

REVIEW

Drug targeting of sphingolipid metabolism: sphingomyelinases and ceramidases

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Sphingolipids represent a class of diverse bioactive lipid molecules that are increasingly appreciated as key modulators of diverse physiologic and pathophysiologic processes that include cell growth, cell death, autophagy, angiogenesis, and stress and inflammatory responses. Sphingomyelinases and ceramidases are key enzymes of sphingolipid metabolism that regulate the formation and degradation of ceramide, one of the most intensely studied classes of sphingolipids. Improved understanding of these enzymes that control not only the levels of ceramide but also the complex interconversion of sphingolipid metabolites has provided the foundation for the functional analysis of the roles of sphingolipids. Our current understanding of the roles of various sphingolipids in the regulation of different cellular processes has come from loss-of-function/gain-of-function studies utilizing genetic deletion/downregulation/overexpression of enzymes of sphingolipid metabolism (e.g. knockout animals, RNA interference) and from the use of pharmacologic inhibitors of these same enzymes. While genetic approaches to evaluate the functional roles of sphingolipid enzymes have been instrumental in advancing the field, the use of pharmacologic inhibitors has been equally important in identifying new roles for sphingolipids in important cellular processes. The latter also promises the development of novel therapeutic targets with implications for cancer therapy, inflammation, diabetes, and neurodegeneration. In this review, we focus on the status and use of pharmacologic compounds that inhibit sphingomyelinases and ceramidases, and we will review the history, current uses and future directions for various small molecule inhibitors, and will highlight studies in which inhibitors of sphingolipid metabolizing enzymes have been used to effectively treat models of human disease.

Abbreviations

aCDase, acid ceramidase; alkCDase, alkaline ceramidase; aSMase, acid sphingomyelinase; C1P, ceramide-1-phosphate; nCDase, neutral ceramidase; NPD, Niemann-Pick disease; nSMase, neutral sphingomyelinase; PM, plasma membrane; S1P, sphingosine-1-phosphate; SL, sphingolipid; SM, sphingomyelin; Sph, sphingosine; SphK, sphingosine kinase

Introduction – background on sphingolipids

Sphingolipids (SLs) were once considered only structural components of the cellular membranes; however, in the mid 1980s, it was found that sphingosine (Sph) exerted biological and biochemical activities through the inhibition of protein

kinase C (Hannun *et al.*, 1986). This was followed by the demonstration of regulated formation of Sph with effects on cell signalling (Merrill *et al.*, 1986; Kolesnick, 1987). Subsequently, it was shown that ceramide functioned as a second messenger (Okazaki *et al.*, 1989). Later, other SLs such as sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), the phosphorylated products of Sph and ceramide, respectively, were shown also to be bioactive lipids, being

involved in the regulation of apoptosis, proliferation, angiogenesis, cell adhesion, differentiation, migration, senescence, and intracellular trafficking (Aguilar *et al.*, 2010). Moreover, ceramide and S1P often exert opposite roles in the cell. Thus, ceramide mediates cell arrest and cell death in response to cell stress, whereas S1P mediates cell survival and proliferation. Therefore, the interconversion of these metabolites through the action of a number of critical enzymes provides for a rich network of cell regulation. As such, these enzymes provide novel approaches to therapeutic strategies that aim to influence SL-mediated biologic and pathobiologic responses.

SLs comprise a family of lipidic compounds derived from (E)-2-amino-4-octadecen-1,3-diol or Sph, the saturated (sphinganine), or the 4-hydroxy forms (phytosphingosine). N-acylation of the sphingoid base with distinct fatty acids of variable length and/or desaturation or other modifications generate diversity of ceramide species. In turn, ceramide is considered the central hub of SL synthesis (Figure 1), and it can serve as substrate for a number of complex SLs. Ceramide is synthesized *de novo* in the endoplasmic reticulum (ER), and is transported to the Golgi apparatus, where it can be transformed to sphingomyelin (SM) by the transfer of a phosphocholine group from phosphatidylcholine to the C1 hydroxyl in ceramide by SM synthases. However, ceramide, in the Golgi apparatus or ER, can also be glycosylated by glucosyl or galactosyl transferases forming the hexosylceramides (HexCer), glucosylceramide (GlcCer) and galactosylceramide respectively. GlcCer serves as the precursor of complex glycosphingolipids, and these glycolipids along with SM are then transported to the plasma membrane (PM), probably primarily through vesicular trafficking. It is not clear how much ceramide or the other 'simple' SLs exist at the PM. In fact, a recent study in our group (Canals *et al.*, 2010) suggested that not much ceramide is present at the PM under unstimulated conditions, and PM ceramide would be mainly generated from hydrolysis from SM and probably from HexCer. As to the complex SLs, once at the PM, these molecules may be transported/recycled to lysosomes by endocytic vesicles, where they can be degraded to ceramide. Ceramide, in turn, can be hydrolysed to Sph by ceramidases, and the resulting Sph is then recycled through the salvage pathway by re-converting to ceramide, or via phosphorylation to S1P. Detailed reviews on the biosynthetic and metabolic pathway of SLs can be found elsewhere (Delgado *et al.*, 2007; Hannun and Obeid, 2008; Gangoiiti *et al.*, 2010).

Functionally, ceramide serves not only as a structural hub but also as an important bioactive molecule and as a key precursor to produce additional bioactive SLs. Ceramide can be generated from the *de novo* synthesis, which implies many enzymatic steps, or from hydrolysis of SM or HexCer. The latter can result in the accumulation of ceramide directly, or indirectly via the salvage pathway which involves further hydrolysis of lysosomal ceramide to Sph followed by re-acylation to ceramide. Ceramide deriving from SM has been widely studied. Some stress-signalling molecules, such as tumour necrosis factor (TNF)- α or interleukin-1 β (IL-1 β) induce an activation of sphingomyelinases (SMases), which can also be activated by other stress stimulus such as exposure to ultraviolet (UV) light or radioactive radiation. These stimuli have been shown to produce an increase of ceramide and subsequent ceramide-dependent responses, such as cell death or cell arrest.

Hydrolysis of ceramide by ceramidases produces another bioactive lipid, Sph, which in turn can be rapidly phosphorylated by sphingosine kinase (SphK) producing S1P. Therefore, the pathways controlling generation of ceramide, Sph and S1P have emerged as key pathways in regulating the formation and interconversion of these bioactive SLs. Importantly, it should be noted that the cellular levels of ceramide are significantly higher than those of Sph, which in turn are significantly higher than those of S1P. Indeed, S1P is bioactive at concentrations two to three orders of magnitude lower than those of ceramide, consistent with their relative cellular concentrations. Thus, even fractional conversion of ceramide to Sph or S1P can have profound cellular effects.

Aberrations in ceramide and bioactive SLs and their metabolism have been linked to various human conditions, including cancer pathogenesis, response to cancer therapeutics, diabetic complications, neurodegeneration, inflammatory responses and ischaemia-reperfusion (heart, liver and brain). Thus, understanding these pathways has significant implications not only to their biochemistry and cell biology, but also for possible therapeutic development.

The present review is focused on the drug targeting of ceramide metabolizing enzymes, notably SMases and ceramidases (Figure 2). Table 1 summarizes the most commonly used inhibitors for those enzymes.

Sphingomyelinases

Neutral SMases (nSMases)

Background. nSMases are a class of phosphodiesterases, which preferentially hydrolyse SM, producing phosphocholine and the bioactive lipid ceramide. These enzymes possess a neutral pH optimum distinguishing them from acid and alkaline SMases. Several genes having nSMase enzymatic activity exist in nature including mammalian, fungal and bacterial versions (Clarke *et al.*, 2006). Pharmacological targeting of mammalian nSMase activity will be discussed with an emphasis on nSMase2.

Diverse stress stimuli such as TNF- α and oxidative stress have been shown to cause an increase in nSMase activity resulting in ceramide production (Adam *et al.*, 1996; Liu *et al.*, 1998). Exactly how this activation occurs is not well understood. Several possibilities exist for the activation of nSMase2, including phosphorylation (Filosto *et al.*, 2011), anionic lipid binding (Marchesini *et al.*, 2003), translocation to the PM, direct redox sensitivity (Liu and Hannun, 1997), protein interaction with factor associated with nSMase activity (Adam-Klages *et al.*, 1996) or embryonic ectoderm development (Philipp *et al.*, 2010), and increased protein levels (Marchesini *et al.*, 2004). nSMase2 contains two hydrophobic domains and multiple palmitoylation sites that anchor it in the membrane (Tani and Hannun, 2007), and it localizes to the inner leaflet of the PM and the Golgi, where a low amount of SM is thought to exist (Marchesini *et al.*, 2007).

The family of nSMases has been implicated in several biological processes including inflammation, cell cycle arrest and apoptosis (further reviewed in Clarke *et al.*, 2006 and Wu

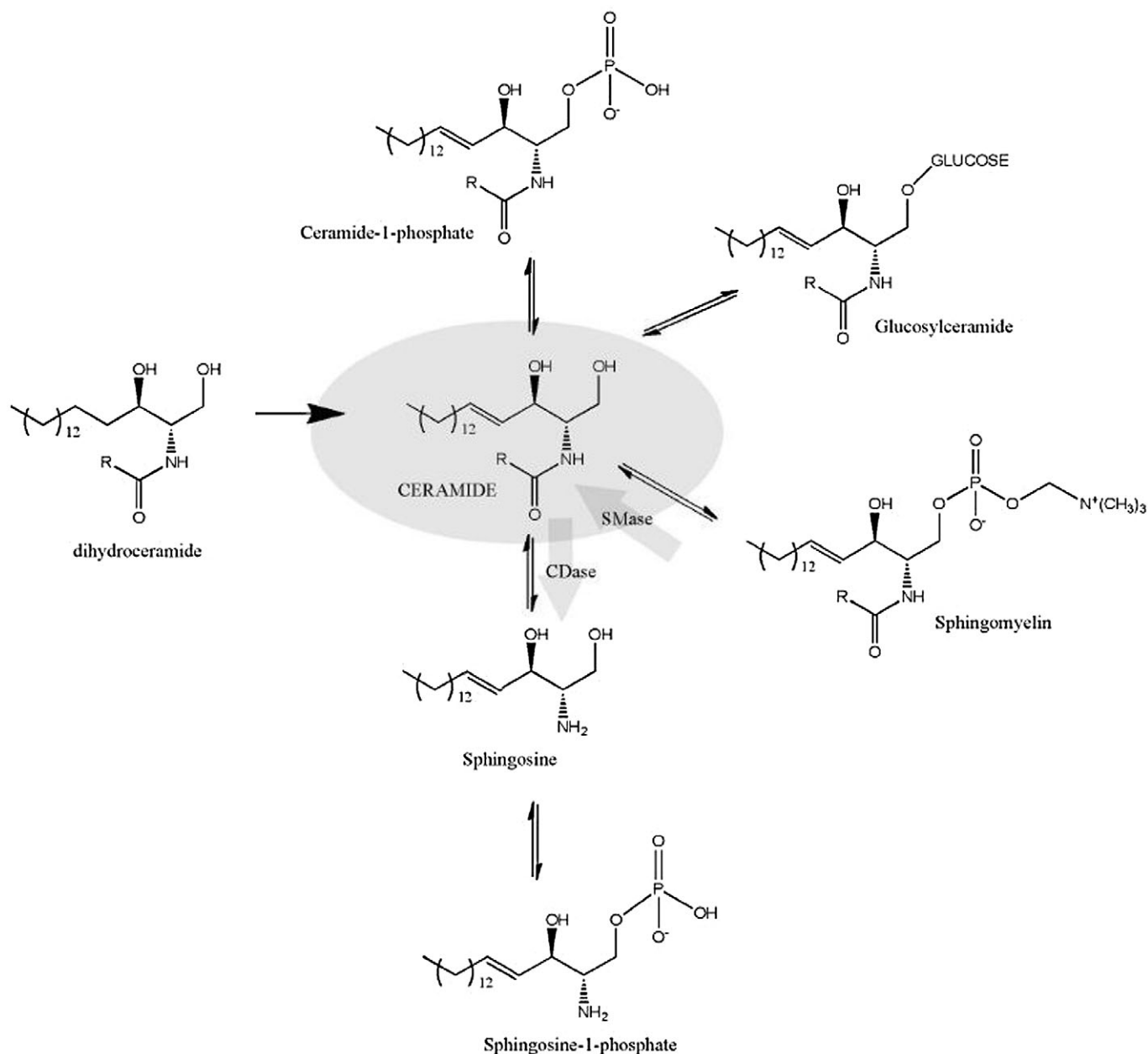


Figure 1

Ceramide is a biosynthetic hub in the sphingolipid (SL) pathway. *De novo* lineal biosynthesis of SLs leads to the irreversible point of reduction of dihydroceramide to ceramide. Ceramide is a central biosynthetic point from where different SLs are synthesized and might be interconverted. Ceramide can be phosphorylated to the bioactive SL ceramide 1-phosphate or glycosylated (or galactosylated, not shown) to glucosyl-ceramide, which is the first step to complex SLs. Ceramide can also be converted to sphingomyelin, an important structural compound of biological membranes. Finally, ceramide can be hydrolysed by ceramidases to sphingosine, which can be phosphorylated to sphingosine-1-phosphate, a pro-survival cellular signal. Sphingomyelinases (SMase) and ceramidases (CDase) enzyme reactions are indicated in the figure. R- different possible acyl chain length.

et al., 2010a). Furthermore, a role for nSMase2 has been demonstrated in heart failure (Adamy *et al.*, 2007), reactive oxygen species-induced lung damage and Alzheimer disease-related neuronal toxicity with implications for neurodegenerative disorders (Haughey *et al.*, 2004; Jana and Pahan, 2004; Lee *et al.*, 2004). Consistent with its role in cell cycle arrest and apoptosis, mutations or deletions have been found in

human leukaemias and in a mouse model of osteosarcoma, suggesting a possible protective role in the context of cancer (Kim *et al.*, 2008). Additionally, from knockout mice studies, it is clear that nSMase2 plays a crucial role in maturation or development of bone during development as these mice display embryonic or juvenile-onset dwarfism (Stoffel *et al.*, 2007).

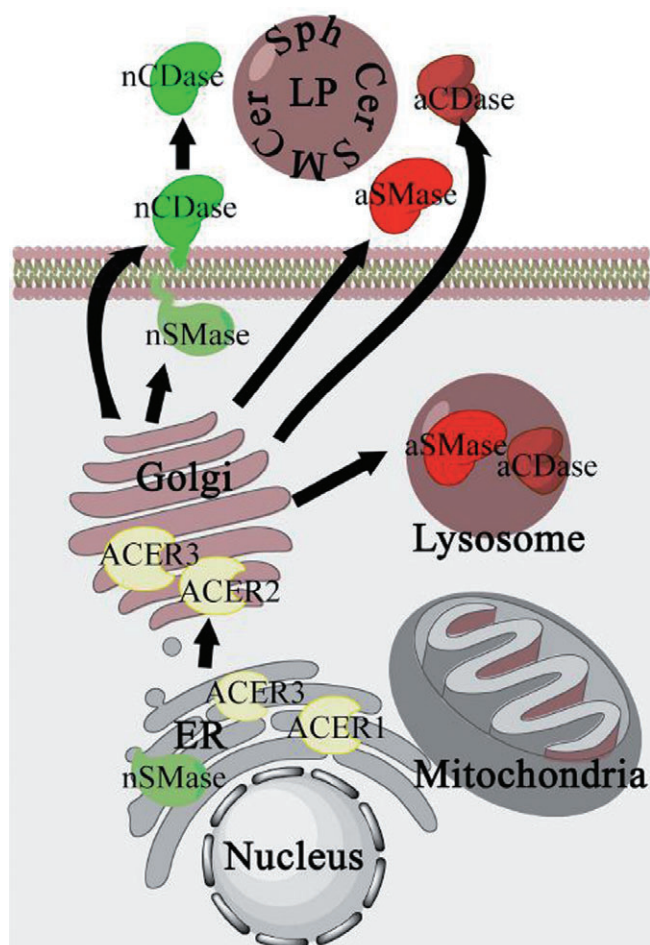


Figure 2

Cellular topology of sphingomyelinases and ceramidases. nSMase have been localized in the endoplasmic reticulum (ER) and attached to the plasma membrane, aSMase, is localized mainly in the lysosomes, and a minor part is also found to be secreted. nCDase is found attached to the plasma membrane, and could have activities in both the inner leaflet and in the intestinal epithelia is attached in the outer leaflet, where can be cleaved and released to the intestinal lumen. Intestinal secreted nCDase is involved in digestion of dietary sphingolipids (SLs). aCDase follow a similar pattern than aSMase, localized mainly in the lysosome, and secreted in to the extracellular matrix. Although less studied, alkCDases have been localized in the ER-Golgi network. Also, mitochondrial sphingomyelinase activities have been described, albeit the frontier between ER and Golgi is diffused, and a specific mitochondria sphingomyelinase have been recently described. The secreted forms of aSMase and aCDase work on the outer leaflet of the plasma membrane, and on lipoproteins (LP) SLs. aCDase, acid ceramidase; aSMase, acid sphingomyelinase; nCDase, neutral ceramidase; nSMase, neutral sphingomyelinase; Sph, sphingosine.

Small molecule inhibitors of nSMase

Because of the emerging roles of nSMase2 in mediating signals related to inflammation and cell death, targeting of nSMase2 as a novel therapeutic intervention point has been sought after. Inhibitors of nSMase have been identified from natural fungal compounds, high-throughput screening and

endogenous cellular substances. Specificity within nSMase isoforms and also between other SMases is a point of emphasis when attempting to implicate a particular nSMase in a biological process especially with the recent identification of mitochondrial-associated nSMase (Yabu *et al.*, 2009; Wu *et al.*, 2010b). It is conceivable that these inhibitors may have different activities and modes of action on each enzyme having nSMase activity.

The earliest nSMase inhibitor to be discovered was Scyphostatin isolated from the fungus *Trichopeziza mollissima*, a discomycete by Tanaka and Nara *et al.* (Nara *et al.*, 1999a,b). Scyphostatin displayed mixed inhibition, affecting both K_m and V_{max} (Nara *et al.*, 1999b), with a 50-fold higher IC_{50} for acid SMase (aSMase), thus demonstrating specificity for nSMase. Another related natural compound with nSMase inhibitory activity is Manumycin A (Arenz *et al.*, 2001b). Manumycin A and Scyphostatin both have an amide bond and a functionalized cyclohexenone ring structure. Several analogues of Scyphostatin and Manumycin A have been synthesized with modifications of the cyclohexenone ring, the amide side chain and the presence of the primary hydroxy (Arenz and Giannis, 2000; Arenz *et al.*, 2001a; Pitsinos *et al.*, 2003). The main element for inhibition appears to be the cyclohexenone ring, regardless of the functional groups such as hydroxyl or epoxy groups. An unsaturated side chain enhances inhibition (Arenz *et al.*, 2001a) and in one case was required for inhibition (Pitsinos *et al.*, 2003). Interestingly, in Arenz *et al.*, the analogue with a saturated side chain displayed no inhibition without preincubation with the enzyme suggestive of irreversible inhibition, whereas the analogue with an unsaturated side chain had almost complete inhibition regardless of preincubation, implicating the side chain as paramount in determining the type of inhibition. The primary hydroxy appears to enhance inhibition, but was not required in analogues lacking an epoxy group on the cyclohexenone ring (Arenz and Giannis, 2000; Arenz *et al.*, 2001a; Pitsinos *et al.*, 2003).

GW4869 was discovered through high throughput screening for small molecule inhibitors of nSMase2 (Luberto *et al.*, 2002). It displayed noncompetitive inhibition with an IC_{50} of 1 μM . This structure does not resemble SM, in agreement with it not competing for the substrate. It was shown to have no inhibition on aSMase. Interestingly, even at high doses, GW4869 was not able to completely inhibit nSMase activity at high levels of phosphatidylserine (PS), but at low PS levels it was able to abolish activity.

In an effort to characterize the structural determinants of SM for recognition by nSMase, Lister *et al.*, showed that the C3-OH group is an important site for substrate recognition due to the finding that C3-H (deoxy-SM) was neither a substrate nor an inhibitor. Conversely, C3-O-methylSM (methyl-SM) was not a substrate, yet could inhibit with an IC_{50} of 50 μM , suggesting that the addition of the methyl group was permissible for binding, but altered the binding in such a way to prevent hydrolysis (Lister *et al.*, 1995).

Amtmann *et al.* synthesized a series of guanidinium lipophilic compounds showing undecylidene-aminoguanidine (C11AG) to be active (Amtmann *et al.*, 2000). Increased lipophilicity correlated with increased inhibition.

Other inhibitory compounds that could be considered natural and also endogenous include glutathione (GSH) and

Table 1

Most used sphingomyelinase and ceramidase inhibitors

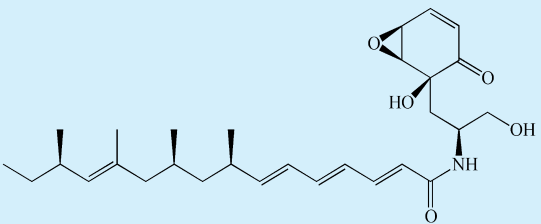
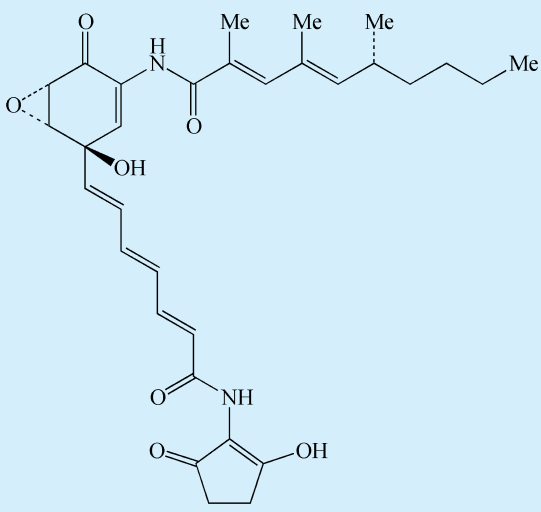
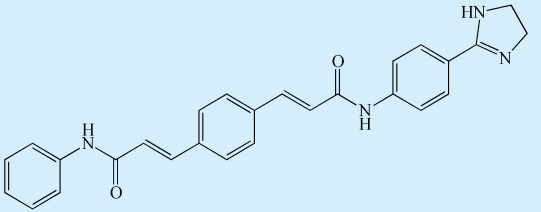
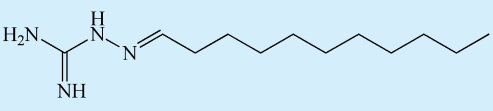
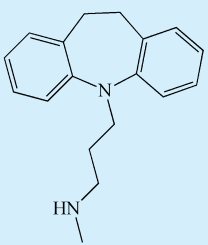
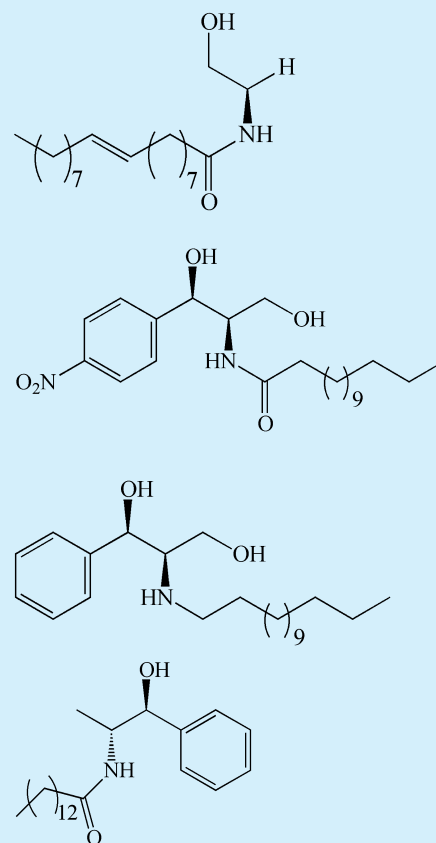
Enzyme targeted	Inhibitor	Comments	
nSMase2	Scyphostatin	Secondary metabolite from fungi can affect aSMase at high concentrations.	
	Manumycin A	Also described as Ras farnesyltransferase, and interleukin-1 converting enzyme inhibitor. Irreversible inhibitors of nSMase.	
	GW4869	Non-competitive. Requires Mg ²⁺ for complete inhibition.	
	C11AG	C11AG was shown to inhibit neutral sphingomyelinase without affecting phospholipase A2, phosphatidylcholine-specific phospholipase C and phospholipase D.	
aSMase/aCDase	Desipramine	Also affects other lysosomal enzymes. Effect blocked by Cathepsin B/L inhibitors.	

Table 1

Continued.

Enzyme targeted	Inhibitor	Comments
aCDase	NOE	Low potency
	B13	Potent for aCDase. Low lysosomal uptake.
	LCL385	Designed for lysosomal uptake. More biological studies needed to confirm effectively
nDCDase /alkCDase	D-e-MAPP	Most used inhibitor. Not selective for neutral or alkaline activities.



aCDase, acid ceramidase; alkCDase, alkaline ceramidase; aSMase, acid sphingomyelinase; C11AG, undecylidene-aminoguanidine; nCDase, neutral ceramidase; nSMase, neutral sphingomyelinase; Sph, sphingosine.

ubiquinol. From a partially purified nSMase fraction, GSH was shown to inhibit nSMase activity in a reversible fashion as dilution of GSH after preincubation with the enzyme resulted in loss of inhibition, even though preincubation significantly increased inhibition. Inhibition was also shown to be noncompetitive. Interestingly, analogues of GSH such as *S*-methyl GSH also inhibited demonstrating that a free sulfhydryl group was not necessary for inhibition (Liu and Hannun, 1997). Moreover, it was shown that GSH was able to partially inhibit TNF α -induced ceramide generation and cell death in MCF-7 cells (Liu *et al.*, 1998). GSH is known to be reduced or effluxed by oxidative agents and stresses (Slater *et al.*, 1995), possibly relieving the inhibition of nSMase and resulting in activation. However, as the oxidized form of GSH, glutathione disulfide (GSSG), also inhibited more potently, it is likely that a decrease in the total levels of GSH/GSSG possibly by efflux would be more likely to result in nSMase activation. Similarly, ubiquinol has been shown to modestly inhibit nSMase isolated from liver PM noncompetitively, which is thought to have roles in PM electron transport and redox regulation (Martin *et al.*, 2001; 2003). Taken together, these antioxidants likely represent an interesting physiologi-

cal means of regulating nSMase, but due to these being endogenous antioxidants with pleiotropic effects, they should not be employed as specific pharmacological inhibitors. Readers are referred to Delgado *et al.* (2006) for further review.

Functional effects of inhibitors of nSMases

Scyphostatin has been used in studies as an anti-inflammatory in response to lipopolysaccharide (LPS) and IL-1 β , particularly for phosphorylation of Jun Kinase (Nara *et al.*, 1999a; Rutkute *et al.*, 2007). In studies performed in cultured hippocampal neurons, scyphostatin was shown to inhibit neurite outgrowth or cell death depending on the time of culture (Brann *et al.*, 1999; 2002). Manumycin A has been used as an antibiotic, antitumour and anti-inflammatory agent (Zeeck *et al.*, 1987; Hara *et al.*, 1993; Sonoda *et al.*, 1998; Bernier *et al.*, 2006); however, due to its additional inhibitory activity on farnesyltransferase, which is important for activation of Ras and related small G proteins, and other targets (Hara *et al.*, 1993), caution should be exercised with its use.

Many studies have used GW4869 in cell culture and *in vivo* studies. Treatment of cells with GW4869 was able to block induction of apoptosis in breast carcinoma and glioma cells (Luberto *et al.*, 2002; Peng *et al.*, 2006). nSMase activity and ceramide levels have been shown to be increased in aged endothelial tissue having decreased vessel relaxation, which was reversible by nSMase inhibition with GW4869 (Smith *et al.*, 2006). It has also been used to demonstrate involvement of nSMase2 in hypoxic pulmonary vasoconstriction and chemotaxis of neutrophils (Cogolludo *et al.*, 2009; Sitrin *et al.*, 2010). A role for nSMase2 in various neurological processes, including synaptic plasticity (Wheeler *et al.*, 2009) and spatial memory (Tabatadze *et al.*, 2010) have been suggested based on results with GW4869.

In regard to methyl-SM, it has been shown to inhibit LPS-induced formation of ceramide, Ras activation, NF- κ B activation and production of nitric oxide (NO) in rat glioma cells (Won *et al.*, 2004). Interestingly, GW4869 was shown not to inhibit NF- κ B activation in response to TNF α (Luberto *et al.*, 2002), contrasting with the above study with methyl-SM. C11AG has been used to show involvement of nSMases in a variety of biologic responses including LPS-induced NO production (Amtmann *et al.*, 2000; 2003; Amtmann and Zoller, 2005).

Potential uses/considerations

From the literature, there appears to be significant potential for the use of nSMase inhibitors in the setting of tissue damage related to inflammation in cardiovascular, pulmonary or neurological systems. However, the full spectrum of use of nSMase inhibitors is only being defined by ongoing studies both in elucidating its physiologic functions and the pathophysiologic implications of this enzyme. In order for nSMase2 to be developed into a therapeutic target, more effort is needed in elaborating its roles in specific disease processes *in vivo* and in cellular studies with the concomitant development and characterization of lead compounds.

aSMase

Background. aSMase (EC3.1.4.12) is a soluble hydrolase that generates ceramide and phosphorylcholine from the cleavage of the phosphodiester bond of SM at an acid pH optimum (Jenkins *et al.*, 2009). Deficiency of aSMase in humans results in Niemann-Pick disease (NPD), a lysosomal storage disorder (LSD) characterized by pathologic accumulation of SM in cells and tissues (Schuchman, 2007). While absence of aSMase in humans leads to an established pathologic state (i.e. NPD), activation of aSMase has been suggested to play an important role in the development and progression of several pathophysiologic conditions, including atherosclerosis, cancer, diabetes, Alzheimers's disease, cystic fibrosis and Wilson disease (Smith and Schuchman, 2008; Jenkins *et al.*, 2009; Schuchman, 2010).

Activation of the aSMase/ceramide pathway has been reported in response to a variety of cellular stresses, many of which overlap activators of nSMase2 and including inflammatory cytokines, UV radiation, pathogen exposure and chemotherapeutic agents, leading to production of the bioactive lipid ceramide (Gulbins and Li, 2006; Stancevic and Kolesnick, 2010). In spite of an expanding body of research,

the precise role of the aSMase/ceramide pathway in the cellular stress response remains unclear, primarily due to paucity of mechanistic studies and the absence of specific potent inhibitors.

The use of cell lines and animals lacking aSMase activity has been integral in the advancement of the field of ceramide signalling. However, cells lacking aSMase develop a subsequent SM storage phenotype that can have indirect effects on cell biology (Lozano *et al.*, 2001). To better understand the precise role of aSMase in specific pathophysiologic processes, small molecule inhibitors are needed to inhibit aSMase acutely, in the absence of SM storage and other indirect effects of chronic aSMase deficiency. Discussed below are several endogenous and exogenous inhibitors of aSMase, their reported effects and mechanism of action, as well as future directions and the need for more specific agents. For a more comprehensive discussion of aSMase inhibitors, readers are referred to reviews by Arenz (2010) and Kornhuber *et al.* (2010).

Small molecule inhibitors of aSMase

Endogenous inhibitors. A variety of endogenous molecules have been shown to inhibit aSMase activity. Phosphate-containing molecules, including inorganic phosphate, nucleotides (e.g. cAMP), dolichol phosphate and phosphoinositides, are known to potently and non-competitively inhibit aSMase activity (Callahan *et al.*, 1983; Watanabe *et al.*, 1983). Later, specific phosphoinositides, PtdIns-3,5-P₂ (Kolzer *et al.*, 2003) and PtdIns-3,4,5-P₃ (Testai *et al.*, 2004), were demonstrated to inhibit aSMase activity in the low μ M range. Importantly, and in contrast, another phosphate-containing lipid, bismonoacylglycerophosphate (BMP) is a potent activator of aSMase (Linke *et al.*, 2001a). Of note, BMP is enriched in lysosomes and the activating effect of BMP requires an acidic pH.

Studies on NPD type C (NPD-C) revealed that another endogenous lipid – cholesterol – potently inhibits aSMase activity (Reagan *et al.*, 2000). While mutations in the *SMPD1* gene that encodes the aSMase protein gives rise to NPD type A and type B, NPD-C results from mutations in NPC-1 and NPC-2 genes that encode proteins involved in cholesterol trafficking within the endo-lysosomal compartment (Sturley *et al.*, 2004). Patients with NPD-C have a functional aSMase deficiency via post-translational inhibition of aSMase activity by cholesterol. Removal of lipoproteins from culture media of NPD-C dermal fibroblasts is sufficient to remove the inhibition of aSMase (Thomas *et al.*, 1989), indicating that exogenous sterols play an important role in the modulation of *in situ* aSMase activity. Moreover, various sterols (e.g. 7-ketocholesterol) have been reported to inhibit aSMase activity *in vitro* (Maor *et al.*, 1995), indicating that sterol derivatives may represent another class of molecules that can be utilized to develop inhibitors of aSMase.

The overall effect of these lipids on aSMase activity *in situ* is likely a function of relative concentrations of each of these molecules in specific subcellular compartments, with additional influence of local pH. In addition, aSMase itself is subject to post-translational modification, which may alter subcellular localization and/or specific activity. Moreover, the relevance of these *in vitro* findings must be considered in the context of intracellular, extracellular and local concentra-

tions. For example, as lysosomes mature, cholesterol concentrations decrease, pH increases, the concentration of BMP increases, thereby promoting a favourable environment for the action of aSMase.

Exogenous inhibitors

In vitro inhibitors. Building on these studies mentioned above, several groups have synthesized compounds based on the structure of SM or of endogenous aSMase regulators. The first class are difluoromethylene analogues of SM, referred to as SMAs (Yokomatsu *et al.*, 2001). Administration of SMA-7 protected mice against dextran sulfate sodium-induced colitis (Sakata *et al.*, 2007a), a model of inflammatory bowel disease. Cell culture studies demonstrated that inhibition of aSMase by SMA-7 disrupted LPS-induced formation of ceramide and disrupted activation of NF- κ B and subsequent release of pro-inflammatory cytokines from macrophages (Sakata *et al.*, 2007a), as well as decreased interleukin-8 production by epithelial cells (Sakata *et al.*, 2007b). The latter effect was related to impaired neutral SMase activation, so the specificity of these SMAs remains unclear. Phosphoinositide-based inhibitors (Roth *et al.*, 2009b), bisphosphonate-based inhibitors (Roth *et al.*, 2009a) and carbohydrate-based inhibitors (Roth *et al.*, 2010) have all been synthesized and characterized, although these inhibitors have not been widely utilized and therefore data on their efficacy is limited.

In vivo inhibitors. Several pharmacologic agents have been shown to induce functional loss of aSMase activity, most notably the tricyclic antidepressants (e.g. desipramine, imipramine and amitriptyline) (Albouz *et al.*, 1981; Kornhuber *et al.*, 2010). These agents inhibit aSMase activity *in vivo* but do not act as *in vitro* enzyme inhibitors. The calmodulin antagonist W-7 (Masson *et al.*, 1989), cocaine (Nassogne *et al.*, 2004), SR33557 (Jaffrezou *et al.*, 1991), and most recently, the S1P receptor antagonist FTY720 (Dawson and Qin, 2011), induce a functional loss of aSMase, along with other cationic amphiphilic drugs (Yoshida *et al.*, 1985). With the tricyclics, the mechanism proposed is that of preventing the interaction of aSMase with anionic lipids in the lysosome, especially BMP, resulting in the enzyme becoming a substrate for lysosomal proteases. For a more comprehensive discussion of functional aSMase inhibitors, readers are referred to the review by Kornhuber *et al.* (2010).

Importantly, these drugs are not specific for aSMase, and have been shown to inhibit acid ceramidase (aCDase) (Zeidan *et al.*, 2006) and lysosomal phospholipases (Pappu and Hostetler, 1984). Importantly, several other lysosomal enzymes, including acid lipase, arylsulfatases A and B, and hexosaminidases, are unaffected by tricyclic compounds (Albouz *et al.*, 1981), suggesting a certain degree of selectivity. The mechanism of tricyclic antidepressant action on aSMase appears to be by promoting proteolysis of mature, lysosomal aSMase (Hurwitz *et al.*, 1994), which distinguishes these agents from other lysosomotropic agents, such as chloroquine, which induce global lysosomal dysfunction and would presumably affect all lysosomal hydrolases.

Current uses, and future directions, for aSMase inhibitors

For several years, anti-inflammatory properties of tricyclic compounds have been reported (Martelli *et al.*, 1967; Roumestan *et al.*, 2007). In recent years, functional aSMase inhibitors have been shown to reduce the severity of several different mouse models of human disease, including cystic fibrosis (Teichgraber *et al.*, 2008; Becker *et al.*, 2010), Wilson disease (Lang *et al.*, 2007), acute lung injury (Yang *et al.*, 2010) and hepatic ischaemia-reperfusion injury (Llacuna *et al.*, 2006). Recently, Roth *et al.* demonstrated that several bisphosphonate compounds potentially inhibited aSMase (Roth *et al.*, 2009a). The most potent of these bisphosphate derivatives exhibited an IC₅₀ of 20 nM towards aSMase. Bisphosphonates, such as zoledronic acid, are already in clinical use for the treatment of osteoporosis, and may also prove to be functional aSMase inhibitors *in vivo*.

However, it is important to emphasize that aSMase represents multiple enzymatic forms that exist in different cellular and extracellular compartments. A common protein precursor (pro-aSMase) is differentially trafficked to form lysosomal aSMase (L-SMase) or secretory aSMase (S-SMase) (Schissel *et al.*, 1998; Tabas, 1999). While L-SMase resides in the endolysosomal compartment, S-SMase is an extracellular enzyme. An additional form of aSMase has been reported in close association with the outer leaflet of the PM where it can form ceramide-rich platforms (Stancevic and Kolesnick, 2010). As future research uncovers the specific roles of these different forms of aSMase, it will likely become increasingly important to target aSMase in a compartment-specific fashion to maximize the therapeutic potential of aSMase inhibitors.

Summary

A variety of endogenous and exogenous molecules regulate the activity of aSMase. While endogenous regulators – including nucleotides, phospholipids, sterols – may serve as a foundation for the development of more selective inhibitors of aSMase in the future, exogenous compounds, namely the functional aSMase inhibitors (e.g. tricyclic antidepressants), are emerging as attractive therapeutic options in the treatment of several pathophysiologic conditions. Clinically approved agents such as tricyclic antidepressants and bisphosphonates represent attractive agents to evaluate the role of aSMase in several pathophysiologic conditions. Re-evaluation of these compounds as functional inhibitors of aSMase signalling may provide an inroad for the treatment and/or improved clinical management of several disease states. However, as noted, these are not totally specific for aSMase, and therefore, an urgent need still exists for the development of potent and selective small molecule inhibitors of this enzyme.

Ceramidases

Background

Thus far, we have described how ceramide is generated from SM by SMases, and how an excess or defect in ceramide or sphingomyelinase activity may result in pathobiologic effects. However, ceramide and S1P have been shown to often exert opposite effects (Cuvillier O *et al.*, 1998; Huwiler and

Pfeilschifter, 2006; Taha *et al.*, 2006b; Canals *et al.*, 2010). There are only two enzymatic steps separating ceramide and S1P: ceramidases provide the first node of this regulatory connection, and SphK the second one. It should not be surprising that both of these enzymes have also been involved in cancer progression and inflammation (Horton, 1999; Nemoto *et al.*, 2009; Pyne and Pyne, 2010; Snider *et al.*, 2010). This section will discuss the ceramidases; the reader is referred to other reviews for discussion of SphKs (Taha *et al.*, 2006a; Pitman and Pitson, 2010).

Five different human genes have been identified that encode proteins that hydrolyse ceramide to Sph and free fatty acid (*N*-acylsphingosine amidohydrolase). They have been grouped as acid, neutral or alkaline ceramidases (EC 3.5.1.23), depending on the optimum pH of their ceramidase activity being pH 4.5 for aCDase (gene name: ASAH1, NAAA), pH 7–9 for neutral ceramidase (nCDase, ASAH2) and pH 8.5–9.5 for alkaline ceramidases (alkCDase, three genes: ASAH3/ACER1, ACER2 and ACER3). A reverse activity has also been described for most of these ceramidases with that for aCDase showing a pH optimum slightly less acidic (5.5) than that of the forward reaction (Okino *et al.*, 2003). Interestingly, and as predicted from their mechanism, the reverse activity of ceramidases uses a free fatty acid as a substrate, unlike ceramide synthases (EC 2.3.1.24) that use acyl-CoAs as substrates. These different ceramidases are found in diverse cellular compartments. Thus, aCDase resides in the lysosomes (Koch *et al.*, 1996), nCDase at the PM, intracellular compartments and secreted in the intestinal lumen (El Bawab *et al.*, 2000) and ACER family in the ER-Golgi network (Mao *et al.*, 2001; Xu *et al.*, 2006).

Acid ceramidase

Acid ceramidase activity, as well as the reverse reaction, was first described in 1963 by Shimon Gatt using rat tissues (Gatt, 1963). In the early 1990s, human aCDase was purified to homogeneity from human urine (Bernardo *et al.*, 1995), and a few years later, the cDNA for the gene was cloned (Li *et al.*, 1998). aCDase has subsequently been purified and characterized from other tissues (Linke *et al.*, 2001b). aCDase consists of a single precursor polypeptide of 53–55 kDa (Ferlinz *et al.*, 2001) that is proteolytically processed into α - and β -subunits that migrate at 13 kDa and 40 kDa respectively. The α -subunit can be reduced to 29 kDa by N-glycanase F treatment (Koch *et al.*, 1996). Of six individual potential *N*-glycosylation sites, five of them are used (Schulze *et al.*, 2007), and some of them are required for correct lysosomal processing or enzymatic activity, and for the formation of the heterodimeric enzyme form (Ferlinz *et al.*, 2001). Purification of human aCDase revealed that at least two β -subunits could be generated for aCDase differing by 2–4 kDa at the C-terminus (He *et al.*, 2003). Although aCDase has been localized mainly in lysosomes, a portion of aCDase is secreted to the medium as a 47 kDa monomer (Bernardo *et al.*, 1995).

Little is known about how the secretory form is regulated and processed. The generation of the lysosomal mature form involves cleavage of the precursor in endosomal/lysosomal compartments, as well as trafficking through the mannose-6-phosphate receptor. Interestingly, mutation of putative *N*-glycosylation sites did not alter the ratio of secreted : lysosomal aCDase (Ferlinz *et al.*, 2001). The use of insect Sf21 cells overexpressing human aCDase showed that the secreted pre-

cursor can be processed to a mature form upon acidification of the cell culture supernatant to pH 4.2–4.3m causing the processing of the precursor and resulting in a homogeneous sample of mature aCDase.

aCDase and aSMase are metabolically consecutive enzymes in SM hydrolysis, and their expression and activity are regulated by each other. For example, aCDase activity enhances aSMase secretion (He *et al.*, 2003). More recent work has shown that secreted aCDase can be found forming a multi-enzymatic complex with aSMase and β -galactosidase (He *et al.*, 2003). The results showed strong enough interaction among these enzymes to allow co-precipitation, and there was significant specificity that other lysosomal enzymes were not detected in those complexes.

Interestingly, as seen for nSMase and aSMase, stress stimuli such as TNF- α also activate aCDase. Other stress stimuli such as UV and ionizing radiation have also been reported to induce aCDase activity. Moreover, aCDase requires anionic lysosomal lipids and SL activator proteins (saposin) as cofactors for efficient hydrolysis of ceramide *in vivo* (Azuma *et al.*, 1994; Linke *et al.*, 2001a).

Abnormal overexpression of aCDase has been related to tumour progression and protection from cell death; on the other hand, deficiency of aCDase results in Farber disease (first described in 1957 by Sidney Farber), an autosomal-recessively inherited LSD resulting in accumulation of ceramide. Farber disease is associated with distinct clinical phenotypes, involving painful swelling of the joints and tendons, pulmonary insufficiency, neurological and general deficient development and a shortened lifespan (Levade *et al.*, 1995; Koch *et al.*, 1996). Several point mutations and exon skipping in the aCDase gene have been identified in Farber disease. Moreover, although certain mutations in the aCDase gene mimic Farber disease, the generation of aCDase knockout mice (Li *et al.*, 2002) resulted in embryonic lethality for the homozygous mice. Furthermore, aCDase has been implicated in other metabolic complications; for example, aCDase was shown to have a role in preventing type 2 diabetes and modulating insulin signalling (Chavez *et al.*, 2005).

Neutral ceramidase

nCDase activity, and the reverse activity, were described as early as 1969 as a ceramide-cleaving activity found in humanduodenal contents, with an optimal pH of 7.6 (Nilsson, 1969), and in 1980, in microsomes from rat liver (Stoffel and Melzner, 1980). nCDases have been cloned from bacteria (Okino *et al.*, 1999; 2010), human (El Bawab *et al.*, 2000), mouse (Tani *et al.*, 2000), rat (Mitsutake *et al.*, 2001), *Drosophila* (Yoshimura *et al.*, 2002), amoeba (Monjusho *et al.*, 2003), Zebra fish (Yoshimura *et al.*, 2004), plants (Pata *et al.*, 2008) and fungus (Tada *et al.*, 2009). Note that the *Aspergillus* and the amoeba nCDases showed an optimum acidic pH. nCDase does not share significant sequence identity with either acid or alkCDases, as well as other amidases, including proteases (Galadari *et al.*, 2006). Northern blotting analysis also revealed that nCDase is expressed ubiquitously (El Bawab *et al.*, 2000), but highly expressed in kidney, liver, heart (Tani *et al.*, 2000) and intestine (Choi *et al.*, 2003). The activity of purified intestinal rat (Olsson *et al.*, 2004) and human (Ohlsson *et al.*, 2008) nCDase, a glycosylated protein of molecular weight of 116 kDa, is not affected by Ca²⁺,

Mg²⁺ or Mn²⁺, but inhibited by Zn²⁺, Fe²⁺ and Cu²⁺ (Galadari *et al.*, 2006). No cations are required to activate nCDase. nCDase was found to be secreted into the intestine lumen for SL digestion, being resistant to pancreatic proteases (Olsson *et al.*, 2004; Ohlsson *et al.*, 2008) and, as with aSMase, was found extracellularly attached to the PM, exposing the catalytic site to the intestinal lumen (Duan *et al.*, 2007). In other tissues and in cell culture, nCDase can be secreted or localized at the PM (Hwang *et al.*, 2005) as a type II integral membrane protein (Tani *et al.*, 2003). In addition, it has also been localized in endosome-like structures (Mitsutake *et al.*, 2001), and in mitochondria in MCF-7 and HEK293 cells (El Bawab *et al.*, 2000). Thus, intestinal and intracellular nCDase are identical enzymes (Duan and Nilsson, 2009). Moreover, nCDase is also highly glycosylated. Even though deglycosylation did not affect its activity, mutants lacking the mucine box or O-glycosylation sites in the mucine box were secreted and not localized at the cell surface in HEK cells (Tani *et al.*, 2003). This is consistent with bacterial and invertebrate nCDase, which lack the mucin box, being secreted proteins (Inoue *et al.*, 2009). Overexpression of nCDase did not result in Sph accumulation in unstimulated human platelets, unless the PM SM was previously hydrolysed (Tani *et al.*, 2005), suggesting that not much ceramide is found at PM in unstimulated conditions. The same conclusion was shown using combination of recombinant bacterial sphingomyelinase and recombinant bacterial ceramidase (Canals *et al.*, 2010).

Galadari *et al.* identified a nCDase motif comprised of six amino acids core (GDVSPN) from a comparison of diverse nCDase sequences, and identified the serine residue (Ser 354) in that hexapeptide as the nucleophile attacking the amide bond of ceramide in the catalytic site. The mutation of that serine, as well as the aspartate or the cysteine of the hexapeptide led to complete loss of nCDase activity (Galadari *et al.*, 2006). However, Inoue *et al.*, based on *Pseudomonas aeruginosa* nCDase crystal structure, suggested that Ser-354 is involved in Zn²⁺ binding (Inoue *et al.*, 2009). The crystal structure of bacterial *P. aeruginosa* nCDase also allowed the identification of the Zn²⁺ and the Mg²⁺/Ca²⁺ binding sites. Modelling the rat sequence on the *P. aeruginosa* crystal structure, other amino acids were identified in rat nCDase to be indispensable for Zn²⁺ binding and formation of the active site (His 175 and Tyr 160), for catalysis (Arg 238) and for the ceramide binding site (Tyr 572). Single mutations of these residues led to the loss of rat nCDase forward and reverse activity (Inoue *et al.*, 2009).

The substrate specificity of nCDase was studied by El Bawab *et al.* (El Bawab *et al.*, 2000), finding that only the natural D-erythro-ceramide isomer was used as substrate of the four stereoisomers of ceramide. Dihydroceramide or phyto-ceramide forms, shortening the length of the alkyl backbone, methylation of the primary or secondary hydroxyl groups resulted in reduction or loss of the nCDase activity (El Bawab *et al.*, 2002).

Physiologically, IL-1 β increased the activity of acid, but also neutral ceramidase in rat hepatocytes (Nikolova-Karakashian *et al.*, 1997) and in renal mesangial cells, describing not only an increase in nCDase activity but also in mRNA levels and protein synthesis. This protein induction by IL-1 β was blocked by the p38 mitogen-activated protein kinase inhibitor SB 202190 (Franzen *et al.*, 2001). Moreover, other cytokines such as TNF and interferon-gamma also increased

activity, mRNA and nCDase protein synthesis, where this elevation was reported to protect cells from cytokine-induced cell death (Zhu *et al.*, 2008).

Alkaline ceramidases

In 1975, it was reported that the hydrolysis of ceramide was observed not only at pH 4.0 but also at pH 9.0 in normal cerebellum (Sugita *et al.*, 1975). Acid and alkCDase activity were also found in fibroblasts and leukocytes (Dulaney *et al.*, 1976), and in different rat tissues (Spence *et al.*, 1986).

In a study in yeast, aimed at molecular identification of enzymes of ceramide metabolism, Mao *et al.* identified two novel alkaline ceramidases (YPC1 and YDC1). These then became the founding members of a novel family of alkCDases. Indeed, three different genes comprise the known human alkCDases, ACER1, 2 and 3, which contain 264 (Sun *et al.*, 2008), 275 (Xu *et al.*, 2006) and 267 (Mao *et al.*, 2001) amino acid, respectively, with a similar molecular weight around 31 kDa. All of them present multiple transmembrane domains. ACER1 is localized in the ER and is mainly expressed in the skin (Mao *et al.*, 2003). ACER2 is localized in the Golgi apparatus and is highly expressed in the placenta and modestly expressed in many other tissues. ACER3 is localized in both ER and Golgi apparatus, and is highly expressed in most tissues, especially in the placenta (Mao and Obeid, 2008). All three ACER activities are enhanced by Ca²⁺ (Mao *et al.*, 2001).

The substrate specificity among aCDases, nCDases and alkCDases was first studied in 1982, finding short acyl-chain ceramides (i.e. C16 acyl chain) being poor substrates for acid, but not for neutral and alkCDases. Both, alkaline and nCDase have been reported to be inhibited by phosphatidylcholine and SM (Yada *et al.*, 1995).

Recently, plasma S1P has been reported to derive from Sph generated from alkCDase activity in erythrocytes. Moreover, this activity is the only ceramidase activity found in erythrocytes. Furthermore, alkCDase has also been shown to be important for erythroid differentiation in K562 erythroleukaemic cells (Xu *et al.*, 2010).

ACER1 is upregulated in epidermal keratinocytes in response to increasing the concentration of Ca²⁺ in tissue culture medium. Using RNAi technology, ACER1 has been implicated in mediating extracellular Ca²⁺-induced differentiation of human keratinocytes. The mechanism by which ACER1 mediates keratinocyte differentiation remains unclear although an increase in the generation of Sph, S1P or both may be involved. ACER2 has been shown to be upregulated in tumours, and its upregulation promotes tumour cell proliferation and survival likely through increasing the generation of S1P and activating the S1P receptor S1P1. Interestingly, over-expression of ACER2 may also result in cell proliferation inhibition or cell death due to an accumulation of Sph, which is highly cytotoxic. ACER2 has also been implicated in the regulation of β 1 integrin maturation and cell adhesion (Sun *et al.*, 2009).

The cytotoxic compound retinoid N-(4-hydroxyphenyl)retinamide (4-HPR), an inhibitor of dihydroceramide desaturase activity (Kravka *et al.*, 2007), has recently been shown to increase ACER2 activity and mRNA, but not ACER 1 or 3. Over-expression of ACER2 (but neither ACER3, nor aCDase nor nCDase) enhanced 4-HPR-induced (same with GT11 another dihydroceramide desaturase inhibi-

tor) dihydroSph formation, and cell death (Mao *et al.*, 2010), suggesting that ACER2 also regulates the levels of dihydro-sphingosine (DHS) as well as DHS-mediated cell death by controlling the hydrolysis of certain dihydroceramides with unsaturated acyl chains. ACER3 prefers unsaturated long-chain ceramides, minor ceramide species in mammalian cells and tissues, so ACER3 knockdown results in an increase in the levels of unsaturated long-chain ceramides (D-*e*-C18:1-ceramide and D-*e*-C20:1-ceramide) in tumour cells. Interestingly, ACER3 down-regulation decreases the levels of other ceramide species while increasing the levels of both Sph and S1P by increasing the expression of ACER2, which hydrolyses most mammalian ceramide species. ACER3 knockdown inhibited cell proliferation by up-regulation of the cyclin-dependent kinase inhibitor p21 (CIP1/WAF1), but also inhibited serum-deprivation induced apoptosis (Hu *et al.*, 2010).

Endogenous inhibitors

The physiologic regulation of ceramidase activity depends on the specific ceramidase (aCDase, nCDase or alkaline) and their localization in the cell. Thus, aCDase has been the most studied ceramidase, shown to be inhibited by anionic lipids such as phosphatidic acid (PA) and phosphatidylserine (PS) for the forward reaction, but these lipids appear to promote the reverse reaction (El Bawab *et al.*, 1999). Conversely, SM activates aCDase in the forward reaction, inhibiting the reverse activity. Zinc cations strongly inhibit the reverse activity. In addition, the product of the forward activity, Sph and oleic acid, as well as other complex SLs such as HexCers have been found to inhibit forward ceramidase activity (Sugita *et al.*, 1975). Furthermore, pH also plays an important regulatory role in ceramidase activity as aCDase can reach both endosome-lysosomal and extracellular matrix compartments. Not only the optimum pH of the enzyme is important for its activity, but also the processing requires low pH to produce the mature form, as seen before.

In vitro studies have shown that nCDase activity was inhibited by reducing agents such as dithiothreitol and β -mercaptoethanol (Galadari *et al.*, 2006). Natural Sph acted as a competitive inhibitor, and the reverse activity of nCDase was inhibited by L-*erythro*-Sph and myristaldehyde in a competitive mechanism (El Bawab *et al.*, 2001). Phosphatidic acid and cardiolipin showed moderate inhibition as well. Interestingly, cardiolipin enhanced ceramidase activity, and, importantly, the reverse activity was not inhibited by fumonisin B1, an inhibitor of the CoA-dependent ceramide synthase enzymes (El Bawab *et al.*, 2001). As seen for aSMase, low concentrations of cholesterol also inhibited intestinal nCDase (Ohlsson *et al.*, 2007).

Small molecule inhibitors of ceramidases

As seen for aSMase, some amphiphilic tricyclic agents such as desipramine, chlorpromazine and chloroquine, also exert an indirect inhibitory effect on lysosomal ceramidase activity by down-regulating the aCDase protein, but not modifying RNA message level. The inhibition by desipramine is blocked by the cathepsin protease inhibitors leupeptin and CA074ME, but not by pepstatin A. This inhibition by the tricyclics has been observed *in vivo* (Elojeimy *et al.*, 2006) but not *in vitro* (Zeidan *et al.*, 2006). Thus, desipramine, induces down-

regulation of aCDase by activation of proteolysis of aCDase by cathepsin B/L (Elojeimy *et al.*, 2006). However, not all lysosomotropic agents have been found to have this effect; ammonium chloride and bafilomycin A1 have no effect on the amount of aCDase protein (Elojeimy *et al.*, 2006). The diacylglycerol analogue, phorbol myristate acetate (PMA) also induced down-regulation of ACER2 (Sun *et al.*, 2009).

Synthetic ceramidases inhibitors

The design and screening of structural analogues of the sphingoid bases and ceramides has led to a few specific inhibitors for ceramidases.

The first aCDase inhibitor described was N-oleylethanolamine (NOE) (Sugita *et al.*, 1975), now better known as an endocannabinoid-related molecule, and has been one of the most used *in vitro* inhibitors for aCDase. *In vitro* studies showed that NOE increased ceramide levels and enhanced apoptosis in L929 cells (Strelow *et al.*, 2000), glioma cells (Hara *et al.*, 2004), primary placenta trophoblast (Payne *et al.*, 1999), and dendritic cells (Kanto *et al.*, 2001). Although those effects were attributed to an increase in ceramide levels, they cannot be specifically attributed to inhibition of aCDase, as NOE, afterwards, was found to inhibit ceramide glucosylation in neuroepithelioma cells as well (Spinedi *et al.*, 1999). Furthermore, structural analogs of NOE were explored as aCDase inhibitors by Fabrias G (Grijalvo *et al.*, 2006), defining NOE as a weak aCDase inhibitor *in vitro* and *in vivo*. Thus, NOE structure was improved for the development of aCDase inhibitors using shorter oxoacyl groups, adding the missing sphingoid alkyl tail and adding also a C3 hydroxyl group. Although the (E)-4,5 double bond was not required for inhibition, a (Z)-4,5 geometry was found to revert the inhibition. Due to its lack of selectivity (NOE can also inhibit neutral and alkaline ceramidase activities), and low potency, the canonical structure of NOE has been avoided for therapeutic use, although structural analogues of it are current candidates for aCDase inhibitors.

Not long after, another ceramidase inhibitor was developed; D-*e*-MAPP [(1S, 2R)-D-*erythro*-2- (N-Myristoylamino)-1-phenyl-1-propanol], was synthesized by Bielawska *et al.* as a ceramide analogue, causing cell cycle arrest in HL-60 cells, elevating intracellular ceramide levels up to 3-fold. *In vitro* studies showed that D-*e*-MAPP inhibited, at low micromolar concentrations, alkaline and neutral ceramidase, but had no effect on aCDase activity. Moreover, its enantiomer L-*e*-MAPP, although undergoing similar cellular uptake, was metabolized and had no effect on ceramidase activity (Bielawska *et al.*, 1996). A recent study demonstrated that D-*e*-MAPP inhibits cellular activities of ACER1-3. Other authors have also reported increases in cellular ceramide levels after D-*e*-MAPP treatment (Raisova *et al.*, 2002; Rodriguez-Lafrasse *et al.*, 2002; Alphonse *et al.*, 2004), and this increase in ceramide levels by D-*e*-MAPP has been involved in apoptosis induction (Rodriguez-Lafrasse *et al.*, 2002; Choi *et al.*, 2003; Lepine *et al.*, 2004) and in radiation treatment, overcoming the resistance to radiation of cancer cells over-expressing ceramidases. Furthermore, D-*e*-MAPP was seen to block the mitogenic effect of oxidized low density lipoprotein (oxLDL), suggesting S1P as the responsible molecule of the mitogenic effect, coming from LDL-ceramide (Auge *et al.*, 1999). Other pathways requiring S1P were also blocked by D-*e*-MAPP, such

as DNA synthesis in smooth muscle cells (Maupas-Schwalm *et al.*, 2004). In other cases, an acid ceramidase inhibitor was required to induce apoptosis, where D-*e*-MAPP had no effect on induction of apoptosis, although ceramide levels increased in both treatments (Payne *et al.*, 1999). In fact, D-*e*-MAPP has been shown to protect cells from apoptosis in response to serum deprivation, the synthetic glucocorticoid dexamethasone, and the herbicide paraquat by preventing the production of Sph. D-*e*-MAPP resulted in blockage of progesterin and adiponectinreceptor (PAQR), involving SLs, and ceramidases downstream of PAQR signalling, an important hormone receptor related to pathological conditions, including obesity, diabetes and coronary artery disease (Kupchak *et al.*, 2009). Other studies have shown D-*e*-MAPP, as well as exogenous short length acyl-ceramides, to enhance the A23187 (Ca²⁺ionophore)-induced release of arachidonic acid, associated with an increase of endogenous ceramide accumulation (Shimizu *et al.*, 2009). Modifications of the structure of D-*e*-MAPP led to B13 ((1R,2R)-2-(N-tetradecanoylamino)-1-(4-nitrophenyl)-1,3-propanediol), a more water soluble form. Interestingly, B13, as shown for D-*e*-MAPP, also increased intracellular levels of ceramide, but unlike D-*e*-MAPP, B13 was shown to be an inhibitor of acid ceramidase (Bielawska *et al.*, 2008).

Although B13 showed a high potency in aCDase inhibition *in vitro*, the effect on the enzyme *in vivo* may not be direct; its neutral nature makes it difficult to accumulate in the lysosome. Thus, B13 was taken as a scaffold to design new ceramidase inhibitor structures, and the uptake by the lysosome was enhanced with a series of lysosomotropic molecules such as LCL204 (also known as AD 2646). Thus, LCL204 significantly decreased cell migration caused by over-expressing aCDase, and it also sensitized head and neck cancer cells to FAS induced apoptosis. LCL204 overcame the resistance to apoptin mediated by the over-expression of aCDase in DU145, PC-3 and LNCaP cells. However, LCL204, although targeted to the lysosomes, was found to eventually destroy the lysosome and to cause degradation of aCDase by cathepsin B/L, and it also inhibited aSMase (Holman *et al.*, 2008), as previously was observed with desipramine. A new family of LCL204 analogues, which also target the lysosome without destabilizing them and without inducing degradation of aCDase were developed, and these include LCL 433, 449, 463, 464, 488 and LCL506 (Bai *et al.*, 2009). Another B13 analogue, LCL385 (Mahdy *et al.*, 2009) showed aCDase inhibition *in vivo*, with similar effects as seen with knocking down aCDase, sensitizing prostatic cancer cells to radiation, and reducing xenografts tumour growth.

A series of modifications in ceramide and Sph were tested on nCDase activity, all stereoisomers of D-*erythro*-ceramide and Sph (L-*threo*, D-*threo*, and L-*erythro* isomers), N-methyl-D-*erythro*-Sph and D-*erythro*-urea-C16-ceramide showed significant inhibitory effects, whereas other ceramide and Sph analogues such as N-methyl ceramide, 1-O-methyl ceramide, *cis*-D-*erythro* ceramide or d N,N-dimethyl-D-*erythro*-sphingosine had no effect. Of note, C1P and S1P stimulated the enzyme (Usta *et al.*, 2001). Diverse detergents such as taurocholate (TC), taurodeoxycholate (TDC), glycodeoxycholate (GDC), and (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) (CHAPS) inhibited *in vitro* nCDase activity (Ohlsson *et al.*, 2007).

Functional effects of inhibitors of ceramidases

Together with aSMase, aCDase has also been related to the pathogenesis of cystic fibrosis, suggesting ceramide accumulation mediating inflammation and cell death in lungs, and enhancing bacterial infection which is the major cause of mortality in CF patients. Thus, CF patients treated with amitriptyline, another tricyclic antidepressant, showed improvement in their lung function (Riethmuller *et al.*, 2009).

aCDase is involved in many cancers such as breast cancer (Ruckhaberle *et al.*, 2008; 2009), prostate cancer (Saad *et al.*, 2007; Holman *et al.*, 2008; Mahdy *et al.*, 2009), leukaemia (Furlong *et al.*, 2008; Shah *et al.*, 2008), colon cancer (Selzner *et al.*, 2001), head and neck squamous cell tumours (Elojeimy *et al.*, 2007), and individuals developing asbestos-induced malignant pleural mesothelioma (Archimandriti *et al.*, 2009). Therefore, one of the important goals to control cancer progression has become the design of drugs targeting aCDase. Endogenous over-expression of aCDase has been shown to reduce ceramide levels and increase S1P levels, and both effects have been related to stimulate cancer progression (Huwiler and Pfeilschifter, 2006). Moreover, resistance to radiation and/or chemotherapy used in cancer treatment has been shown to be caused by induction of aCDase synthesis in some cancer cell culture models (Mahdy *et al.*, 2009), thus pharmacological inhibition of aCDase sensitizes cancer cells to radiation and chemotherapy. The aCDase inhibitor B13 induced up to 90% apoptosis in colon cancer cells (Liu *et al.*, 2008) and reduction of colon tumor growth in nude mice (Selzner *et al.*, 2001). Furthermore, the B13 analogue, LCL 385, sensitized prostate cancer cells to radiation, and also decreased growth of xenograft tumour (Mahdy *et al.*, 2009), and the analogue LCL204 sensitized head and neck squamous cell tumours to Fas-induced cell death in both *in vitro* and *in vivo* systems (Elojeimy *et al.*, 2007).

On the other hand, the role of nCDase in cancer has not been studied. However, nCDase has been implicated in protective role in inflammation (Franzen *et al.*, 2001) and proliferation, as down-regulation of nCDase led to cell cycle arrest. ACER2 up-regulation has been implicated in tumour cell proliferation and survival whereas the role of ACER1 and ACER3 in cancer has not been studied.

Drug targeting of human ceramidases summary

The protein structure, the pH optimum, and the sub-cellular localization and topology of the ceramidases create, for one single reaction, different possible physiologies. Ceramidases have been implicated in many diseases, including various types of cancer, thus rendering ceramidases as attractive targets for drug developing. Thus, inhibitors of aCDase and nCDase have been shown to be promising drugs to overcome cell death resistance after prolonged anti-cancer treatments. Nevertheless, at the moment there are no inhibitors that combine specificity and the ability to reach the right cell compartment. For example, desipramine is used to inhibit aCDase, but it also inhibits aSMase, and other lysosomal enzymes. The inhibitor B13 is not efficient enough to reach the lysosome; encouragingly, other molecules such as LCL385 have been developed to solve this issue, and more studies are needed to show their *in vivo* specificity. There are no highly

specific inhibitors for neutral or alkCDases, although D-e-MAPP seems to inhibit the latter enzymes, having no effect in the acidic form. More research is needed for selective inhibitors that would help to understand not only the function of different ceramidases, but also their development into more efficacious and promising therapeutics to control ceramide-related pathologies.

Discussion

Dysregulation of SL metabolism has been reported for several human disease states; therefore, drugs that target enzymes of SL metabolism are attractive candidates for therapeutic development. Because ceramide has emerged as a key bioactive SL, enzymes of ceramide metabolism have emerged as key cellular regulators in stress responses. Enhanced accumulation of ceramide has been implicated in neuro-degeneration, diabetic complications, and increased responses to ischaemia/reperfusion injury. Therefore, targeting enzymes of ceramide production is an attractive target to ameliorate these conditions. Reciprocally, insufficient ceramide levels may allow cancer cells to escape cell death and to acquire resistance to chemotherapeutics; therefore, enhancing ceramide formation, primarily by inhibiting ceramidases (and other enzymes of ceramide metabolism, such as glucosylceramide synthase) are emerging as targets in cancer therapy.

Classically, ceramide has been studied as a single effector molecule; however emerging evidence clearly demonstrates that the generation or hydrolysis of ceramide (and bioactive lipids in general) might have different effects depending on where this ceramide is produced or further metabolized. For that reason, developing specific drugs against individual and distinct enzymes and targeted to the right compartment has emerged as a key and important goal that will help to understand ceramide function depending on its cellular localization and topology. This, in turn, should lead to more specific and rational therapeutics.

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Conflict of interest statement

None.

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