSLs, their synthesis is also dependent on the availability of ceramide generation. Indeed, ganglioside generation, which is involved in cell death after exposure to inflammatory cytokines or chemotherapeutic drugs, is dependent on the activation of specific SMases that provide the ceramide needed for ganglioside synthesis, as recently shown in fenretinide-induced killing of neuroblastoma cells [19].

In addition to these classical pathways, recent observations have provided evidence for a novel path in which ceramide is generated from GSLs at the plasma membrane. Using conditions in which sugar units of GM3 were detached, Valaperta *et al.* showed the formation of ceramide at the plasma membrane in human fibroblasts [20]. Indeed, the production of ceramide from GM3 ocurred under conditions that blocked endocytosis or lysosomal activity, and the overexpression of the plasma membrane ganglioside sialidase

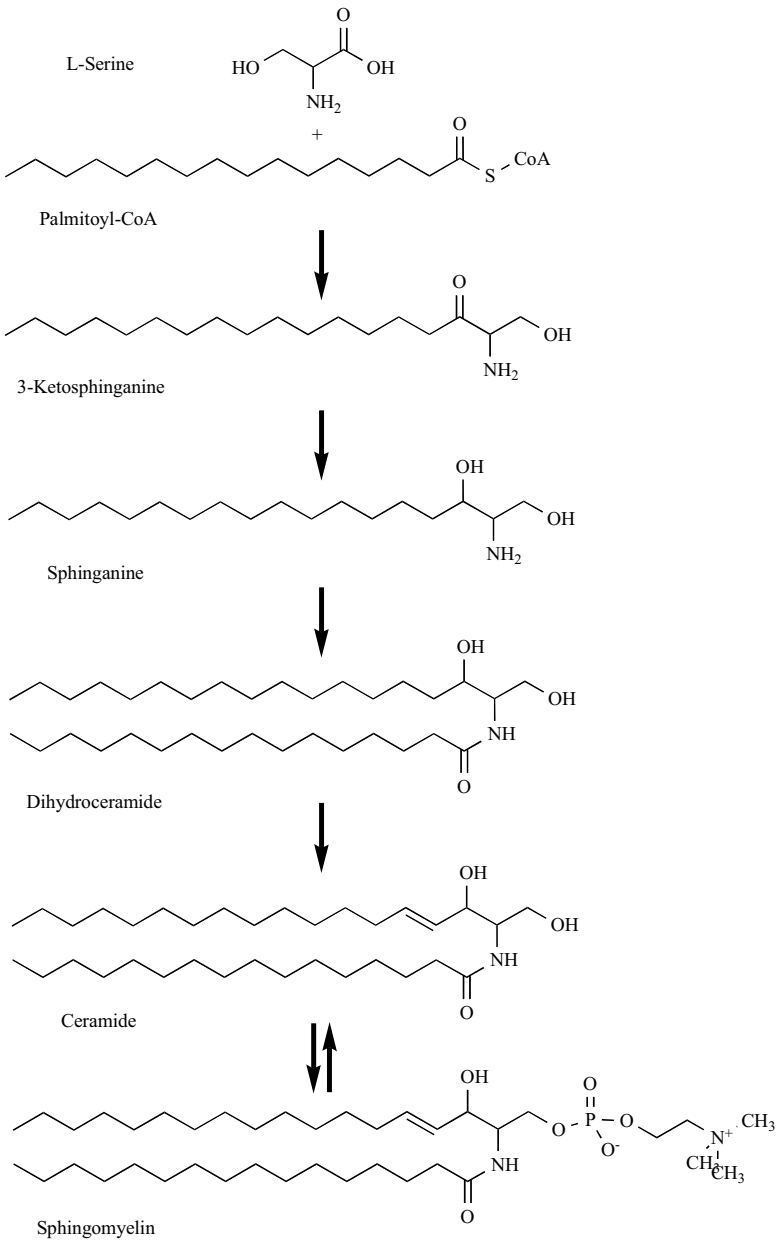

Fig. (2). Schematic diagram of ceramide generation. Among different enzymes involved, serine palmitoyltransferase (SPT) and ceramide synthase (CS), in the *de novo* pathway, and neutral and acid sphingomyelinases (NSMase and ASMase), through hydrolysis of sphingomyelin, are considered as the major enzymes in ceramide production.

Neu3 correlated with a higher production of ceramide in the plasma membrane. Moreover, the description of CS and reversed CDase in mitochondria [21, 22], complete the map of cellular sites where ceramide can be generated, and indicate a high versatility in the metabolism and action of GSLs.

SPHINGOLIPIDS IN CELL DEATH REGULATION

Sphingolipids function as second messengers in different cellular processes such as cell proliferation, differentiation and cell growth. In particular, the involvement of ceramide in the stress response and in cell death has attracted a great deal of interest in the characterization of these events at the cellular and molecular levels. A fundamental issue established in this regard was the fact that ceramide levels increase before the onset of cell death [1, 3, 23], implying that its generation is a cause rather than a consequence of cell death.

Activation of Prodeath Pathways

As the subcellular pathways of cell death are being uncovered [24-26], sphingolipids have been described to promote cell death pathways dependent on the participation or recruitment of ER and/or mitochondria. For instance, C2-ceramide, a cell permeable ceramide analogue, has been

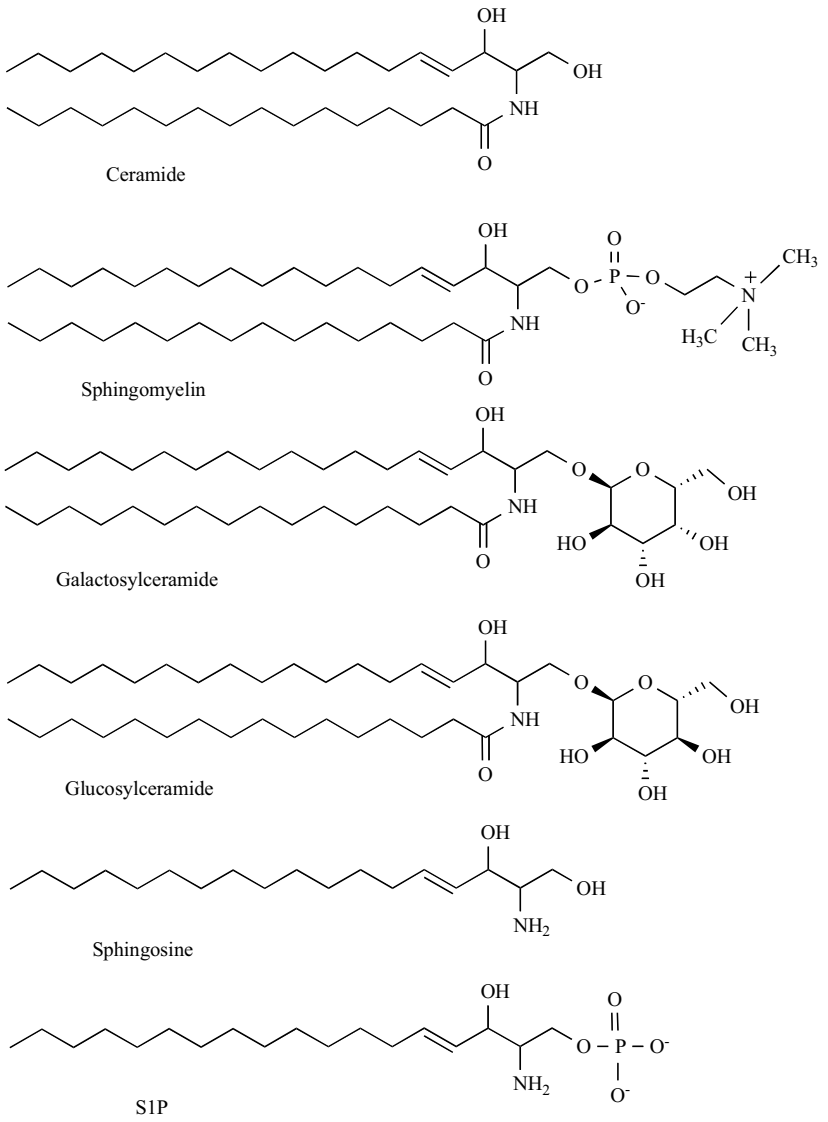

Fig. (3). Schematic diagram of ceramide biotransformation. Ceramide is the backbone of GSLs that along with ceramide and other derivatives are critical cell membrane constituents. Among numerous enzymes involved, ceramidases (CDases) and sphingosine kinases (SKs) are critical in ceramide elimination and S1P formation, while glucosylceramide synthase (GCS) catalyzes the first step in ceramide glucosylation and ganglioside formation.

shown to induce ER stress-mediated factors such as ATF-4, CHOP and TRB3 expression in astrocytoma U87MG cells and subsequent cell death [27]. Furthermore, cannabinoid-induced apoptosis of tumor cells was mediated by an early increase in ceramide levels from the induction of SPT, the enzyme responsible for the de novo synthesis of ceramide from serine and palmitoyl-CoA as mentioned above, and the enhancement in ceramide levels in the ER activated the ER-mediated apoptotic pathway. In addition to this novel pathway of ceramide-induced death, a great deal of evidence has demonstrated a major role of mitochondria in ceramide-induced cell death. For instance, ceramide has been shown to disrupt electron flow at complex III, resulting in enhanced ROS generation, which facilitates cytochrome c release and caspase activation [28-30]. Apoptotic stimuli, such as death ligands (e.g., Fas and TNF), chemotherapeutic agents or ionizing radiation, activate specific SMases, although the precise intracellular site of ceramide generation by individual SMases remains to be clearly established. In line with this, sphingomyelin was thought to be located almost exclusively in the outer leaflet of the plasma membrane, however recent evidence indicated that this lipid is also present in other compartments, such as mitochondria, and the *in situ* generation of ceramide within this organelle by enforced mitochondrial targeting of bacterial SMase induced apoptosis in MCF7 cells [31].

The contribution of individual SMases to apoptosis remains to be fully established and probably depends on several conditions, such as the kind of apoptotic stimuli used and the cell type studied [5]. For instance, the role of ASMase in TNF-mediated hepatocellular apoptosis has been

recently observed showing the resistance of ASMase$^{-/-}$ mice to endogenous or exogenous TNF-induced liver damage *in vivo* [32, 33]. Furthermore, we revealed that ceramide played a critical role in the liver damage induced by ischemia/reperfusion though a mechanism involving JNK activation and mitochondrial migration of Bim_L, a proapoptotic protein of the Bcl-2 family [34]. Consistent with these findings on TNF apoptosis, ASMase has been shown to mediate Fas-induced death in hepatocytes but not in lymphocytes [35]. In addition, upregulation of ASMase-generated ceramide has been implicated in pulmonary cell apoptosis and emphysema [36], and in pulmonary edema in acute lung injury [37]. These latest results, added to the previous observations pointing to ceramide as a key factor in radiation- and chemotherapy-induced cell death, as well as in the developmental death of oocytes [38, 39], have positioned this sphingolipid as potencial target of new therapeutic strategies.

In addition to the evidence for a direct role for ceramide in the regulation of stress induced cell death, ceramide provides the carbon backbone for the synthesis of complex glycosphingolipids (GSLs), such as gangliosides. In particular, ganglioside GD3 (GD3), a sialic acid-containing GSL, has attracted considerable attention due to its emerging role as a cell death effector. As with ceramide, the cellular levels of GD3 increase in response to Fas or TNF [40, 41], whereas the down regulation of GD3 synthase, the enzyme responsible for GD3 synthesis from its precursor GM3, prevents Fas-, TNF- or [beta]-amyloid-induced cell death [40, 42, 43].

Most of GD3 is present at the plasma membrane in resting hepatocytes, however, in response to TNF, exogenous ASMase or ionizing radiation, GD3 undergoes a dramatic redistribution that involved its disappearance from the plasma membrane and its trafficking to mitochondria [44]. Once in mitochondria, GD3 enhances the production of reactive oxygen species (ROS), resulting in the opening of the cyclosporin A-sensitive permeability transition pore and the release of cytochrome C [41].

Regulation of Survival Pathways

In addition to the active role of GD3 in promoting mitochondrial-dependent apoptosis, GD3 interferes with the nuclear translocation of active members of NF-κB thus suppressing the activation of NF-κB-dependent gene induction including antiapoptotic genes [45]. This effect is not shared with ceramide or other sphingolipids, since the presence of specific sugar residues in the backbone of ceramide is required to block the nuclear translocation of NF-κB [45]. The exploitation of this dual role of GD3 in apoptosis, as mitochondrial ROS stimulator and NF-κB-inactivating agent, has been recently shown in HepG2 cells, a tumor cell line highly resistant to current cancer therapy [46]. However, this proapoptotic role of GD3 is not common to all cancer cells. For instance, GD3 is highly expressed in melanomas in comparison with melanocytes and has been used as a target for the immune therapy of melanomas [47]. This apparent controversy may depend on the acetylation status of GD3, since O-acetylated GD3 has been reported to have antiapoptotic activity. In fact, cells resistant to the overexpression of the GD3 synthase actively convert de novo synthesized GD3 to 9-O-acetyl-GD3, while prevention of 9-O-acetyl-GD3 accumulation reconstitutes GD3 responsiveness and apoptosis [48]. Nevertheless, the specific role of GD3 in different cell types and the expression of related enzymes remain to be fully evaluated.

Due to the sphingolipid participation in cell death pathways, sphingolipid intracellular levels are tightly regulated. Several catabolic pathways are induced by the same stimuli in order to eliminate pro-apoptotic lipids to preserve cell integrity. For instance, CDases and/or SKs, which transform ceramide into S1P, are activated by numerous external stimuli including inflammatory cytokines, radiation or chemotherapeutic agents. Intracellular S1P mobilizes Ca^{2+} from internal stores independently of inositol trisphosphate, in addition to triggering signaling pathways that lead to cell proliferation and survival. The existence of these opposing signaling pathways controlled by the dynamic balance between the intracellular concentration of S1P and ceramide has been suggested to be an important factor in the determination of mammalian cells' fate. The capacity to modulate cell survival by intervention on this rheostat has been observed in different pathologies ranging from developmental or chemotherapy-induced apoptosis in oocytes to lung emphysema or septic shock [1, 3]. For instance, the induction of ceramide-mediated apoptosis by the anticancer drug doxorubicin in unfertilized mouse oocytes is blocked by S1P. The protective effect of S1P in female germ cells was not mimicked by lysophosphatidic acid or by an equimolar concentration of dihydro-S1P, and was not influenced by treatment of the cells with pertussis toxin. Therefore, this outcome appeared entirely independent of a family of five cognate G protein-coupled receptors, designated S1P(1–5)Rs that can interact with S1P.

In addition to S1P as an intracellular suppressor of apoptosis, a recent function of S1P in cancer biology has been revealed through its action as a ligand of S1PRs [49]. By binding to S1PRs, S1P regulates cell functions such as angiogenesis and vascular maturation. Notably, diverse growth and proangiogenic factors that have been implicated in cancer progression, including EGF, VEGF, and PDGF, stimulate and translocate SphK1 to the plasma membrane, resulting in local formation of S1P [49] and activation of S1P receptors (S1PRs). Neutralizing S1P with a specific monoclonal antibody has been shown to be an effective means to retard the progression of deadly and multiresistant cancers such as lung, breast, melanoma, and ovarian cancers in murine xenograft and allograft models, probably by preventing the pro-angiogenic effects of the blood-borne S1P [9].

Collectively, these results indicate that S1P may have a role in protection against ceramide-mediated apoptotic death, as well as in promoting cell growth, transformation and cancer progression through interaction with S1PRs.

INTERVENTION ON CERAMIDE METABOLISM AS A STRATEGY TO MODULATE CELL DEATH

The data showing the divergent biological action of closely related sphingolipids and their involvement in different pathologies suggest that the control of the intracellular levels of specific sphingolipids may be a novel strategy to manage different clinical settings. Keeping in mind that the

participation of each particular sphingolipid seems to depend on the cell type and the stimulus studied, interfering with sphingolipidic metabolism may be of medical interest. In this regard, we will cover the chemical and structural characterization of enzyme inhibitors, in special those that control more than one bioactive sphingolipid such as the proteins involved in the ceramide-S1P rheostat, or chemically synthesized sphingolipids with anti- or pro-apoptotic properties, that may find a niche in the future as specific therapies for diseases mediated by an unbalanced ceramide to S1P ratio.

Control of Ceramide Production

The *de novo* ceramide biosynthesis and the sphingomyelin hydrolysis by SMases constitute major mechanisms for ceramide generation in response to a wide variety of stimuli. In general, the latter provides a means for the almost instantenous ceramide increase at the expense of sphingomyelin stores, while the former accounts for a late but sustained ceramide generation. Despite these kinetic differences in ceramide generation, it is uncertain whether these two mechanisms occur in different cellular locations. The most commonly used compounds to inhibit these enzymes are shown in Fig. (**4**) and Fig. (**5**).

1) De Novo Ceramide Synthesis

SPT Inhibitors

SPT, a pyridoxal 5'-phosphate-dependent enzyme located in the ER catalyzes the limiting step in the *de novo* pathway of ceramide synthesis. Myriocin (ISP-1), a natural compound obtained from the fungus *Isalia sinclairii* exhibit a chemical structure similar to that of sphingosine (Fig. **4**). It is an extensively used SPT inhibitor in mammalian cells with IC_{50} values in the nanomolar range, in spite of being a potential immunosuppressant due to the induction of apoptosis of T cell lines [36, 50, 51]. L-cycloserine and β-chloroalanine are synthetic inhibitors (Fig. **4**), which have been used to inhibit SPT in intact cells. However, they exhibit a wide-range activity towards many pyridoxal 5'-phosphate-dependent enzymes, and their SPT inhibitory activity requires a concentration in the millimolar range, which hampers their use to specifically modulate ceramide levels *via* SPT. Other fungal metabolites with similar structure to myriocin such as sulfamisterin or NA255 have been isolated [52, 53]. Of note, NA255 has been shown to disrupt the association of Hepatitis C virus nonstructural (NS) viral proteins on the lipid rafts, suggesting a role as a new anti-HCV replication inhibitors [53]. Whether this function relies on their ability to prevent sphingolipid biosynthesis remains to be critically established.

CS Inhibitors

Fumonisins, a family of mycotoxins produced by *Fusarium verticillioides* are common fungal contaminants of corn and some other grains, and exhibit CS inhibitory activity [54]. Fumonisin B_1 (FB_1, Fig. **4**), the most prevalent member of this family of mycotoxins, is toxic to the liver, kidney and brain in humans and exhibits carcinogenic potential, at least in animal tests, according to the National Center for Toxi-

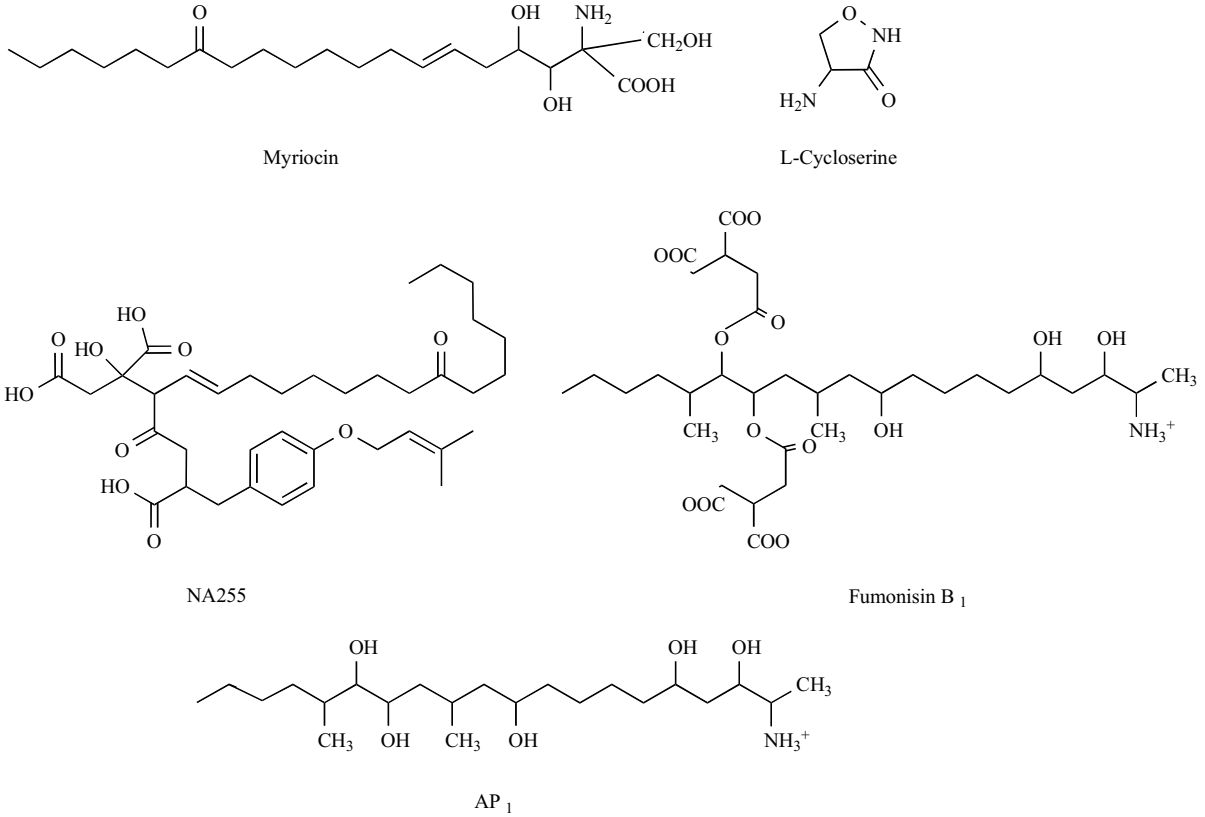

Fig. (4). Chemical structures of some SPT and CS inhibitors.

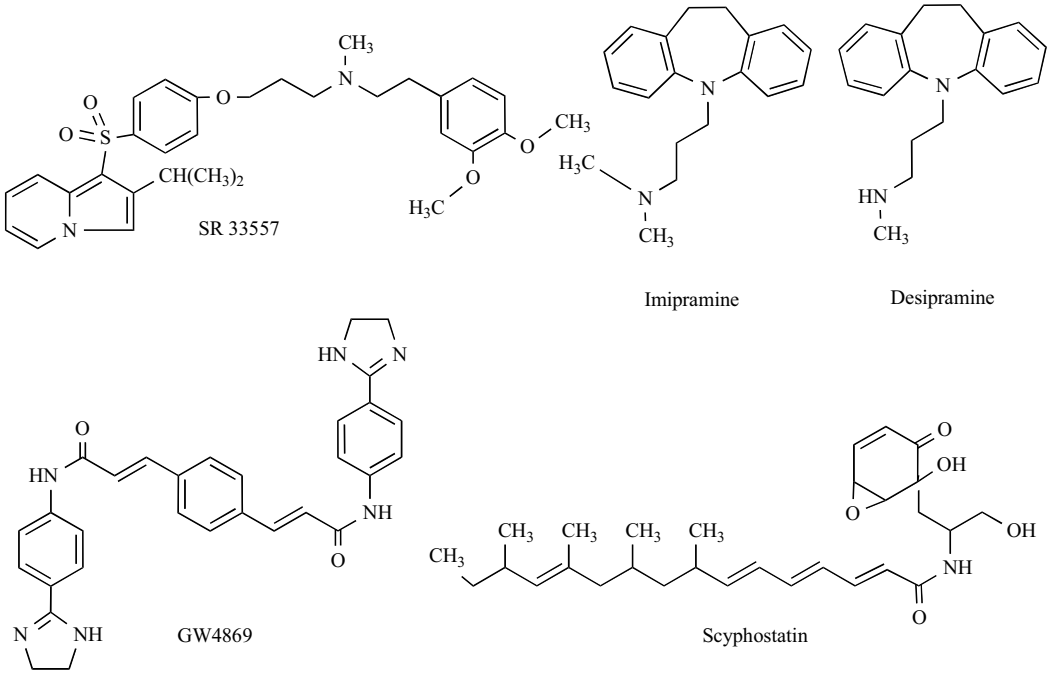

Fig. (5). Chemical structures of some ASMase and NSMase inhibitors.

cologic Research [55]. FB_1 contains a long-chain aminopentol backbone with two ester-linked tricarballylic acids. Apparently, its aminopentol backbone (AP_1) competes for binding of the sphingoid base substrate, while the anionic tricarballylic acids interfere with binding of the fatty acyl-CoA. Through this mechanism FB_1 inhibits CS activity, prevents ceramide accumulation and protects cells against drug-induced cell death [3, 5, 36]. However, the onset of FB_1-induced toxicity, mainly in liver, not related to the reduced biosynthesis of ceramide and complex GSLs precludes the general use of this compound [50]. The recent description of different mammalian genes encoding diverse CS may account for the specificity of CS to incorporate particular acyl-CoAs into ceramide, suggesting that the isolation of new CS inhibitors with specificity towards individual CS may open the door to regulate the *de novo* formation of individual ceramides.

2) Ceramide Generation from Sphingomyelin Hydrolysis

SMase activation in response to many different stimuli occurs in a rapid fashion, and thus constitutes a quick and efficient mechanism to generate ceramide from existing sphingomyelin pools in membranes. As indicated above, among the different described SMases, the neutral and the acidic enzymes which are encoded by independent genes and located in different cell locations share major roles in cell signaling and cell death regulation.

ASMase Inhibitors

Tricyclic antidepressants such as imipramine and desipramine are among the earliest reported ASMase inhibitors (Fig. **5**). In 1981, Albouz *et al.* [56] described the effect of desipramine on the ASMase activity in cultured murine neuroblastoma cells and human fibroblasts. The inhibition of ASMase caused by desipramine is due to the interference with the binding of ASMase to the lipid bilayer that precludes the access of the enzyme to its membrane-bound substrate [57]. In addition, the displacement of the glycoprotein ASMase from the inner membrane of late endosomes and lysosomes by desipramine or imipramine renders it susceptible to proteolytic cleavage by lysosomal proteases. Nevertheless, cationic amphiphilic drugs such as desipramine or imipramine, may also affect other metabolic pathways. In fact, it is already known that these compounds alter P-glycoprotein activity and hence may be of potential use in the reversal of multidrug resistance [58]. Therefore, to better charactize whether the effects of these drugs in cellular metabolism are due to the putative inhibition of ASMase, the use of more specific loss-of-function approaches such as siRNAs or genetic deletion should be implemented. In this regard, recent observations in L929 murine fibroblasts indicated that desipramine inhibited ACDase in addition to blocking ASMase, and this resulted in inhibition of PGE2 production by TNF [59]. Although this would suggest an unspecific role of desipramine in affecting ACDase, there seems to be a potential physical interaction between ACDase and ASMase based on the observations that overexpression of ACDase in human skin fibroblasts results in ASMase stimulation, and furthermore, ASMase can co-precipitate with ACDase using anti-ceramidase antibodies [60]. In addition, SR33557, (2-isopropyl-1-((4-(3-*N*-methyl-*N*-(3,4-dimethoxy-b-phenethyl) amino) propyloxy) benzenesulphonyl)) indolizine (Fig. **5**), a different ASMase inhibitor belonging to the indolizin sulfone class has been shown to be effective in inhibiting p53-independent apoptosis in human glioma cells in response to gamma-radiation [61]. More recently, natural compounds, such as PtdIns(3,4,5)P3 and PtdIns(3,5)P2, that contain the 3-phosphoinositide moiety

have been described as novel non-competitive inhibitors of A-SMase [62, 63]. Interestingly, it has been suggested that these two lipids may be found specifically in the raft microdomains of membranes, where a pool of ASMase has been suggested, thus providing the molecular basis as a physiological regulator of the ASMase involved in receptor clustering. Additional studies will be required to clarify the biological significance of these observations.

NSMase Inhibitors

Scyphostatin, isolated from a culture broth of the discomycete *Trichopeziza mollissima* by Ogita and co-workers in 1997, is a potent and specific inhibitor of NSMase (Fig. **5**). Structurally, scyphostatin contains a highly oxygenated cyclohexene ring and an aminopropanol side chain linked to a C20 unsaturated fatty acid moiety [64]. Its use has allowed the characterization of the participation of NSMase in p75 neurotrophin receptor and TNF signaling, at least in some cell types [65]. Recently a procedure for scyphostatin chemical synthesis has been described, that may facilitate its commercial production and use in future studies [66]. GW4869 is another noncompetitive inhibitor of NSMase recently reported [67].

However, several proteins from different genes possess NSMase activity. At present, three genes encoding NSMases have been identified and several others remain to be established. Interestingly, C11AG may be a NSMase inhibitor specific for some NSMase isoforms. C11AG may stimulate CD95-induced apoptosis in T-cells by selectively suppressing a NSMase activity at pH 7.0, whereas the activity at pH 8.0 is unaffected [68]. Regardless of the potential interest of this compound, the existence of new products with inhibitory activity specific for each different NSMase may help to reduce off-target effects and to characterize the biological action of each individual enzyme.

3) Ceramide Biotransformation

Being ceramide the backbone for numerous GSLs species, several enzymes are required in its biotransformation. Among them, those enzymes involved in ceramide deacylation and S1P formation, CDases and SKs, respectively; and those needed for ganglioside formation, in special GCS, have been frequently studied, since these particular enzymes not only participate in the assembly of essential sphingolipids for cellular structures, but also play a major role in the processing of ceramide induced by cytotoxic stimuli. The compounds frequently used to inhibit their activity are shown in Figs. (**6**) and (**7**).

Ceramidase Inhibitiors

The ceramide analog (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (D-erythro-MAPP) and N-oleoylethanolamine (NOE) have been currently considered as specific inhibitors of the neutral/alkaline [69] and acidic ceramidase isoenzymes [61, 70], respectively. However, several studies suggest that in certain cell lines and, particularly, in *in vivo*, both may act as general CDase inhibitors with different degree of affinity for each protein, and even may affect other sphingolipidic enzymes, but only when used at high concentrations [71]. Therefore, although D-e-MAPP and NOE may be used as fairly specific inhibitors in many experimental procedures, to guarantee that effects are due to the inhibition of neutral or acid CDase activity, it is wise to perform parallel experiments using genetic knock down strategies targeting either enzyme. In this regard, we have recently reported the role of ACDase inhibition in the therapy of liver cancer [72]. The treatment of several hepatoma cells with NOE enhanced the therapeutic efficacy of daunorubicin both *in vitro* and reduced the tumor growth *in vivo*, similar to the effect observed after silencing ACDase with siRNA [72]. A similar outcome has been reported with the use of another ceramidase inhibitor, (1R,2R)-2-N-myristoylamino-1-(4-nitrophenyl)-1,3-propandiol (B13) (Fig. **6**). B13 is significantly more active than D-MAPP despite their similar chemical structure, probably due to the enhanced solubility of B13 compared with D-MAPP [73]. Interestingly, B13 is also a good ACDase inhibitor in some cell types, reducing tumor growth in prostate and colon cancer xerographs without affecting healthy tissue [73, 74]. These novel results draw attention to CDases, in particular to ACDase, as a potential therapeutic target in cancer therapy.

Sphingosine Kinase Inhibitors

SK may be another interesting enzyme to control cell death because its product, S1P, has been linked to numerous proliferative and survival pathways. Although most pharmacological studies to date to inhibit SK activity with D-erythro-N,N-dimethylsphingosine, DL-threo-dihydrosphingosine, and N,N,N-trimethylsphingosine (Fig. **6**), have reported interesting results, these compounds are known to affect multiple lipid and protein kinases [75]. For this reason, more selective inhibitors are required. In this regard, using recombinant human SK1 to screen a library of synthetic compounds, a panel of SK1 inhibitors were recently identified [76]. Among them, 4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol (SKI-II) (Fig. **6**), displays the best oral bioavailability and antitumor activity.

Another alternatives that have been lately explored in cancer therapy are based on the neutralization of S1P by an anti-S1P antibodies or the antagonism of S1P-induced effects by preventing its binding to S1PRs by 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol (FTY720) (Fig. **6**). FTY720 is a sphingosine analog with immunosuppressant properties that becomes phosphorylated *in vivo*. FTY720 interacts with S1Prs resulting in their internalization and desensitization due to the inhibition of their recycling to the cell surface. Through this mechanism, FTY720 markedly inhibited tumor-associated angiogenesis and decreased tumor cell proliferation, inhibiting metastatic tumor growth in a mouse model of melanoma [77]. Of note, similar results have been observed based on anti-S1P antibody therapy [9]. In this seminal work, Visentin *et al.* showed that a biospecific monoclonal antibody against S1P reduced tumor progression and tumor mass in murine xenograft and allograft models by inhibiting blood vessel formation and arresting tumor-associated angiogenesis.

GCS Inhibitors

GCS catalyzes the first glycosylation step in the biosynthesis of glycosphingolipids, including glucosylceramide, lactosylceramide, and gangliosides, that play an essential role in cell development, cell death, tumor progression, and

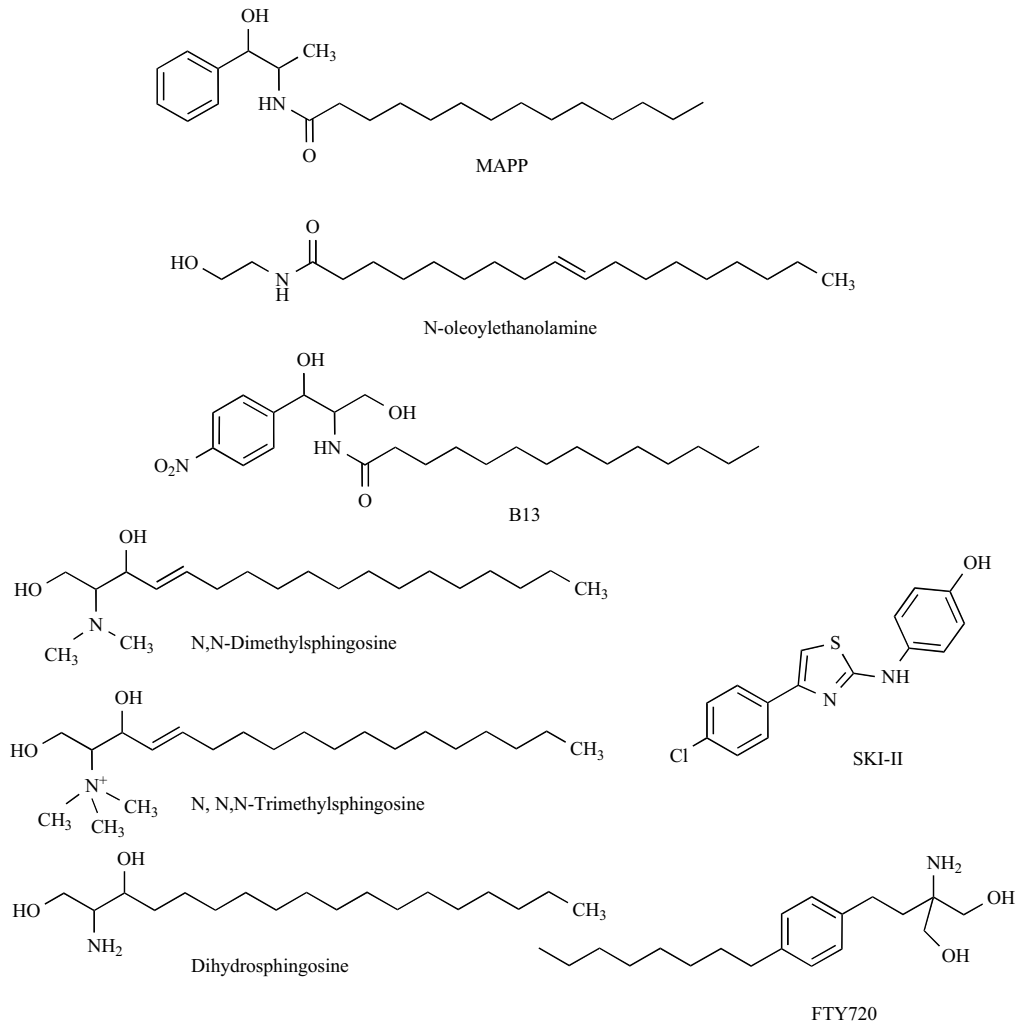

Fig. (6). Chemical structures of some CDases and SKs inhibitors.

pathogen/host interaction. This fact and the existence of numerous glycosphingolipid lysosomal storage diseases, resulting from the inheritance of defects in the genes encoding the enzymes required for catabolism of GSLs within lysosomes, have encouraged the research of GCS inhibitors for many years [78]. Among them, (R,R)-(D-threo)-isomer of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and analogs such as 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) (Fig. **7**) have been widely used as GCS inhibitors [78, 79].

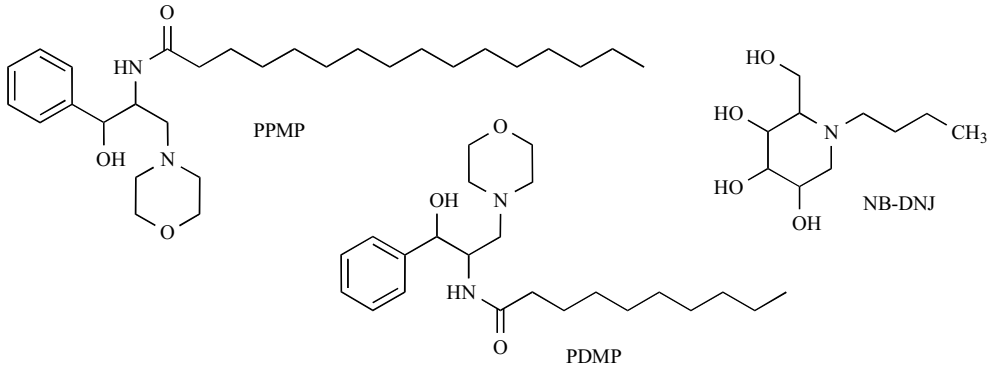

Fig. (7). Chemical structures of some GCS inhibitors.

However, it was discovered that many of these compounds raised cell ceramide levels independently of the GlcCer depletion. The dissociation of GlcCer depletion from ceramide accumulation may be necessary for the development of GCS inhibitors as potential drugs for inherited GSL storage diseases, but this drawback may be secondary when used in cancer therapy. However, different groups have questioned the use of PDMP and analogs as chemosensitizers, at least based only on their inhibition of GCS.

N-(n-butyl)-1,5-dideoxy-1,5-imino-D-glucitol, also known as N-butyl-deoxynojirimycin, NB-DNJ or miglustat (Fig. **7**), belongs to another family of GCS inhibitors [80]. This imino sugar, although it is not the most effective GCS inhibitor *in vitro*, does not cause ceramide elevations and apoptosis in healthy tissue and has been licensed for oral therapy in patients with mild to moderate type 1 Gaucher's disease who cannot take enzyme replacement therapy with imiglucerase. *In vivo* NB-DNJ administration to animals was shown to deplete the hepatic content of GSLs and gangliosides [81]. Yet, continuous administration of NB-DNJ in humans has many side effects and may exist a risk of reproductive toxicity as reported in rodents [82]. However, short-term treatment of NB-DNJ or other GCS inhibitors may be helpful to potentiate the cytotoxic effects of specific chemotherapeutic agents.

CONCLUDING REMARKS AND THERAPEUTIC PROSPECTS

A large body of evidence available during the last decade has widened our view on the biological roles of sphingolipids besides their structural function as key membrane constituents. Indeed, sphingolipids and GSLs are now considered as second messengers that control or participate in a wide range of cellular processes including cell death regulation and in the stress response. As a consequence from this novel activity, it is increasingly recognized the role of sphingolipids in the disease pathogenesis, suggesting that their modulation could be of therapeutic relevance. Some of the compounds already used in controlling sphingolipid metabolism are summarized in Table **1**. However, due to their key structural function in cell membranes and their participation in the organization of specific membrane microdomains, their pharmacological modulation must be carefully considered in order to be applied as potential therapy with minimal side effects due to putative membrane perturbations. As inferred from this basic formulation, the pharmacological intervention may be especially effective in specific pathologies or clinical settings in which changes in sphingolipid metabolism has been causally linked to disease pathogenesis. An interesting example is the multidrug resistance phenotype that is developed by cancer cells. In addition to the reported increased capacity for ceramide glycosylation, several steps in sphingolipid metabolism have been described in multidrug resistant cells, exhibiting an aberrant sphingolipid composition [83, 84]. Thus, a detailed characterization of sphingolipid metabolism must first be accomplished before pharmacological inhibitors of sphingolipid regulated enzymes are used in cancer therapy. This requisite may imply the use of combinatorial strategies to enforce the accumulation of proapoptotic sphingolipids with the added benefit of reducing those with survival properties as exemplified by targeting CDases and SKs. Indeed, their inhibition has shown promising prospects in the treatment of liver or prostate cancer both *in vitro* and *in vivo* [72-74]. The use of pharmaceutical products through modulation of sphingolipid metabolism in cancer treatment is a current approach that may undergo an accelerating growth and interest in the field as more specific inhibitors are developed.

Table 1. List of the Pharmacological Agents more Frequently Used in the Control of Sphingolipid Metabolism Classified by their Intracellular Targets and Potential Effects on Sphingolipid Levels

Inhibition of ceramide production:	SPT inhibitors:	
	Myriocin	(36)
	NA255	(53)
	CS inhibitors:	
	Fumonisin B_1	(19, 36)
	AP_1	(54)
	ASMase inhibitors:	
	SR33557	(61)
	Imipramine	(34)
	Desipramine	(19)
	NSMase inhibitors:	
	GW4869	(67)
	Scyphostatin	(65)
Inhibition of S1P formation and induction of ceramide/sphingosine accumulation:	CDase inhibitors:	
	MAPP	(69)
	N-oleoylethanolamine	(61, 70)
	B13	(73, 73)
	SK inhibitors:	
	N,N-Dimethylsphingosine	(1)
	SKI-II	(76)
	FTY720	(77)
Inhibition of GlcCER/gangliosides formation and induction of ceramide accumulation:	GCS inhibitors:	
	PDMP	(19, 79)
	NB-DNJ	(79, 80)

In addition, since sphingolipids are involved in many other diseases including acute hepatitis, diabetes, transplantation or neurological disorders, the prospect of sphingolipid modulation as specific therapy may go beyond just cancer therapy. In line with this, ceramide generation from ASMase activation has been shown to mediate TNF-induced hepatocellular apoptosis [32, 33], and hence the pharmacological inhibition of ASMase may be an attractive target with potential relevance in liver diseases including fulminant liver failure, steatohepatitis or hepatic ischemia reperfusion injury [32-34, 85]. As the mechanisms of sphingolipids in cell death regulation are uncovered, different and specific compounds may be available soon to modulate specific diseases caused or aggravated by particular perturbations in the regulation of sphingolipids, thus increasing the efficacy of current treatments or opening the prospects for novel approaches in the medical management of numerous human diseases.

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ABBREVIATIONS

ASMase = Acidic SMase

CDase = Ceramidase

CS = Ceramide synthase

ER = Endoplasmic Reticulum

GCS = Glucosylceramide synthase

GlcCer = Glucosylceramide

GSL = Glycosphingolipid

NSMase = Neutral SMase

SK = Sphingosine kinase

SMase = Sphingomyelinase

SPT = Serine palmitoyltransferase

S1P = Sphingosine-1-phosphate

S1PR = S1P receptor

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