# Ceramide Involvement in Apoptosis and Apoptotic Diseases

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**Abstract**: Apoptosis is implicated in a number of diseases, including neurodegenerative diseases and AIDS. More and more, evidence is accumulating pointing to the critical role of ceramides in the induction of apoptosis. The present review summarizes (i) the molecular basis and regulation of the apoptotic machinery, (ii) the molecular role of ceramides in the induction or execution of apoptotic pathways, and (iii) evidence linking ceramide generation to various apoptotic diseases. Additionally, this review discusses putative therapeutic approaches inhibiting ceramide production in apoptotic diseases.

**Keywords**: Neurodegenerative disease, AIDS, ceramide, sphingomyelinase, extrinsic apoptotic pathway, intrinsic apoptotic pathway, sphingomyelinase inhibitor.

## INTRODUCTION

Apoptosis or programmed cell death occurs when a cell specifically activates an internally encoded suicide program as a result of either internal or external signals. Apoptosis is needed to destroy cells that represent a threat to the integrity of the organism, and to control cell number and proliferation as part of normal development. Failure to regulate apoptosis can lead to pathologies such as cancer and neurodegenerative diseases. The current review will give a short overview of the two major pathways leading to apoptosis, more information can be found in following reviews [1-5]. More and more, it is becoming clear that ceramide generation is a near universal feature of apoptosis. Therefore, this review will focus on (i) the molecular role of ceramides in the apoptotic machinery, (ii) ceramide metabolism in apoptotic diseases and (iii) compounds targeting ceramide-content of a cell and their use as therapeutics in apoptotic diseases.

## APOPTOTIC PATHWAYS

Apoptotic cell death occurs mainly via two different pathways: cell death receptor (or extrinsic) and mitochondrial (or intrinsic) pathways. Both apoptotic systems rely on the activation of various cysteinyl aspartate-specific proteases (caspases), ultimately leading to nuclear damage and cell death (Fig. **1,I**). The caspases are classified as initiators or executioners, depending on their point of entry into the apoptotic cascade. The initiator caspases, such as caspase-9 and caspase-8 are the first to be activated in the intrinsic and extrinsic apoptotic pathway, respectively (see below), and in their turn activate the executioner caspases, such as caspase-3 [reviewed in 6].

### (A) The Extrinsic Apoptotic Pathway

The extrinsic pathway involves the active induction of apoptosis through ligation of members of the death receptor family, such as Fas (also called CD95) or TNF R1 (tumor necrosis factor receptor 1). Clustering of the death receptors on the cell membrane during their interaction with their ligand (FasL or TNF, respectively) leads to the formation of the DISC (death-inducing signaling complex), comprising the death receptor, a linker molecule FADD (Fas-associated death domain protein) and procaspase-8. In the DISC complex, caspase-8 is activated and subsequently activates caspase-3, which is the central executioner of the apoptotic program. Activated caspase-3 induces proteolysis of target proteins, like poly(ADP-ribose) polymerase and specific transcription factors, leading to cell death.

### (B) The Intrinsic Apoptotic Pathway

The intrinsic pathway is triggered by changes of mitochondrial integrity, which can be initiated by various signaling molecules such as $Ca^{2+}$, reactive oxygen species (ROS, such as $H_2O_2$) and ceramide (Cer) generation as response to various stresses (see further). Loss of mitochondrial integrity leads to release of mitochondrial pro-apoptotic factors in the cytosol, such as Smac/Diablo, HtrA2/Omi, Apoptosis inducing factor (AIF), Endonuclease G (EndoG) and cytochrome *c* (Cyt c). Upon release in the cytosol, Cyt c binds Apaf-1 (apoptotic protease-activating factor) and dATP, together with procaspase-9 (this complex is referred to as apoptosome). Subsequent activation of caspase-9 leads in his turn to activation of caspase-3.

It is important to note that the Bcl-2 family of proteins, located in the outer mitochondrial membrane, is considered the key regulator in precipitation of the intrinsic apoptotic pathway [reviewed in 5,7-9]. The relative amounts of pro-apoptotic Bcl-2 proteins (such as Bax, Bak, Bad, Bid, Bik, Bim) and anti-apoptotic Bcl-2 proteins (such as Bcl-2 and Bcl-$X_L$) in the mitochondrial membrane (referred to as Bcl-2/Bax rheostat) control release of pro-apoptotic factors from mitochondria. In a prosurvival scenario, Bax/Bak are inactivated e.g. by binding to Bcl-2/Bcl-$X_L$. Following a death signal, pro-apoptotic Bax/Bak are activated and upon oligomerization, insert into the outer mitochondrial

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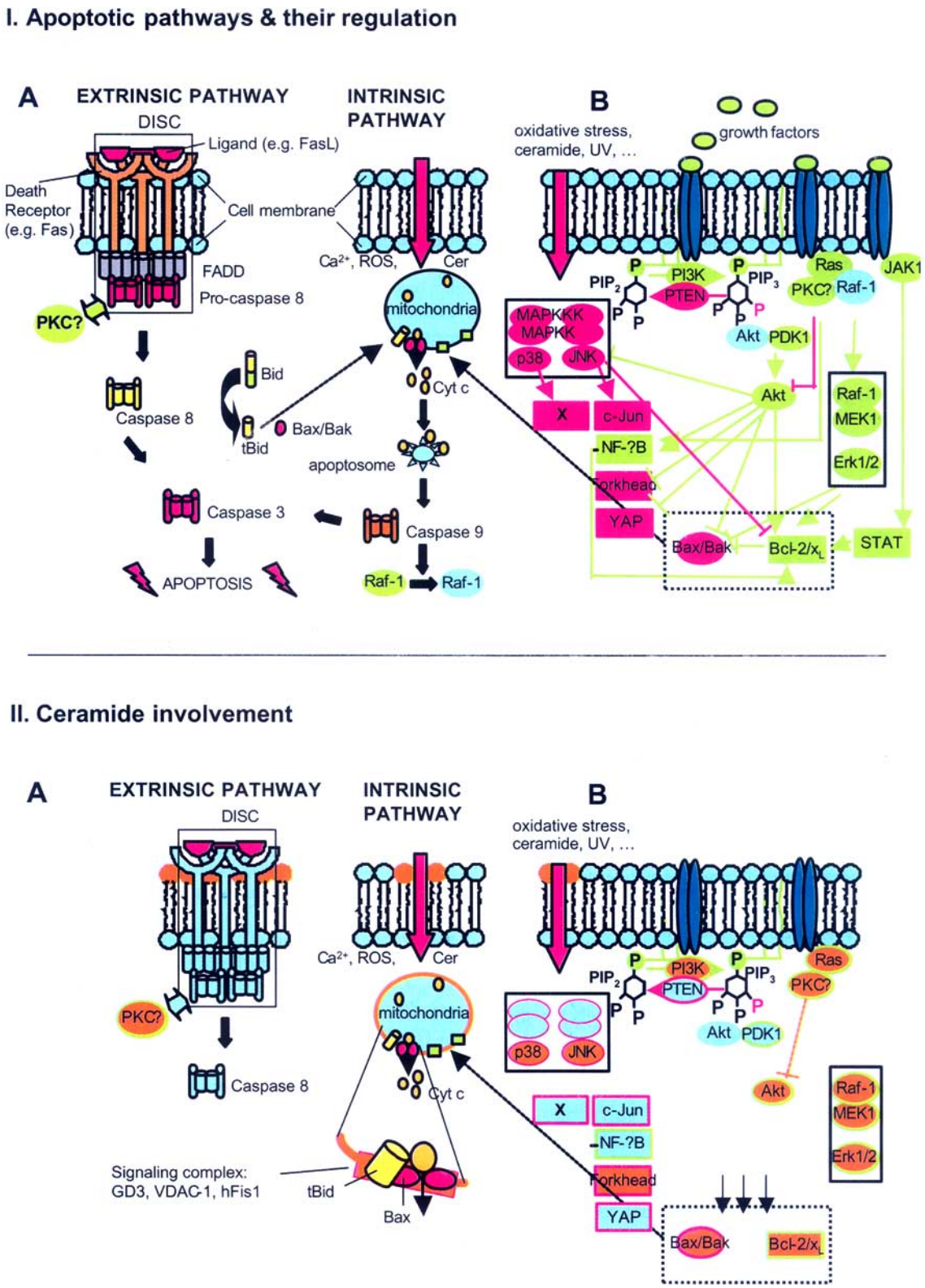



**Fig. (1). Panel I.** Schematic representation of *A*, instrinsic and extrinsic apoptotic pathways and *B*, the regulation of the Bcl-2 balance. The Bcl-2 balance (depicted in box with dashed lines) is influenced/regulated by various kinases and phosphatases (represented by ovals) and transcription factors (represented by boxes). Downstream transcription factors of p38 are represented by 'X'. Red and green colors represent pro-apoptotic and anti-apoptotic scenarios, respectively. MAPK signaling cascades are boxed, see text for more detailed explanation; **Panel II**. Ceramide (Cer) generation (orange) and effect of Cer on different steps of the apoptotic pathways (scheme is a simplified version of panel I). Kinases, phosphatases, transcription factors and apoptotic effector molecules that are known to be influenced by Cer are in orange. See text for more explanation.

membrane. This leads to mitochondrial permeabilization (via formation and opening of permeability transition pore (PTP) in the inner membrane or mitochondrial apoptosis-induced channel (MAC) in the outer membrane) and in release of Cyt c and other pro-apoptotic factors into the cytosol [reviewed in 10,11].

Both apoptotic pathways are linked, as the mitochondrial pathway is often required to amplify the relatively weak death-receptor induced apoptotic signal [12]. In hepatocytes, caspase-8 (extrinsic pathway) interacts with the intrinsic apoptotic pathway by cleaving Bid (a pro-apoptotic member of the Bcl-2 family) to form an active fragment tBid. tBid translocates from the cytosol to the mitochondria, where it binds to the specific mitochondrial lipid cardiolipin [13], resulting in destabilization of the lipid bilayer. The translocation of tBid to the mitochondria is associated with the activation of outer mitochondrial membrane proteins Bax/Bak and the release of Cyt c from the mitochondria.

Oxidative stress (induced by $H_2O_2$) can result in activation of both intrinsic or extrinsic pathway, via activation of sphingomyelinase, which results in increased Cer levels [14]. As further explained in this review, Cer can influence both regulation and progression of apoptotic pathways. Tripathi and Hildeman demonstrated that pro-apoptotic $H_2O_2$ is involved in suppression of anti-apoptotic Bcl-2 levels, sensitizing cells to the apoptotic effects of pro-apoptotic protein Bim [15]. Interestingly, ROS are also implicated in the extrinsic apoptosis pathway through modulation of FasL levels: ROS can induce FasL expression through altering $Ca^{2+}$ signaling, but whether or not this effect is mediated by $O_2^-$ or $H_2O_2$ and how these molecules specifically alter $Ca^{2+}$ signaling is not yet clear [15].

## REGULATION OF APOPTOTIC PATHWAYS

At least three key pathways originating at receptor tyrosine kinases activated by growth factors are known to interfere with apoptotic signal transduction and promote cell survival, namely (i) JAK kinases, (ii) mitogen-activated protein kinase (MAPK) cascade involving Raf-1 and ERK1/2 and (iii) PI3K/Akt signaling pathway [reviewed in 16]. In contrast, (i) JNK MAP kinase and (ii) p38 MAP kinase are involved in promotion of apoptosis, following survival-factor withdrawal, induction of oxidative stress or Cer generation [reviewed by 17]. MAPK signaling cascades involve activation of MAPK (such as ERK1/2, JNK or p38) through a sequential kinase cascade that includes MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) (Fig. **1,I**), MAPK cascades are represented by boxes). In the following paragraph, these signaling cascades and their effect on Bcl-2/Bax rheostat and apoptotic machinery will be reviewed. In addition, emphasis will be put on kinase C, regulating several of the above mentioned signaling pathways. Note that this review focuses on regulatory signaling pathways that can be affected by Cer (see further).

### (A) Anti-Apoptotic Signaling Pathways

(i) Activation of so-called Janus kinases (JAK), after binding of cytokines to their receptor, triggers activation of signal transducer and activator of transcription (Stat) proteins. Stats are a family of transcription factors that bind to γ-IFN activation site (GAS) motifs, which may modulate Bcl-2/Bax rheostat [18] (Fig. **1,I**).

(ii) Activation of the Raf-1 kinase activity is tightly regulated and involves targeting to the membrane by Ras and phosphorylation by various Ras-activated kinases [19]. Raf-1 belongs to MAPK cascade consisting of Raf-1 (MAPKKK), mitogen-induced extracellular kinase 1 (MEK-1) (MAPKK), and extracellular-regulated kinase (Erk1/2) (MAPK). Raf-1 kinase interferes with apoptosis induction by either directly influencing Bcl-2 function or controlling levels of Bcl-2 via MEK-1 Erk1/2 kinase activation. Cornelis and coworkers recently demonstrated that apoptosis of hematopoietic cells upon growth factor withdrawal is associated with caspase-9 mediated cleavage of Raf-1 [20] (Fig. **1,I**).

(iii) Third pathway frequently activated by growth factor receptor stimulation is the phosphatidylinositol 3-kinase (PI3K) pathway, leading to activation of protein kinase B (PKB), also denoted Akt. When IGF-1, insulin, and other growth factors bind to their membrane tyrosine kinase receptors, PI3K is activated and phosphorylates the membrane phospholipids phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3) [reviewed in 21]. This leads to recruitment of Akt and its activator, 3-phosphoinositide-dependent protein kinase-1 (PDK1), to the cell membrane. This colocalization of Akt and PDK1 causes the latter to phosphorylate and activate the former. Activation of Akt (i) controls the expression level of Bcl-2, as well as activity of pro-apoptotic Bad. Phosphorylated Bad is prevented to translocate to the mitochondria and participates in the release of pro-apoptotic factors [16] (Fig. **1,I**). Akt also (ii) negatively regulates transcription factors (such as forkhead (FKHRL1) and YAP) that direct the expression of several cell death genes, and (iii) promotes NF-κB-mediated transcription of genes that encode pro-survival proteins. Activation of Akt can be inhibited by phosphatases such as PTEN, SHIP, PP2A (protein phosphatase 2A).

### (B) Pro-Apoptotic Signaling Pathways

(i) c-Jun N-terminal kinase (JNK) is also called stress activated protein kinase (SAPK). The activation of JNK is mediated through a sequential kinase cascade that includes MKK4/7 (MAPKK) and MLKs (MAPKKK) [reviewed by 22-24] (Fig. **1,I**). Upon activation (via various stimuli such as $H_2O_2$ or TNF), JNKs phosphorylate and activate the transcription factor c-Jun, which is the critical mediator of the pro-apoptotic machinery. Activated c-Jun leads to upregulation of cell death genes, such as Bim. In addition, JNK phosphorylates (i) anti-apoptotic proteins Bcl-2 and Bcl-$X_L$, resulting in their inactivation, and (ii) pro-apoptotic Bim, leading to activation of Bax/Bak-mediated Cyt c release.

(ii) The activation of p38 MAPK is mediated through a sequential kinase cascade that includes MKK3/6 (MAPKK) and e.g. ASK1 (MAPKKK) [reviewed by 25]. Furthermore, it has been shown that the upstream activating MAP kinases of JNK can also activate p38, explaining why p38 and JNK are often co-activated. Downstream substrates of the p38 group MAP kinases include protein kinases and various transcription factors (Fig. **1,I**).

### (C) Modulation of Signaling Cascades by Protein Kinase C

Members of the protein kinase C (PKC) family of serine-threonine kinases have been shown to play an important role in both, inhibition and stimulation of apoptosis [reviewed in 26]. At present, at least 10 PKC isoforms have been cloned. They have been divided into three subfamilies that differ in structure and dependencies on specific activators (such as $Ca^{2+}$, phosphatidylserine (PS) and diacylglycerol (DAG)) [26]. PKC-α, PKC-βII and PKC-δ are pro-apoptotic lamin B kinases, resulting in phosphorylation of lamin B and subsequent degradation. Lamin B is a key nuclear polypeptide belonging to the nuclear matrix and its phosphorylation leads to caspase-6-mediated proteolytic degradation and nuclear lamina disassembly. However, nuclear PKC isoforms, such as anti-apoptotic PKC-ζ have been shown to protect cells from apoptosis, acting downstream of PI3K and PDK1 in the PI3K/Akt survival pathway [27,28] and/or upstream of Raf-1 [29]. In addition, at least a fraction of PKCζ has been shown to interact with DISC components upon Fas activation, thereby conferring significant protection against FasL-induced apoptosis in Jurkat cells [30] (Fig. **1,I**). However, PKCζ can also suppress cell growth of certain cell types, including human embryonic kidney cells, and act as a negative regulator of Akt [reviewed by 31]. Since PKCζ is a direct binding target of ceramide (see further), it is likely that PKCζ may regulate ceramide-induced apoptosis.

## CERAMIDE AND APOPTOSIS

In recent years, it has become increasingly clear that ceramides/sphingolipids play an important role in apoptosis and cellular regulation [reviewed in 32-34]. Sphingolipids comprise a family of lipid molecules that include the long chain bases sphingosine, sphinganine and their related phosphorylated derivatives, ceramides, gangliosides, neutral glycolipids and sulfatides among others [reviewed in 35]. Sphingolipids, similarly to sterols, are important constituent membrane lipids that are critical for the normal function and viability of eukaryotic cells. They play important structural roles in cell recognition and adhesion and in the formation of functional lipid microdomains, termed lipid rafts. Lipid rafts are highly enriched in sterols and sphingolipids; their distinct lipid composition accounts for the partitioning of specific proteins involved in signal transduction pathways, cell adhesion, and other cell polarity processes [reviewed by 36]. In addition, sphingolipid precursors/metabolites, namely, ceramides (Cer) and Cer phosphate (CerP), long-chain bases (LCBs) and long-chain base phosphates (LCBPs) have been shown to affect cell growth, differentiation, and death. Cer and LCBs usually inhibit proliferation and promote apoptosis, while the further metabolites CerP and LCBP stimulate growth and suppresses apoptosis. Because these metabolites are interconvertible, it has been proposed that it is not the absolute amounts of these metabolites but rather their relative levels that determines cell fate. This balance is maintained by a group of sphingolipid metabolic enzymes, which can be referred to as the Cer/LCBP rheostat. The intracellular level of Cer is regulated by *de novo* synthesis through activation of serine-palmitoyl transferase, the rate limiting enzyme in Cer synthesis [reviewed by 37]. However, Cer levels can also arise from hydrolysis of sphingomyelin through the neutral and acid sphingomyelinase (SMase) as further discussed below.

### (A) Sphingolipid Metabolic Enzyme

Cer can be formed through SMase-dependent catabolism of sphingomyelin (SM), as well as by *de novo* synthesis (Fig. **2**) [reviewed by 37-39]. *De novo* Cer biosynthesis requires the coordinated action of serine palmitoyltransferase and Cer synthase to generate Cer. This process begins with the condensation of serine and palmitoyl-CoA. Alternately, this pathway may re-utilize sphingosine released by sequential degradation of more complex sphingolipids for Cer synthesis. SMases, specialized enzymes with phospholipase C activity, hydrolyze the phosphodiester bond of SM. Several isoforms of SMase can be distinguished by their different pH optima, and some but not all of these molecules have been identified at the molecular level. Neutral SMase (nSMase) and acid SMase (aSMase) are rapidly activated by diverse stress stimuli (including oxidative stress) and promote an increase in cellular Cer levels over a period of minutes to hours. Alkaline SMase activity is found in the intestinal mucosa and bile and does not appear to participate in signal transduction.

Ceramide clearance can occur through activation of (i) sphingomyelin synthase, resulting in production of SM, (ii) glucosylceramide synthase, resulting in production of glucosylceramide which is the precursor for synthesis of complex sphingolipids such as gangliosides (such as GD3, see further) and sulfatides, or (iii) ceramidases leading to sphingosine production (Fig. **2**).

This review will only focus on the molecular mechanisms of action of pro-apoptotic Cer and GD3 in different stages of the two apoptotic pathways (Fig. **1,II**). More detailed information can be found in following reviews [31,40,41].

### (B) Pro-Apoptotic Ceramide and GD3

#### *Effects on extrinsic apoptotic pathway*

Early events required for induction of apoptosis by death receptor family (e.g. Fas) are preassociation of Fas, the formation of DISC and clustering of Fas in distinct membrane domains. Grassme and coworkers showed that aSMase functions upstream of DISC formation, mediating CD95 clustering in Cer-enriched membrane platforms [42]. Apparently, activation of the death receptors by their specific ligands results in translocation of aSMase from intracellular

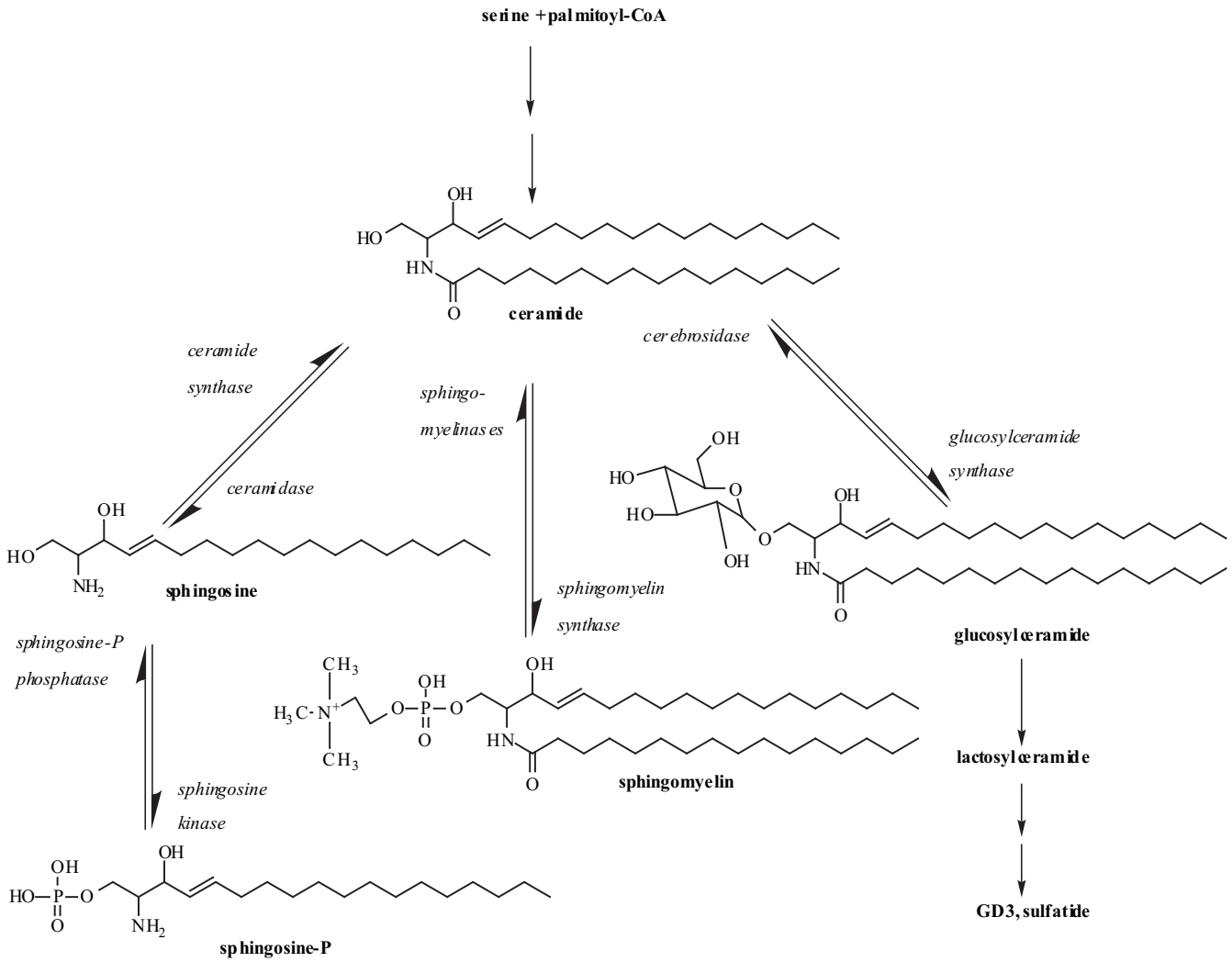



**Fig. (2).** Ceramide (Cer) biosynthesis and metabolism. Cer can be generated through *de novo* synthesis or catabolism of SM, glucosylceramide and sphingosine. Cer can be converted to (i) glucosylceramide via glucosylceramide synthase and further to complex sphingolipids (e.g. GD3); (ii) sphingomyelin via sphingomyelin synthase, or (iii) sphingosine via ceramidase.

stores to the extracellular leaflet of the plasma membrane, where it co-localizes with sphingolipids in rafts [reviewed by 43,44]. Within 1 min following activation of the death receptor family members, Cer is generated in lipid rafts in which death receptors reside. This generation of Cer results in the fusion of small rafts to large platforms or membrane macrodomains, hence clustering the death receptors. This is due to the unique biochemical properties of Cer, being the capability to self-associate through hydrogen bounding. Subsequently, caspase-8 is activated in the DISC complexes, and then triggers the entry into the execution phase of apoptosis.

### *Effect on Intrinsic Apoptotic Pathway*

Mitochondria, key players in the orchestration of death signals, contain small amounts of various sphingolipids, including pro-apoptotic ganglioside GD3 and Cer. As outlined by van Blitterswijk and coworkers, there are a number of indications that Cers are mediators of apoptosis at the mitochondrial level [41]. Apparently, short-chain as well as long-chain (natural) Cer induce Cyt c release from mitochondria, leading to apoptosis. Cer does not trigger a particular mechanism for Cyt c release, but increases the permeability of the mitochondrial outer membrane (via Cer channel formation) for a number of small proteins, including Cyt c [45]. Recently, Stoica and coworkers showed that Cer (using exogenous C(2) ceramide as well as inducing endogenous ceramide accumulation using inhibitors of glucosylceramide synthetase) induced the translocation of certain, but not all, pro-apoptotic mitochondrial proteins: Cyt c, Omi, SMAC, and AIF were released from the mitochondria, whereas EndoG was not [46].

Apart from Cer, the Cer-derived glycosphingolipid (ganglioside) GD3 is implicated in mitochondrion-dependent apoptosis [reviewed by 41]. In contrast with natural Cer, GD3 appears to act on the mitochondrial permeability transition pore, secondary to the generation of ROS, eventually leading to Cyt c release [47,48]. Additionally, Garofalo and coworkers demonstrated the presence of microdomains in mitochondria composed of ganglioside GD3, the voltage-dependent anion channel-1 (VDAC-1) and the fission protein hFis1. In this multimolecular signaling complex, pro-apoptotic Bcl-2 family proteins (t-Bid and Bax) were recruited. Disruption of such lipid microdomains

prevented mitochondria depolarization induced by GD3 or t-Bid. These data point to a scenario in which mitochondria-associated lipid microdomains can act as regulators and catalysts of cell fate [49].

### *Effect on Regulatory Kinases*

Past years, more and more evidence is accumulating indicating that Cer activates a number of protein kinases and phosphatases involved in stress-signaling pathways [reviewed in 31,38,40,41,50]. Cer has been shown to directly regulate (i) stress-activated protein kinases (SAPKs, such as pro-apoptotic JNKs), (ii) kinase suppressor of Ras (KSR), which is a conserved component of the Ras pathway that acts as a molecular scaffold to promote signal transmission from Raf-1 to MEK and MAPK, (iii) various PKC isoforms (such as PKC-ζ), (iv) c-Raf-1, (v) phospholipase $A_2$, (vi) cathepsin D, and (vii) Cer-activated protein phosphatases (CAPPs). These CAPPS, such as protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), dephosphorylate (i) Bcl-2 proteins, rendering them inactive and (ii) anti-apoptotic kinases, including PKB/Akt, PI3K and ERK1/2. In this respect, Stoica and coworkers showed that Cer-induced neuronal apoptosis was associated with (i) dephosphorylation of ERK1/2, Akt, Bad, forkhead transcriptional factor (FKHR), and glycogen synthase 3-b; (ii) hyperphosphorylation of pro-apoptotic p38 MAP kinases; and (iii) induction of the mitochondria-dependent pathway [46,51]. In addition, Cer was also found to specifically prevent the membrane recruitment of Akt to the PI3K-product $PIP_3$, leading to Akt dephosphorylation and inactivation [52]. A direct binding of Cer to proteins/enzymes however, has only been found for cathepsin D, PLA2, c-Raf, PKCα, -β, and -ζ. Cer-activated PKC-ζ has been shown to directly bind and subsequently inactivate various kinases such as Akt [reviewed by 41].

## APOPTOSIS-RELATED DISEASES

Apoptosis is implicated in a number of diseases, including neurodegenerative diseases and AIDS, which are characterized by apoptosis of neuronal cells and T cells, respectively. In the following paragraph, evidence will be summarized regarding Cer generation and onset of these diseases.

### (A) Neurodegenerative Diseases

#### *Alzheimer's Disease*

Alzheimer's disease (AD) is a major neurodegenerative illness defined as dementia accompanied with amyloid plaques and neurofibrillary tangles. Amyloid plaques are extracellular aggregates of amyloid-β (Aβ) protein, a 40–43 amino acid proteolytic fragment derived from the amyloid precursor protein (APP), whereas tangles consist of intracellular aggregates of the microtubule-binding protein Tau. In between the soluble and fibrillar (β-sheeted) forms of Aβ, different intermediates with different levels of toxicity have been identified (and referred to as ADDL's, protobrils etc). Although it remains controversial whether apoptosis is the main mechanism of neuronal cell death in AD, it has been shown that several forms of the Aβ peptide are apoptotic and cytotoxic to neuronal cells. Aβ-induced neuronal cell death may occur through a mitochondrial-dependent pathway [reviewed in 53]. In addition, Morishima and coworkers demonstrated that Aβ also activates JNK in cortical neurons, resulting in phosphorylation and activation of the c-Jun transcription factor [54].

Interestingly, fibrillar Aβ peptides were recently shown to induce the activation of nSMase via NADPH oxidase-mediated superoxide production, and concomitantly, the production of Cer in human primary neurons and oligodendrocytes [55,56]. Also Cutler and coworkers reported alterations in sphingolipid metabolism during normal brain aging and in the brains of AD patients that resulted in accumulation of Cer [57]. In accordance with these findings, Satoi and coworkers found that Cer was significantly increased in the cerebrospinal fluid (CSF) in patients with AD as compared to neurological control patients [58]. In addition, Han and coworkers demonstrated that sulfatides were depleted up to 93% in gray matter and up to 58% in white matter from all examined brain regions from AD subjects, whereas the content of Cer was elevated more than three-fold in white matter and peaked at the stage of very mild dementia [59]. All these data suggest a sequence of events in the pathogenesis of AD in which Aβ induces membrane-associated oxidative stress, resulting in perturbed Cer and cholesterol metabolism which, in turn, triggers an apoptotic cascade in neuronal cells. Recently, Costantini summarized current knowledge regarding Cer and its role as a signaling molecule connecting aging to AD [60].

#### *Ischemia*

Ischemic white matter (WM) lesions represent an age-related neurodegenerative condition that appear as hyperintense signals on magnetic resonance (MR) images. Chronic cerebral ischemia is likely to be the cause of these ischemic WM lesions, which are responsible for the cognitive decline and gait disorders in the elderly. Using a rat model of chronic cerebral ischemia, Ohtani and coworkers demonstrated that Cer level in astroglia were increased as a result of downregulation of glucosylceramide synthase (GCS) and an upregulation of aSMase activity [61]. Nakane and coworkers also demonstrated that lethal forebrain ischemia stimulates SM hydrolysis and Cer generation in the gerbil hippocampus [62]. Since aSMase-deficient mice are known to be more resistant to acute ischemic insults than the wild-type mice, the observed elevated Cer-levels in acute cerebral ischemia may directly induce apoptosis [63]. Moreover, also nSMase has been implicated in Cer-generation in ischemia in mice. Soeda and coworkers showed that administration of nSMase inhibitors (see further) significantly reduced Cer formation and apoptosis and reduced significantly the size of the cerebral infarcts, compared to the control mice [64].

### (B) AIDS

Human immunodeficiency virus type 1 (HIV-1) induces a dramatic depletion of CD4+ T cells in infected individuals finally leading to AIDS (acquired immunodeficiency syndrome). This massive loss of CD4 T-lymphocyte appears to result from apoptosis in both, directly infected cells and in uninfected bystander cells. It appears that the principal

pro-apoptotic molecule encoded by HIV-1 is a part of the envelope glycoprotein complex, namely gp120. Addition of soluble gp120 to lymphocytes causes rapid intrinsic apoptosis, via stimulation of the p53 pathway and p38 MAPK [reviewed by 65]. HIV-infected cells are characterized by enhanced SM breakdown and accumulation of intracellular Cer [66,67]. Moreover, patients with AIDS have significantly higher lymphocyte-associated Cer levels than healthy individuals [66]. In general, HIV-1-infected individuals develop AIDS within several years, but about 5% remain healthy over an extended time (and are known as long-term nonprogressors) [68]. Interestingly, De Simone and coworkers observed that HIV-1-infected long-term nonprogressors have less elevated lymphocyte-associated Cer levels than subjects with evolving disease [66,69,70]. Remarkably, this is paralleled by a lower frequency of apoptotic CD4 and CD8 cells in long-term nonprogressors than in patients with AIDS.

## ANTI-APOPTOTIC COMPOUNDS THAT REDUCE CERAMIDE LEVELS

Based on the above reviewed data regarding the correlation between Cer generation and onset of various apoptotic diseases, one can envisage that therapeutic strategies aimed at downregulating intracellular Cer levels may slow the progression of these diseases [reviewed in 71]. Elevated Cer levels may induce/modulate extrinsic and/or intrinsic apoptotic pathways following one or more of the regulatory pathways described above. The following paragraph will focus on compounds that reduce Cer content and hence, can provide a cure for various apoptotic diseases. Since oxidative stress results in SMase activation and accumulation of Cer (see above), compounds with antioxidant properties (such as vitamin E, curcumin, melatonin, Coenzyme Q10 and specific plant extracts [reviewed in 72-74]) are used in various conditions including neurodegenerative diseases. Such compounds will not be discussed in the current review. We will mainly focus on compounds that directly inhibit SMase activity. Other (theoretical) approaches that result in reduced ceramide content could be (i) upregulation of glucosylceramide synthase activity or (ii) physical protection of SM against degradation. (i) Upregulation of glucosylceramide synthase activity (GCS) has been reported in multidrug-resistant tumor cell lines [75,76]. The only naturally occurring compounds that are known to upregulate glucosylceramide synthesis are basic fibroblast growth factor (bFGF) and laminin. bFGF affects GCS activity, possibly via post-translational modification of GCS [77]. bFGF has no effect on the rate of sphingomyelin synthesis. It is currently not clear how binding of bFGF (and laminin) to their respective cell surface receptors leads to post-translational modification of GCS. (ii) Compounds that would protect SM from breakdown by SMase, e.g. *via* physical interaction/sequestration of SM, are hitherto not reported.

### (A) Acidic Sphingomyelinase Inhibitors

In this paragraph, inhibitors of sphingomyelinase activity (e.g. carnitine) and drugs that induce degradation of sphingomyelinase (e.g. desipramine) will be discussed. Chemical structures of these compounds are depicted in (Fig. **3**). If possible, chemical structures of the inhibitors are presented in the same configuration as sphingomyelin.

L-carnitine is a naturally occurring quaternary amine zwitterion (β-hydroxy-γ-(trimethylammonium)-butyrate; Fig. (**3B**)) and is required for transformation of free long-chain fatty acids into acylcarnitines, and for their subsequent transport into the mitochondrial matrix, where they undergo β-oxidation for cellular energy production. Carnitine concentrations vary from 0.1 to a few mM, depending on the tissue. Highest concentrations of carnitine are found in cardiac and skeletal muscle. Other functions of carnitine are protection of membrane structures, stabilizing a physiologic coenzyme-A (CoA)-sulfate hydrate/acetyl-CoA ratio, and reduction of lactate production. On the other hand, numerous *in vitro* experiments have demonstrated that carnitine and its derivatives acetylcarnitine (Fig. **3B**) and propionylcarnitine have anti-apoptotic properties [78-80], most likely by preventing SM breakdown and consequent Cer synthesis; this effect seems to be specific for aSMase [reviewed in 81].

The use of a simple metabolite like carnitine as an anti-apoptotic agent is very attractive since it is likely to be relatively non-toxic compared to synthetic inhibitors. The chemotherapeutic effects of L-carnitine have already been demonstrated in AIDS [82,83] and Alzheimer's disease [84]. Recently, Fringuelli and coworkers synthesized various new carnitine derivatives and amino acid analogues (including (R)-4-acetoxy-2-pyrrolidinone, *N*-(2-oxopiperidin-3-yl)dodecanamide and *n*-propyl $N^2$-[(benzyloxy)carbonyl]-α-asparaginate; (Fig. **3B**). All these derivatives inhibited Fas-induced apoptosis of human Jurkat T-cell line [85].

Desipramine, a tricyclic antidepressant drug (Fig. **3B**), has been shown to reduce SMase activity by accelerating its degradation [86]. Desipramine was found to induce rapid intracellular degradation of mature aSMase when added to cultured human skin fibroblasts in the micromolar range, concomitantly abolishing the enzymatic activity. Desipramine was shown to prevent the increase in Cer levels and to inhibit apoptosis of various cell lines after treatment with apoptosis-stimulating compounds (such as macrophage colony-stimulating factor deprivation in bone marrow-derived macrophages [87]; histon deacetylase inhibitor/perifosine-treatment of human leukemia cells [88]; and Cr(VI)-induced activation of apoptosis in CHO cells [89]). Imipramine is another tricyclic antidepressant drug (Fig. **3B**) with inhibitory activity against aSMase: it was shown to cause accumulation of SM [90,91]. In addition, a novel tricyclic compound, NB6 (Fig. **3B**), was shown by Deigner and coworkers, to inhibit transcription of aSMase [92]. Various *in vitro* studies demonstrated its anti-apoptotic activity: NB6 was shown to decrease Cer generation and inhibit pro-apoptotic protein kinases (e.g. p38 MAPK and JNK) in response to oxidized lipoproteins in arterial smooth muscle cells [93,94]. However, no *in vivo* data regarding the anti-apoptotic potential of these tricyclic drugs currently exist.

### (B) Neutral Sphingomyelinase Inhibitors

The membrane neutral magnesium-dependent SMase (nSMase) is distributed primarily in the brain. Selective

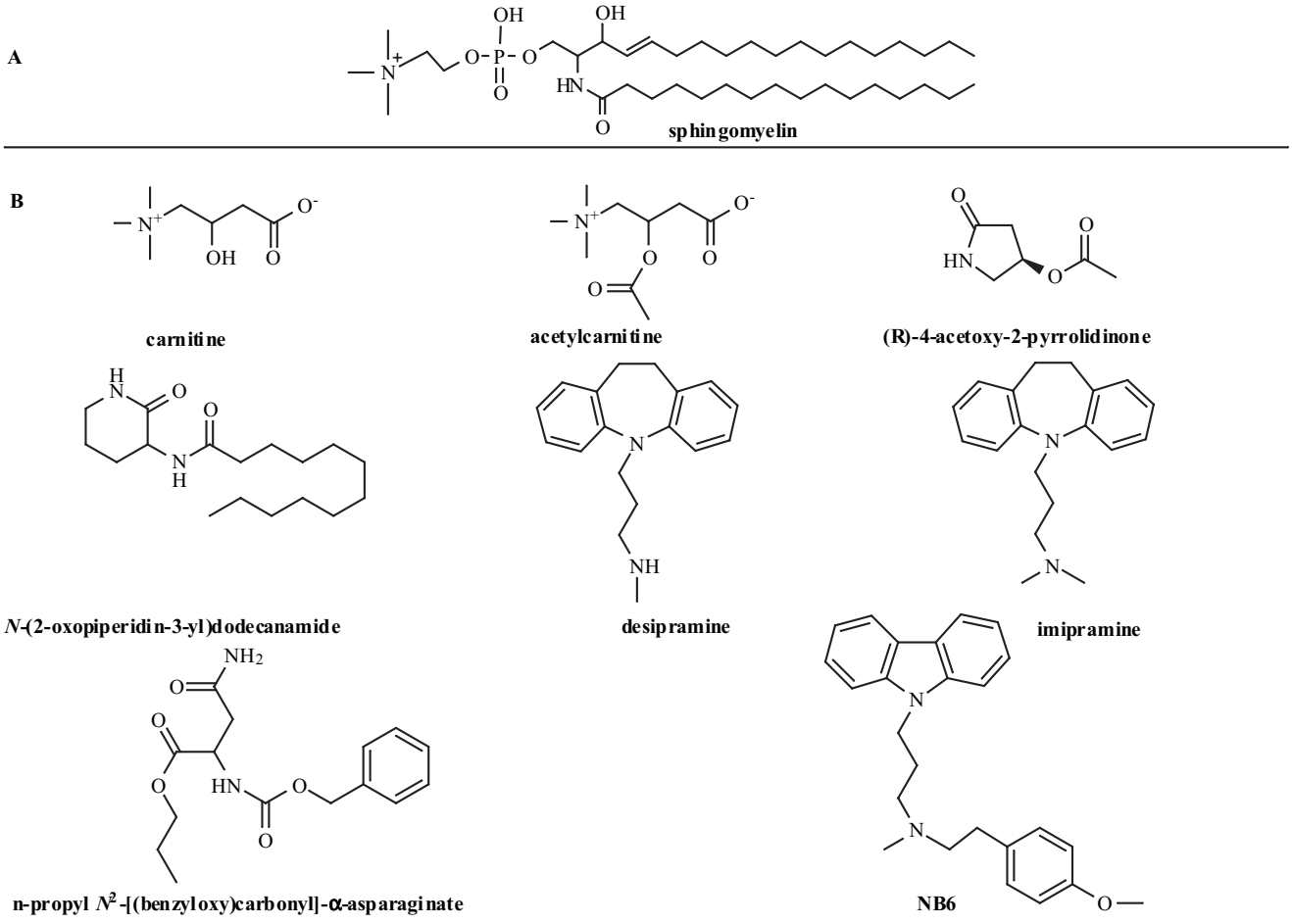
nSMase inhibitors present significant potential to prevent neuronal cell-death caused by cerebral ischemia and neurodegenerative diseases.

Scyphostatin (Fig. **3C**), isolated from the mycelial extract of the fungus *Trichopeziza mollissima*, is the first specific potent low-molecular-weight reversible inhibitor of the nSMases and was shown to possess anti-apoptotic properties [95]. Claus and coworkers recently synthesized a scyphostatin desepoxy analogue (referred to as scyphostatin analogue 14, (Fig. **3C**) [96]. This analogue is a chemically and metabolically stabilized compound lacking the epoxy function of the natural congener and carrying a palmitic acid group instead of the native trienoyl residue. An evaluation of the biological activity of scyphostatin analogue 14 revealed nSMase inhibition in several *in vivo* test systems (monocytes, macrophages, hepatocytes) [96]. Arenz and coworkers reported on the synthesis of the spiroepoxide 2 (Fig. **3C**), which was synthesized as another analogue of the natural product scyphostatin [97]. Spiroepoxide 2 inhibits nSMase, but contrary to scyphostatin, it is an irreversible inhibitor of this enzyme. In addition, Arenz and coworkers further synthesized two analogues of spiroepoxide 2 (Fig. **3C**) in which the primary hydroxy group was replaced by hydrogen or a phenyl group [98]. The inhibitory potency of these two epoxides towards nSMase was drastically reduced, compared to the known inhibitor spiroepoxide 2, indicating that the primary hydroxy group is crucial for nSMase inhibition. In addition, these investigators also identified the antibiotic manumycin A (Fig. **3C**) as a potent irreversible nSMase inhibitor [99].

Luberto and coworkers performed a high throughput screen for nSMase [100]. One inhibitor discovered in the screen was GW4869 (Fig. (**3C**)). This compound inhibited specifically nSMase at 1 μM, not only *in vitro* but also in a cellular model: GW4869 was shown to partially inhibit TNF-induced activation of nSMase in MCF7 cells [100]. GW4869 was able, in a dose-dependent manner, to significantly protect from cells from apoptosis, and consequently, of Cyt c release and caspase-9 activation.

Soeda and coworkers synthesized a series of difluoromethylene analogues of sphingomyelin (SMAs), replacing the long alkenyl chain and the phosphodiester moiety of SM by a phenyl and an isosteric difluoromethyl-enephosphonic acid, respectively (e.g. SMA-7, Fig. **3C**). These SMAs were shown to inhibit the enhanced nSMase activity in the serum/glucose-deprived neuronally differentiated pheochromocytoma cells, thereby suppressing the apoptotic sequence involving Cer formation [64]. Furthermore, *in vivo* studies showed that SMA-7 significantly reduced infarcted area caused by occlusion of the middle cerebral artery (MCA) in mice [64].

Finally, Taguchi and coworkers designed and synthesized hydrolytically stable analogues of sphingomyelin as nSMase inhibitors. These novel analogues replace the phosphodiester moiety of sphingomyelin with carbamate and urea moiety,



**(Fig. 3). contd.....**

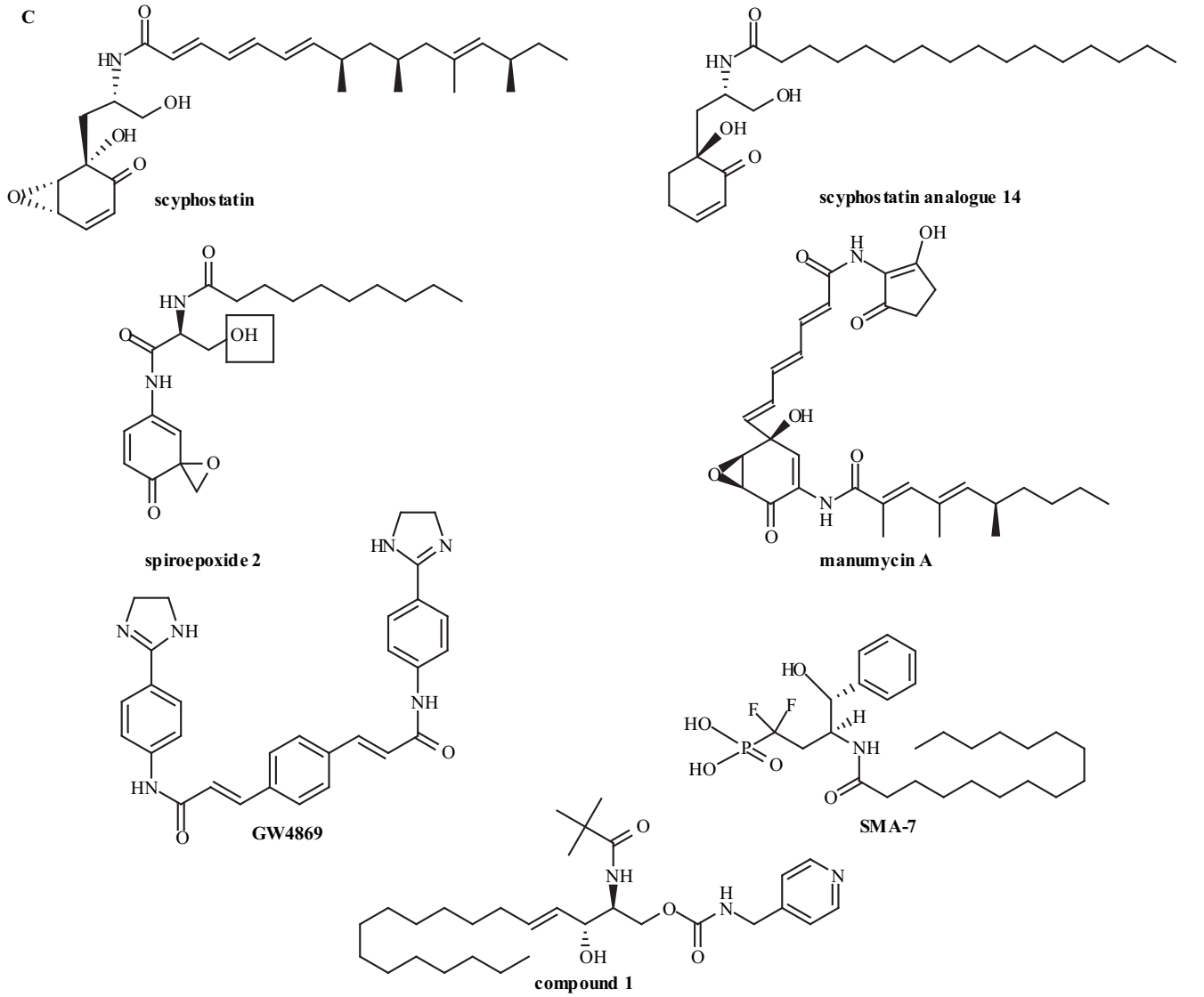



**Fig. (3).** Chemical structures of *A*, sphingomyelin, *B*, acid sphingomyelinase inhibitors and *C*, neutral sphingomyelinase inhibitors. For comparison, chemical structures of the inhibitors are presented in the same configuration as sphingomyelin (if possible). Primary hydroxyl group of spiroepoxide 2 is boxed, see text for explanation.

resulting in inhibition of neutral sphingomyelinase. Compound 1 (Fig. **3C**) prevented Cer generation and apoptotic neuronal cell death in a model of ischemia, based on organotypic hippocampal slice cultures [101].

## CONCLUSIONS

It is clear that Cer induce the apoptotic machinery in various cell types, and that Cer action affects different regulatory and executioner stages of apoptotic pathways. Moreover, Cer generation seems to be an early event in onset of various apoptotic diseases. Inhibition of Cer generation by compounds that act on SMase action seems to counteract apoptosis, not only *in vitro* but in various *in vivo* cell systems and animal models. Apparently, the current SMase inhibitors either act directly on SMase, or induce degradation or modulate transcription of SMase. In many cases, the structure of these inhibitors resembles parts of the SM structure: some inhibitors resemble the choline ceramide head group, such as carnitine and its derivatives; whereas other inhibitors resemble the acyl chain of SM (e.g. scyphostatin analogue 14 and SMA-7), or the sphingoid base (e.g. compound 1). Some SMase inhibitors seem to display no resemblance to SM structure, as in case of GW4869 and tricyclic inhibitors. It is clear that more studies are needed to unravel the precise way of SMase inhibition by different classes of inhibitors. In addition, novel compounds targeting SMase activity or alternatively, GCS activity, should be identified. Theoretically, protection of SM from breakdown, e.g. via physical interaction of SM with a chemical compound, could be a novel target to identify new anti-apoptotic compounds.

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| TNF | = | Tumor necrosis factor |
| DISC | = | Death-inducing signaling complex |
| ROS | = | Reactive oxygen species; cytochrome *c* |
| Cyt c; MEK-1 | = | Mitogen-induced extracellular kinase 1 |
| ERK | = | Extracellular-regulated kinase |
| PI3K | = | Phosphatidylinositol 3-kinase |
| PKB/Akt | = | protein kinase B |
| JNK | = | c-Jun N-terminal kinase |
| HSP | = | Heat shock protein |
| Cer | = | Ceramide |
| LCB | = | Long-chain base |
| nSMase | = | Neutral sphingomyelinase |
| aSMase | = | Acid sphingomyelinase |
| SM | = | Sphingomyelin |
| AD | = | Alzheimer's disease |
| PKC | = | Protein kinase C |

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