Small Molecule Inhibitors of Ceramidases

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Abstract
The equilibrium between the pro-apoptotic ceramide and pro-vital sphingosine-1-phosphate is considered to be decisive for cell death or survival. The different ceramidases thus play key roles in cell fate and might offer attractive targets for pharmacological intervention. Although until recently only moderately active inhibitors have been described, first in vivo experiments suggest activity against cancer cell survival and multi-drug resistance. Here, we provide a brief overview on the different ceramidases, and we will review the known inhibitors and current strategies for further inhibitor development.

Introduction
Besides their role as structural components of eukaryotic plasma membranes, sphingolipids are involved in cellular signaling. Gangliosides and sphingomyelin are the major sphingolipids of plasma membranes. After endocytosis, degradation of these membrane components yields ceramide and its further catabolites sphingosine and sphingosine-1-phosphate (Fig. 1). In addition, ceramide, but not sphingosine or sphingosine-1-phosphate is produced during de novo biosynthesis of sphingolipids. Ceramide has been shown to trigger inflammation, cell cycle arrest and apoptosis, while sphingosine-1-phosphate is pro-vital and triggers proliferation in many cell types. The conversion of ceramide to sphingosine-1-phosphate has been termed “sphingolipid rheostat”, due to its putative role in cell fate and it includes ceramidases and sphingosine kinases [1]. In addition, cellular ceramide concentrations are significantly influenced by different sphingomyelinases that are capable of rapidly producing ceramide upon stimulation. Inhibition of the sphingomyelinases thus
may result in protection from cell death and inflammation.[2, 3] On the other hand, inhibition of ceramidases should increase cellular ceramide and might be a strategy to induce cell death in cancer cells or in hyper-proliferative tissue. Ceramidases (CDases) are a heterogeneous family of ubiquitous N-acylsphingosine amidohydrolases, enzymes that catalyze the cleavage of ceramides into sphingosine and fatty acids. To date, five human ceramidases encoded by five distinct genes have been cloned. They are generally categorized by their pH optima for activity and subcellular localization: the acid ceramidase (ASAH1); neutral ceramidase (ASAH2); alkaline ceramidase 1 (ACER1/ASAH3); alkaline ceramidase 2 (ACER2/ASAH3L); and alkaline ceramidase 3 (ACER3/APHC/PHCA).

Alkaline ceramidases

Three different AlkCDase genes have been identified. The protein products of these genes have a similar molecular weight of approximately 31 kDa, with a pH for optimal activity between 8.5 and 9.5. All isoforms localize to the ER-Golgi network [4, 5]. The activity of all three AlkCDases is enhanced by the presence of the Ca\(^{2+}\) cation [4]. Recent studies demonstrated that AlkCDase is the only ceramidase present in erythrocyte and it is instrumental for the generation of sphingosine (SPH) and sphingosine 1-phosphate (S1P). Furthermore, AlkCDase has been found to be important for erythroid differentiation in K562 erythroleukaemic cells [5]. Alkaline ceramidase 1 (ACER1/ASAH3) is highly expressed in the skin and favors very long chain unsaturated ceramides as substrates. ACER1 plays an important role in mediating the Ca\(^{2+}\)-induced growth arrest and differentiation of epidermal keratinocytes [6, 7]. Alkaline ceramidase 2 (ACER2/ASAH3L) is mainly expressed in the placenta and favors long or very long chain ceramides over dihydroceramide and phytoceramide as substrates [8, 9]. ACER2 was found to play a protective role in cell survival during serum-deprivation by mediating the balance between SPH and S1P in HeLa cells. ACER2 has also been implicated in the regulation of Bcl-2 protein expression [10]. Alkaline ceramidase 3 (ACER3/APHC/PHCA) localizes to both the ER and Golgi apparatus and is highly expressed in most tissues, especially the placenta [11]. ACER3 favors long-chain (but not very long) unsaturated ceramides as substrates [4, 7, 12]. It has been suggested that ACER3 may act as a house-keeping enzyme responsible for the catabolism of a specific group of ceramides, in order to maintain basal cellular levels of sphingosine (SPH), dihydrosphingosine (DHS) or phytosphingosine (PHS) and their phosphates [4]. Interestingly, downregulation of ACER3 decreases the levels of other ceramide species and increases the cellular levels of both sphingosine (SPH) and sphingosine-1-phosphate (S1P) due to increased expression of ACER2 which hydrolyzes most mammalian ceramide species. Additionally, knockdown of ACER3 not only inhibited cell proliferation by up-regulation of the cyclin-dependent kinase inhibitor p21, but also inhibited serum-deprivation-induced apoptosis [12].
Acid ceramidase

Acid ceramidase activity (ACDase/ASAH1) activity was first reported in the rat brain by Shimon Gatt et al. [13]. The enzyme was subsequently purified to homogeneity from human urine [14], the cDNA for the gene was cloned [15] and characterized from other tissues [16]. The aCDase is expressed in form of a polypeptide of 395 amino acids in length (53-55 kDa) that undergoes proteolytic self-cleavage into two subunits: an α-subunit (13 kDa) and a β-subunit (40 kDa) [17]. ACDase localizes in the lysosomes from which a portion is secreted extracellularly [14, 18]. No cations are required for aCDase activity; however, anionic lysosomal lipids and sphingolipid activator proteins are required as cofactors for the efficient hydrolysis of ceramide in vivo [14]. The enzyme has an optimal pH of 4.5 [19] and favors medium chain unsaturated ceramides as substrates [14]. Some amphiphilic tricyclic agents such as desipramine, chlorpromazine and chloroquine can indirectly inhibit lysosomal ceramidase activity by down-regulating aCDase protein expression, but do not alter the levels of aCDase mRNA. Such inhibitory effects of tricyclic agents have only been observed in vivo or in intact cells [20]. Desipramine can induce downregulation of aCDase by activating its cathepsin-mediated proteolysis [20]. The major drawback of using tricyclic compounds is that they are non-selective, and in addition to inhibiting aCDase, they also inhibit aSMase [2, 21] and other lysosomal phospholipases [22]. Genetic mutations within the ASAH1 gene can cause a dramatically reduced activity of aCDase leading to a lysosomal storage disorder, named Farber disease [23]. Interestingly, the attempt to produce ASAH1 (-/-) mice failed [24], and aCDase was subsequently shown to be essential for early embryonic development in mice [25]. In contrast, heterozygous knock-out mice had a normal life span but developed a lipid storage phenotype in several organs [24]. A recent study with conditional ASAH1 knock-out mice confirmed an important role of this enzyme in female fertility by promoting oocyte survival during maturation of follicles [26].

A whole body of evidence suggests that aCDase plays a role in tumor formation or progression. The enzyme is up-regulated in different prostate cancer cell lines, which subsequently renders tumor cells resistant to chemo- and radiotherapy, resulting in disease progression and cancer relapse. Indeed, inhibition of aCDase has been shown to sensitize prostate cancer cells to chemo- and radiotherapy, reduce tumor growth and prevent cancer relapse [27-30]. Overexpression of aCDase in prostate cancer cells was associated with increased lysosomal density and increased levels of autophagy, accompanied by enhanced resistance to C2- ceramide [31]. Recently it was demonstrated that in prostate tumors, overexpressed aCDase results in S1P-mediated activation and nuclear expression of Ets1 [32]. Ets-1 in turn promoted prostate cancer invasion by triggering the over-expression and secretion of cathepsin B. In another study, aCDase has been found to be more highly expressed in metastatic than in the non-metastatic prostate cancer cells. Moreover, knockdown of ASAH1 in the metastatic cells caused an accumulation of ceramides, an inhibition of clonogenic potential, an increased requirement for growth factors, and last but not least an inhibition of tumorigenesis and lung metastases [33]. The aCDase has also been shown to promote the nuclear-cytoplasmic trafficking of PTEN in human prostate tissue, through sphingosine 1-phosphate-mediated activation of Akt signaling [34]. These events are associated with the promotion of tumor formation, cell proliferation, and resistance to therapy. In human pancreatic cancer cells, induced de novo biosynthesis of ceramide resulted in pronounced cytostatic effects, but not cell death. In such cells, inhibition of aCDase however induced cell death, suggesting aCDase as a therapeutic target in pancreatic cancer [35]. ACDase is also upregulated in non-small cell lung cancer (NSCLC) patients with acquired resistance to choline kinase α (ChoKα) inhibitors. Inhibition of aCDase was found to overcome the resistance to choline kinase α inhibition, and to sensitize lung cancer cells to ChoKα inhibitors [36]. Furthermore, Akt-2 and aCDase have been shown to cooperate to induce cell invasion and confer resistance to apoptosis. Combination of Akt and aCDase inhibitors synergistically inhibit cell viability/proliferation more effectively than single-agent treatments [37]. Moreover, aCDase has been described as an estrogen-dependent enzyme that might provide...
prognostic information in ER-positive breast cancers [38]. In another study, aCDase was downregulated in response to tamoxifen, suggesting that aCDase plays a crucial role in mediating the anti-estrogen activity of tamoxifen in treatment of breast cancer [39].

In contrast to the concept of aCDase as a promoter of cancer progression, it also was reported that low expression of aCDase can be associated with tumor progression in ovarian cancer, suggesting a role of sphingosine as a tumor suppressor [40].

Neutral ceramidase

Neutral ceramidase (NCDase/ASAH2) activity was first described in the human duodenum by Nilsson et al. [41]. NCDase has been cloned from bacteria [42], Drosophila [43], human [44], mouse [45], rat [46] Zebra fish [47] and several other species. NCDase was recently reviewed by Ito M. et al. [48]. NCDase confers 782 amino acids [49]. Although being highly glycosylated, deglycosylation does not affect nCDase activity [50, 51]. NCDase localizes to the outer leaflet of the plasma membrane (PM) [52] or it is secreted into the intestinal lumen [53]. Northern blotting demonstrated that nCDase is ubiquitously expressed [44], with high levels observed in the intestine, kidney, liver and heart and low levels in the brain, lungs, spleen, skeletal muscle and testis [45, 54]. Interestingly, both secreted intestinal and intracellular nCDase are identical enzymes [55]. NCDase has a broad optimal pH, ranging from 7-9, and no cations are required for activity [44]. The activity of nCDase purified from the rat or human intestine is not affected by Ca$^{2+}$, Mg$^{2+}$ or Mn$^{2+}$, but is inhibited by Zn$^{2+}$, Fe$^{2+}$ and Cu$^{2+}$ [53]. NCDase favors the natural D-erthro-ceramide isomer as a substrate over other isomers of ceramide, and has low or no affinity for the hydrolysis of dihydroceramide, phytoceramide, methylated ceramide and shorter ceramides [44]. Galadari et al. identified a nCDase motif comprised of a six amino acids (GDVSPN), and found that the serine residue (Ser 354) of this hexapeptide acts as the nucleophile to attack the amide bond of ceramide bound to the catalytic site [56].

A recent study identified a putative promoter region of 200 bp in the 5′-UTR of the human nCDase gene. Single mutation of an individual site within the putative promoter decreased reporter activity by up to 50% [57]. Overexpression of nCDase was found to inhibit apoptosis in response to TNF-α stress stimuli in rat primary hepatocytes, by regulation of ceramide and S1P in these cells [58]. In INS-1 cells, a chronic activation of nCDase is probably providing protection from cytokine-induced cell death and toxicity [59]. In addition, inflammatory stimuli (IL-1β) led to early sphingomyelinase activation and elevated ceramide in mesangial cells, followed by a later increase in nCDase, counteracting the sphingomyelinase-mediated ceramide production [60]. Other studies showed that overexpression of nCDase also results in increased generation of SPH and S1P by mediating the hydrolysis of ceramides in the plasma membrane and in extracellular space. However, in these reports it remained unclear whether, besides decreased ceramide, increased generation of SPH and S1P by nCDase has any effects on cell survival or apoptosis [61, 62]. Indeed, nCDase knock-out mice have a normal life span and do not have any obvious abnormalities, but are deficient in intestinal ceramide degradation [63]. Snider, A. et al. recently reported that loss of nCDase results in an unexpected increase in S1P generation in inflammatory bowel disease, and suggests that nCDase may actually protect against inflammation [64]. Furthermore, it has been shown that retinoic acid induces down-regulation of nCDase and accumulation of ceramide in SH-SY5Y cells, leading to cell-growth arrest and differentiation, while sphingosine or sphingosine 1-phosphate are unaffected [65]. Gemcitabine-treatment of polyoma cells has been shown to down-regulate nCDase expression leading to cell cycle arrest, through elevating the levels of ceramide [66].

As detailed above, ceramidases play a crucial role for final cellular response, cell homeostasis, and normal cell development through controlling the ceramide/sphingosine-1-phosphate (S1P) rheostat, and may also confer resistance to drugs and radiation. Therefore, ceramidase inhibitors have excellent potential for development as new anticancer drugs.
Saied/Arenz: Ceramidase Inhibitors

Herein, we review ceramide-derived inhibitors and other structurally unrelated inhibitors of different ceramidases (Fig. 2).

**Structural analogues of ceramides as ceramidase inhibitors**

NOE (N-oleoyl-ethanol amine) was the first ceramide-mimicking CDase inhibitor to be described [67]. The compound increases cellular levels of ceramide and induces apoptosis in different cell lines. However, in some studies NOE was shown to not only inhibit aCDase but also the glucosylation of ceramide in CHP-100 neuroepithelioma cells [68]. Moreover, it has been reported that both acidic and alkaline ceramidases in keratinocytes are inhibited by NOE [69]. Subsequently, NOE was defined as a weak and unselective aCDase inhibitor (with a $K_i$ of 500 µM) and its ability to inhibit aCDase in vitro and in vivo was not always reproducible [70]. Nevertheless, the endocannabinoid-related molecule NOE served as a scaffold for the design of other aCDase inhibitors. Fabrias and co-workers developed a series of several amides of differently 2-substituted aminoethanols in order to improve the selectivity and inhibitory potency of NOE [70-72]. Detailed structure activity relationship (SAR) studies revealed that the inhibitory efficacy of NOE in vitro and in intact cells was enhanced by suitable modifications of the functional groups. DM102, one of the NOE analogues developed, was found to dose dependently inhibit aCDase activity in intact cells with an IC$_{50}$ value of approximately 15µM, and to exert a cytotoxic effect in A549 cells with a LD$_{50}$ value of about 40 µM. Moreover, 50 µM DM102 elevated the levels of ceramide, resulting in induction of cell cycle arrest and apoptosis [72]. In light of these results, DM102 was further biochemically investigated. Inhibition of aCDase using DM102 was found to sensitize DU-145 prostate cancer cells to N-[4-hydroxyphenyl]-retinide (4-HPR), a potent (dihydro) ceramide-generating anticancer agent. Indeed, combined exposure to DM102 and 4-HPR synergistically improved the therapeutic efficacy of 4-HPR, by enhancing caspase activity,

![Fig. 2. Selection of previously described ceramidase inhibitors. Bottom row: compounds un-related to ceramide.](image-url)
and increasing the levels of (dihydro)-ceramide and reactive oxygen species (ROS) by 6- and 30-fold, respectively [73].

The ceramide analogue D-erythro-2-(N-myristoylamo)-1-phenyl-1-propanol (D-e-MAPP) was developed by Bielawska et al. in 1992 as a lipophilic aromatic ceramide analogue, and was found to elevate the endogenous cellular levels of ceramide by up to 3-fold, inhibit cell growth and induce apoptosis in HL-60 cells [74]. In further investigations, D-MAPP was reported to selectively inhibit alkCDase isolated from HL-60 human promyelocytic leukemia cells extract with an IC₅₀ of approximately 1-5 µM. The compound resulted in concentration- and time-dependent growth suppression accompanied by G0/G1 phase cell cycle arrest. On the other hand, L-MAPP, the enantiomer of D-e-MAPP had no effect on alkCDase. Instead, L-e-MAPP has been shown to be a substrate for alkaline ceramidase and is metabolized in a manner similar to C₁₆-ceramide via N-deacylation [75]. D-MAPP was also reported to function as a moderate inhibitor of acid ceramidase (IC₅₀ of 500 µM) in human melanoma and HaCat keratinocytes, in which D-MAPP induces apoptosis by increasing the endogenous levels of ceramide [76].

Further investigation in active lipophilic aromatic ceramide analogues led to the discovery of the more water soluble ceramidase inhibitor B13 [74, 76]. B13 was found to be a potent and selective aCDase inhibitor with an IC₅₀ of 10 µM in human melanoma and HaCat keratinocytes in which it led to elevation of the endogenous ceramide levels and induced apoptosis [76, 77]. We recently found that B13 exhibited an inhibitory activity towards purified recombinant human nCDase with a similar potency [78]. Our results may offer an explanation for the previous study by Bai et al., who reported that B13 significantly increased the cellular ceramide levels but did not affect aCDase activity under the conditions tested, probably due to inefficient delivery to the lysosomes [79]. Nonetheless, B13 has been shown to prevent growth of different human metastatic colorectal cancer cell lines. These effects were accompanied by increased levels of ceramide, activation of various pro-apoptotic molecules, such as caspases and with release of cytochrome c [80]. B13 also induced apoptotic cell death in different cultured prostate cancer cells and increased the sensitivity of androgen-insensitive prostate cancer xenografts tumors to radiation, which finally led to a significant reduction in tumor size [81].

In first attempts to improve the inhibitory potency of B13, the amide group was isosterically replaced with urea or an N-alkylamine group, which increased both the inhibitory effect against ceramidase and also cytotoxicity [82]. The obvious poor penetration of the lysosomes by B13 prompted Bielawska and co-workers to developed different series of B13 and D-e-MAPP analogues that are modified to localize in different cellular compartments. Several novel lysosome- and mitochondrion-directed analogues (LCL-analogues) showed enhanced rates of subcellular enrichment and a generally higher in situ CDase inhibition than the parent compounds [77, 79, 83]. Another series of cationic ceramide analogs were synthesized that preferentially targeted the mitochondria [84-86]. Noteworthy, some similar compounds have been previously described as inhibitors for sphingolipid biosynthesis by Gatt and co-workers [87]. Among the LCL-series, LCL204, LCL385 and LCL85 have been investigated further in detail. LCL85 an analogue of D-MAPP directed to mitochondria, displayed a more potent growth inhibitory effect (IC₅₀ = 2.3 µM), more promising anticancer activity (GI₅₀ -5.30) and lower toxicity in MCF7 breast carcinoma cells compared to the parent compound D-MAPP [77, 83]. Short-term exposure of LCL85 in combination with photodynamic therapy (PDT) in mouse squamous carcinoma cells led to distinct effects on the sphingolipid profile, enhanced autophagy, and induced activation of caspase-3 without leading to cell death. In contrast, long-term exposure to LCL85/ PDT enhanced overall cell death [88]. Very recently, LCL85 was shown to induce proteasomal degradation of the cIAP1 and xIAP proteins and to sensitize metastatic human breast and colon cancer cells to Fas-mediated apoptosis, which suppressed metastasis in vivo [89].

LCL204 (also known as AD 2646) has been shown to increase the sensitivity of head and neck squamous cancer cells (HNSCC) cells to FAS-induced apoptosis both in vitro and in vivo in a xenograft mouse model [90]. Additionally, LCL204 decreased the cellular levels of...
sphingosine and selectively increased C₁₄-, C₁₆-, and C₁₈-ceramide levels in DU-145 prostate cancer cells [91]. **LCL204** also dose- and time-dependently reduced the viability of Jurkat leukemic cells, accompanied by accumulation of endogenous ceramide and caspase activation [92]. Another analogue of **B13**, **LCL385**, was found to inhibit aCDase in vitro and sensitized PPC-1 prostate cancer cells to radiation and to significantly reduced xenograft tumor growth in nude mice [93]. However, further biochemical investigation revealed that **LCL204** and probably **LCL385** induced lyosomal permeabilization and proteolytic degradation of aCDase in a cathepsin-dependent manner [79] and also inhibited aSMase [91].

Based on these observations and in an attempt to develop novel aCDase inhibitors without lyosomal permeabilization activity, a second generation of novel lysosomotropic ω-N-amino analogues of the **B13** scaffold was developed and their aCDase inhibitory potentials were demonstrated to be high in vitro and in intact cells. **LCL464**, a representative second generation analogue, was found to inhibit aCDase activity in vitro (IC₅₀ ≈ 50 µM) with significant lower potency than **B13**; however, **LCL464** exhibited a significantly more potent inhibitory towards lysosomal aCDase than **B13** in MCF-7 cells. These observations suggest that the structural modification in **LCL464** is at the cost of **per se** inhibitory potency, which is however over-compensated by lyosomal enrichment. **LCL464** induced a potent and early inhibition of cellular aCDase which was associated with a decreased level of sphingosine, a specific increase in the contents of C₁₄- and C₁₆-ceramide and induction of apoptosis via activation of executioner caspases. **LCL464** also promoted higher levels of caspase-dependent apoptotic cell death in a wide range of different cancer cell lines. In contrast to **LCL204**, **LCL464** was shown to target lysosomal aCDase without destabilizing or degrading the enzyme [79]. In an effort by our group to improve the inhibitory potency of **B13** in vivo, we identified alternative sites for introduction of basic moieties into **B13** scaffold without interfering with the aCDase-inhibitory effect in vitro. Indeed, we found that the introduction of a weakly basic isostere (pyridine group) in the aromatic region of the **B13** scaffold generated a new analogue (**DP24c**) with a higher inhibitory effect against recombinant human aCDase compared to **B13**. However, it remains unclear whether the pyridine group, a weakly basic group, is indeed ensuring lysosomal targeting [94].

Furthermore, our group has carried out several detailed SAR studies on existing CDase inhibitors (**B13**, **D-e-MAPP**, **LCL464** and NOE) in order to determine which structural features are critical for ceramidase inhibition and also preferential enzyme selectivity. In order to investigate the effect of different electronic substituents in the phenyl ring on the inhibitory potency and enzyme selectivity, a detailed SAR study was performed using two sets of amide- and sulfonamide-based inhibitors that partially resemble the structure of **B-13** or **D-e-MAPP**. Our in vitro experiments revealed that while the electronic contribution of different substituents in the phenyl ring had negligible effects on inhibitory potency or enzyme selectivity, the hydrophobicity or steric effects of longer alkyl chains (Me, n-Pr, n-Bu or t-Bu) groups at the phenyl ring were found to be important for enhancing the selectivity towards aCDase over nCDase. In addition, replacement of the amide group with a sulfonamide further enhanced the inhibitory effects of **B13** and **D-MAPP** analogues [78]. Indeed, the sulfonamide analogue of **B13** (**KPB-70**) was significantly more potent than **B13** over the entire range of concentrations tested, with an IC₅₀ value for the inhibition of nCDase less than half of that of **B13**. In another report, we developed a second series of compounds based on structural modifications of the known ceramidase inhibitors **B13** and **LCL464**. Replacement of the p-nitro group of the **B13** scaffold with a primary amino group created a molecule (**KPB-67**) which potently elevated the endogenous levels of ceramide and consequently induced apoptotic cell death in MDA-MB-231 breast cancer cells. Noteworthy, this modification did not negatively affect the inhibitory potency towards aCDase and nCDase in vitro [95].

A family of structural analogues of ceramide and sphingosine were developed by Usta et al., in order to investigate the structural features essential for nCDase inhibition in vitro. The primary and secondary hydroxyl groups, the C₄-C₅ double bond, the trans-configuration of the C₄-C₅ double bond, and the NH-protons from either the amide of ceramide or the amine of sphingosine were found to be important features for nCDase inhibition in vitro. Moreover,
urea-ceramide ($C_{10}$-urea-Cer) and ceranine ($C_{16}$-ceramine) were identified as competitive inhibitors of nCDase [82].

Moreover, in a recent study, Camacho and co-workers identified novel potent, specific inhibitors of aCDase from a series of small ceramide analogs modified at the amide linkage with thiol reactive functions (an α-halocarbonyl unit or an α,β-double bond Michael acceptor moiety). Among the ceramide analogues developed, the compounds RBM1-12, RBM1-13, and SABRAC were the most potent aCDase inhibitors in intact FD10X cells (ranging from 50 to 70% inhibition) and also in the in vitro assay (with IC$_{50}$ values of 0.53 μM, 11.2 μM and 52 nM, respectively). These compounds were found to also time-dependently inhibit aCDase activity, suggesting a covalent modification of the enzyme. In contrast, cysteine proteases like papain were not affected. Both SABRAC and RBM1-12 were potent inhibitors of aCDase in intact PC-3/Mc cells and also elevated the levels of ceramide, and reduced the growth and clonogenic ability of these highly metastatic cells, however, the levels of sphingosine were unaffected. Surprisingly, in contrast, RBM1-13, which inhibited ACDase in FD10X cells, failed to inhibit aCDase in PC-3/Mc cells [96].

Ceramidase inhibitors that are not structural analogues of ceramide

Inoue et al. identified ceramidastin, which is structurally related to the rubratoxins, isolated from *Penicillium sp.* Mer-f17067, as a novel bacterial ceramidase inhibitor (with an IC$_{50}$ value of approximately 12 μM). Interestingly, ceramidastin had no effect on human ceramidase, and does not possess anti-microbial or antifungal activity. These properties suggest that ceramidastin may have potential for treating atopic dermatitis exacerbated by bacterial infection, with a low possibility of inducing drug resistance [97]. Draper et al. screened a compound library and identified a new class of quinolinone-based compounds (cerenib-1 and cerenib-2) as novel inhibitors of human ceramidase activity. Both cerenib-1 and cerenib-2 dose-dependently inhibited ceramidase activity in cell-based assays (with an IC$_{50}$ of 28 μM and 55 μM, respectively), and led to an accumulation of ceramides and a reduction in the levels of sphingosine and sphingosine-1-phosphate (S1P). Cerenib-1 and cerenib-2, both alone and in combination with paclitaxel, also inhibited cell proliferation and induced cell cycle arrest and cell death in a human ovarian cancer cell line [98].

In a very interesting study, Piomelli et al. identified a new class of substituted 2,4-dioxopyrimidine-1-carboxamides like 25a as highly potent non-competitive inhibitors of aCDase during screening of a commercial chemical library [99]. Indeed, preliminary studies by the same group identified carmofur, an anti-neoplastic drug currently used in the clinic to treat colorectal cancer, as the first nanomolar inhibitor of intracellular aCDase activity (IC$_{50}$= 29 nM, for rat recombinant aCDase). Interestingly, this inhibitory effect has been demonstrated to be an essential component of the anti-proliferation effect of carmofur and is independent of the ability of carmofur to generate 5-fluorouracil (5-FU). Consistent with these results, structural modifications to the carmofur scaffold produced a set of novel aCDase inhibitors that act synergistically with standard anti-cancer drugs to inhibit cancer cell proliferation. Later on, a detailed SAR study was performed by the same group to investigate the structural features of the uracil scaffold that are essential for aCDase inhibition in vitro [100]. Substitution at position N3, a 1-carboxamide alkyl chain of six to eight methylene units, and an electron withdrawing group at position 5 of the uracil ring identified to be beneficial to achieve potent aCDase inhibition. Moreover, the C$_{2}$-C$_{6}$ double bond of the uracil ring and the unsubstituted nitrogen in the 1-carboxamide moiety are mandatory structural features for aCDase inhibitory activity. Accordingly, the same study identified the first single-digit nanomolar inhibitors of recombinant rat aCDase (e.g., 25a, IC$_{50}$ of approximately 4 nM). However, the inhibitory potencies of these novel inhibitors have not yet been investigated in intact cells [100].

Elyahu et al. investigated the effect cystatins on aCDase activity, which led to identification of cystatin SA (cysSA) as a novel physiological peptide-based inhibitor of
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Ceramidase Activity

Interestingly, cysSA was found to affect activity of aCDase without affecting the cleavage of aCDase precursor. In vitro kinetic analysis of purified, recombinant aCDase and cysSA suggested a non-competitive type of inhibition with a $K_i$ of approximately 5 µM. Co-transfection of the full-length cDNAs encoding cysSA with the aCDase cDNA into cells inhibited endogenous aCDase activity and increased the levels of ceramide, while transfection of cells with siRNA targeting cysSA elevated aCDase activity and reduced the levels of ceramide. Subsequently, two short peptides were developed and also were shown to inhibit aCDase activity in vitro [101].

Ceramidase Assays

For the development and identification of novel ceramidase inhibitors, powerful assays will be of utmost importance. Several methods have been developed to evaluate ceramidase activity both in vitro and in situ. The first ceramidase assays were based on the hydrolysis of ceramide substrates labeled with $[^3]H$ or $[^14]C$ in the fatty acid and required separation of the non-hydrolyzed ceramide from the fatty acid in the reaction product by extraction and/or thin layer chromatography (TLC) [102, 103]. Since ceramide is very hydrophobic, high concentrations of detergent are required for delivery of the radioactive substrate. The drawback of time-consuming separation is accompanied by the advantage of using the natural substrate and the high validity of thus-performed assays.

A number of fluorescent spectroscopic ceramidase assays have been developed to avoid the use of radioactive substrates (Fig. 3). Such assays mostly make use of ceramide analogues with fluorophores attached to either the fatty acid or the sphingosine part. The ceramide activity can be measured by monitoring the release of the fluorescent molecule from the substrate by TLC separation and fluorimetry or by HPLC coupled with fluorescence detection. The fluorophores 7-nitro-2,1,3-benzoxadiazol (NBD), 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY), coumarin and diphenylhexatriene are most commonly used.

Initially, Cer-C$_{6}$-NBD was used for the determination of both acid and alkaline ceramidase activity in vitro and in situ. However, further investigations demonstrated that Cer-C$_{12}$-NBD was hydrolyzed at a higher rate by both neutral ceramidase and alkaline ceramidase from B16 melanoma cells than Cer-C$_{6}$-NBD in both in vitro and in vivo assays [104, 105]. Moreover, Cer-C$_{12}$-NBD was also found to be hydrolyzed at a higher rate than radioactive $[^14]C$-labeled ceramide by alkaline neutral ceramidase, while it was relatively resistant to acid ceramidase.

He X. et al. reported a novel method for determination of acid ceramidase activity in different cell lysates using (Cer-C$_{12}$-BODIPY) as a substrate. This assay was found to be more sensitive than the radioactive substrate assays, as it enabled determination of femtomole quantities of the product and accurate measurement of acid ceramidase activity [106]. An alternative approach for determination of ceramidase activity is the evaluation of the produced sphingosine by post-reaction derivatization with fluorescent compounds and its determination by HPLC and fluorescence detection. Two derivatizing agents have been reported for this approach: O-phthalic aldehyde (OPA) and naphthalene-2,3-dialdehyde (NDA) [14, 107]. The advantage of this approach is the use of un-modified natural ceramide as a substrate.

As an alternative to HPLC-based assays, Nieuwenhuizen and co-workers reported the first homogenous assay for alkaline ceramidase from Pseudomonas aeruginosa. This assay uses the fluorescence-quenched ceramide analogue Cer-C$_{12}$-pyrene, in which the fatty acid is labeled with a fluorescent pyrene group, while the sphingoid part is coupled to a Dinitrobenzoic acid quencher. The non-fluorescent probe (Cer-C$_{12}$-pyrene) becomes fluorescent upon hydrolysis of its N-acyl bond, and ceramidase activity can be assayed by detecting the release of fluorescent pyrene using a fluorimeter [108].

Recently, Bedia et al. developed a ceramidase probe in which a cumarin dye is conjugated to the aminodiol moiety of a dihydroceramide derivative (Cer-C$_{16}$-coumarine). This probe was found to be efficiently hydrolyzed by ceramidases both in vitro and in cultured cells in a
microtiter plate layout, and releases an aminodiol group which is then chemically oxidized by periodate to release umbelliferone (7-hydroxycoumarin), which can be easily detected by fluorimetry and thus be used for homogenous high-throughput assays (HTS) [109, 110]. Since a post-reaction treatment with periodate is necessary for the liberation of the fluorescent hydroxycoumarin, it is incompatible with real-time assays and does not provide spatial information [111].

We recently developed a set of singly-labeled fluorescent ceramide analogues, in which instead of the polar NBD-dye the more lipophilic Nile red (NR)-dye is attached to the fatty acid or sphingosine part, respectively. We investigated these analogues as substrates for recombinant acid and neutral ceramidases in micellar assays. Our kinetic studies revealed that for acid ceramidase there was no preference for NR-substitution at either the acyl- or the sphingosyl-part; however, for neutral ceramidase the ceramide molecules with acyl-substituted NBD (Cer-C_{12}-NBD) or Sph-substituted NR dyes (NR-Cer-C_{16}) were the better substrates, respectively [112]. In a subsequent step, we used the observed preferences to develop doubly-labeled fluorescent ceramide analogues as FRET probes to enable real-time determination of ceramidase activity. In these FRET probes, a NBD-dye (donor) and NR-dye (acceptor) are used as a donor-acceptor fluorescent pair to create a FRET effect. Hydrolysis of the FRET probe separates the donor-acceptor pair at the cost of the FRET effect. As a result, the NBD-fluorescence increases, while the NR fluorescence is decreasing. In contrast to simple turn-on probes, such a setup allows for ratio-imaging and thus should enable time- and spatially-resolved assays of ceramidase activities. The NR-Cer-C_{12}-NBD, was found to be efficiently hydrolyzed by recombinant acid and neutral ceramidases. However, in cultured cells the probe was rapidly enriching to Golgi apparatus and no cleavage was observed [113]. This limitation may be overcome in future by the development of a liposomal transporter. For a more detailed review on labeled chemical biology tools for sphingolipid research see also [114].

**Conclusion**

There is ample evidence for a role of different ceramidases in regulation of cell fate and for a therapeutic potential of ceramidase inhibitors in the treatment of human cancers. Very recently, the field experienced a significant boost by the development and identification of highly potent ceramidase inhibitors. Ceramide is a rather simple but very hydrophobic molecule with not more than four heteroatoms. Therefore, it seems almost impossible to develop ceramide analogues with high inhibitory potency and selectivity for a distinct enzyme at the same time. In our opinion, future potent ceramidase inhibitors will be allosteric compounds that do not structurally resemble ceramide. It is clear that presently.

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**Fig. 3.** Modified ceramides as probes for measuring ceramidase activities.
such compounds cannot be rationally designed. Therefore, the quest for such compounds will have to rely on high throughput screening. Very recently, a number of steps into such direction have been made. In contrast to ceramide-derived inhibitors, the novel ceramidase inhibitors like Carmofur and Ceranib appear to be more drug-like and thus have the potential to act more specifically. Carmofur (HCFU) has been used as inhibitor of thymidylate synthetase in the treatment of human cancers for many years and therefore appears to be an invaluable tool for \textit{in vitro} and \textit{in vivo} studies on the role of aCDase, provided that controls for thymidylate synthase inhibition are made. However, a more detailed characterization of the new compounds seems necessary and may provide further support to the concept of inhibiting ceramidases for medical purpose.

**Disclosure Statement**

The authors have no conflicts of interest to disclose.

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**References**

1. Maceyka M, Payne SG, Milstien S, Spiegel S: Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. Biochim Biophys Acta 2002;1585:193-201.
2. Arenz C: Small molecule inhibitors of acid sphingomyelinase. Cell Physiol Biochem 2010;26:1-8.
3. Canals D, Perry DM, Jenkins RW, Hannun YA: Drug targeting of sphingolipid metabolism: sphingomyelinases and ceramidases. Br J Pharmacol 2011;163:694-712.
4. Mao C, Xu R, Szulc ZM, Bielawski A, Galadari SH, Obeid LM: Cloning and characterization of a novel human alkaline ceramidase. A mammalian enzyme that hydrolyses phytoceamide. J Biol Chem 2001;276:26577-26588.
5. Xu R, Sun W, Jin J, Obeid LM, Mao C: Role of alkaline ceramidases in the generation of sphingosine and its phosphate in erythrocytes. FASEB J 2010;24:2507-2515.
6. Houben E, Holleran WM, Yanginuma T, Mao C, Obeid LM, Rogiers V, Takagi Y, Elias PM, Uchida Y: Differentiation-associated expression of ceramidase isoforms in cultured keratinocytes and epidermis. J Lipid Res 2006;47:1063-1070.
7. Sun W, Xu R, Hu W, Jin J, Crellin HA, Bielawski J, Szulc ZM, Thiers BH, Obeid LM, Mao C: Upregulation of the human alkaline ceramidase 1 and acid ceramidase mediates calcium-induced differentiation of epidermal keratinocytes. J Invest Dermatol 2008;128:389-397.
8. Sun W, Jin J, Xu R, Hu W, Szulc ZM, Bielawski J, Obeid LM, Mao C: Substrate specificity, membrane topology, and activity regulation of human alkaline ceramidase 2 (ACER2). J Biol Chem 2010;285:8995-9007.
9. Xu R, Jin J, Hu W, Sun W, Bielawski J, Szulc Z, Taha T, Obeid LM, Mao C: Golgi alkaline ceramidase regulates cell proliferation and survival by controlling levels of sphingosine and S1P. FASEB J 2006;20:1813-1825.
10. Adams JM, 2nd, Pratipanawatr T, Berria R, Wang E, DeFranco RA, Sullards MC, Mandarino LJ: Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes 2003;53:25-31.
11. Mao C, Obeid LM: Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate. Biochim Biophys Acta 2008;1781:424-434.
12. Hu W, Xu R, Sun W, Szulc ZM, Bielawski J, Obeid LM, Mao C: Alkaline ceramidase 3 (ACER3) hydrolyzes unsaturated long-chain ceramides, and its down-regulation inhibits both cell proliferation and apoptosis. J Biol Chem 2010;285:7964-7976.
13 Gatt S: Enzymatic hydrolysis of sphingolipids. I. Hydrolysis and synthesis of ceramides by an enzyme from rat brain. J Biol Chem 1966;241:3724-3730.

14 Bernardo K, Hurwitz R, Zenk T, Desnijck RJ, Ferlinz K, Schuchman EH, Sandhoff K: Purification, characterization, and biosynthesis of human acid ceramidase. J Biol Chem 1995;270:11098-11102.

15 Li CM, Hong SB, Kopal G, He X, Linke T, Hou WS, Koch J, Gatt S, Sandhoff K, Schuchman EH: Cloning and characterization of the full-length cDNA and genomic sequences encoding murine acid ceramidase. Genomics 1998;50:267-274.

16 Linke T, Wilkening G, Sadeghlar F, Mozoll H, Bernardo K, Schuchman E, Sandhoff K: Interfacial regulation of acid ceramidase activity: Stimulation of ceramide degradation by lysosomal lipids and sphingolipid activator proteins. J Biol Chem 2000;276:5760-5768.

17 Shtraizent N, Eliyahu E, Park JH, He X, Shalgi R, Schuchman EH: Autoproteolytic cleavage and activation of human acid ceramidase. J Biol Chem 2008;283:11253-11259.

18 Ferlinz K, Kopal G, Bernardo K, Linke T, Bar J, Breiden B, Neumann U, Lang F, Schuchman EH, Sandhoff K: Human acid ceramidase: processing, glycosylation, and lysosomal targeting. J Biol Chem 2001;276:35352-35360.

19 He X, Okino N, Dhami R, Dagan A, Gatt S, Schulte H, Sandhoff K, Schuchman EH: Purification and characterization of recombinant, human acid ceramidase. Catalytic reactions and interactions with acid sphingomyelinase. J Biol Chem 2003;278:32979-32986.

20 Elojeimy S, Holman DH, Liu X, El-Zawahry A, Villani M, Cheng JC, Mahdy A, Zeidan Y, Bielwaska A, Hannun YA, Norris JS: New insights on the use of desipramine as an inhibitor for acid ceramidase. FEBS Lett 2006;580:4751-4756.

21 Kolter T, Sandhoff K: Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid activator proteins and anionic lysosomal lipids. Annu Rev Cell Dev Biol 2005;21:81-103.

22 Pappu A, Hostetler KY: Effect of cationic amphiphilic drugs on the hydrolysis of acidic and neutral phospholipids by liver lysosomal phospholipase A. Biochem Pharmacol 1984;33:1639-1644.

23 Koch J, Gartner S, Li CM, Quinlent LE, Bernardo K, Levan O, Schnabel D, Desnijck RJ, Schuchman EH, Sandhoff K: Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. Identification of the first molecular lesion causing Farber disease. J Biol Chem 1996;271:33110-33115.

24 Li CM, Park JH, Simonaro CM, He X, Gordon RE, Friedman AH, Ehleiter D, Paris F, Manova K, Hebildiker S, Fuks Z, Sandhoff K, Kolesnick R, Schuchman EH: Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage disease in heterozygotes. Genomics 2002;79:218-224.

25 Eliyahu E, Park JH, Shtraizent N, He X, Schuchman EH: Acid ceramidase is a novel factor required for early embryo survival. FASEB J 2007;21:1403-1409.

26 Eliyahu E, Shtraizent N, Shalgi R, Schuchman EH: Construction of conditional acid ceramidase knockout mice and in vivo effects on oocyte development and fertility. Cell Physiol Biochem 2012;30:735-748.

27 Saad AF, Meacham WD, Bai A, Anelli V, Elojeimy S, Mahdy AE, Turner LS, Cheng J, Bielawska A, Bielawski J, Keane TE, Obeid LM, Hannun YA, Norris JS, Liu X: The functional effects of acid ceramidase overexpression in prostate cancer progression and resistance to chemotherapy. Cancer Biol Ther 2007;6:1455-1460.

28 Cheng JC, Bai A, Beckham TH, Marsson ST, Yount CL, Young K, Lu P, Bartlett AM, Wu BX, Keane BJ, Armeson KE, Marshall DT, Keane TE, Smith MT, Jones EE, Drake RR, Bielawska A, Norris JS, Liu X: Radiation-induced acid ceramidase confers prostate cancer resistance and tumor relapse. J Clin Invest 2013;123:4344-4358.

29 Liu X, Cheng JC, Turner LS, Elojeimy S, Beckham TH, Bielawska A, Keane TE, Hannun YA, Norris JS: Acid ceramidase upregulation in prostate cancer: role in tumor development and implications for therapy. Expert Opin Ther Targets 2009;13:1449-1458.

30 Gouaze-Andersson V, Flowers M, Karimi R, Fabrias G, Delgado A, Casas J, Cabot MC: Inhibition of acid ceramidase by a 2-substituted aminoethanol amide synergistically sensitizes prostate cancer cells to N-(4-hydroxyphenyl) retinamide. Prostate 2011;71:1064-1073.

31 Turner LS, Cheng JC, Beckham TH, Keane TE, Norris JS, Liu X: Autophagy is increased in prostate cancer cells overexpressing acid ceramidase and enhances resistance to C6 ceramide. Prostate Cancer Prostatic Dis 2011;14:30-37.
Saied/Arenz: Ceramidase Inhibitors

32 Beckham TH, Lu P, Cheng JC, Zhao D, Turner LS, Zhang X, Hofman S, Armeson KE, Liu A, Morrison T, Hannun YA, Liu X: Acid ceramidase-mediated production of sphingosine 1-phosphate promotes prostate cancer invasion through upregulation of cathepsin B. Int J Cancer 2012;131:2034-2043.

33 Camacho L, Meca-Cortés O, Abad JL, García S, Rubio N, Díaz A, Celià-Terrassa T, Cingolani F, Bermudo R, Fernández PL, Blanco J, Delgado A, Casas J, Fabriás G, Thomson TM: Acid ceramidase as a therapeutic target in metastatic prostate cancer. J Lipid Res 2013;54:1207-1220.

34 Beckham TH, Cheng JC, Lu P, Shao Y, Troyer D, Lance R, Marrison ST, Norris JS, Liu X: Acid ceramidase induces sphingosine kinase 1/S1P receptor 2-mediated activation of oncogenic Akt signaling. Oncogenesis 2013;2:e49.

35 Morad SA, Messner MC, Levin JC, Abdelmageed N, Park H, Merrill AH, Cabot MC: Potential role of acid ceramidase in conversion of cytostatic to cytotoxic end-point in pancreatic cancer cells. Cancer Chemother Pharmacol 2013;7:1:635-645.

36 Ramírez de Molina A, de la Cueva A, Machado-Pinilla R, Rodriguez-Fanjul V, Gomez del Pulgar T, Cebrian A, Perona R, Lacal JC: Acid ceramidase as a chemotherapeutic target to overcome resistance to the antitumoral effect of choline kinase α inhibition. Curr Cancer Drug Targets 2012;12:1:617-624.

37 Berndt N, Patel R, Yang H, Balasise SM, Sebti SM: Akt2 and acid ceramidase cooperate to induce cell invasion and resistance to apoptosis. Cell Cycle 2013;12:2024-2032.

38 Ruckhäberle E, Holtrich U, Engels K, Hanker L, Gärtner R, Metzler D, Karn T, Kaufmann M, Rody A: Acid ceramidase 1 expression correlates with a better prognosis in ER-positive breast cancer. Clinimetric 2009;12:502-513.

39 Morad SA, Levin JC, Tan SF, Fox TE, Feith DJ, Cabot MC: Novel off-target effect of tamoxifen--inhibition of acid ceramidase activity in cancer cells. Biochim Biophys Acta 2013;1831:1657-1664.

40 Hanker LC, Karn T, Holtrich U, Gärtner R, Rody A, Heinrich R, Ruckhäberle E, Engels K: Acid ceramidase (AC)-a key enzyme of sphingolipid metabolism--correlates with better prognosis in epithelial ovarian cancer. Int J Gynecol Pathol 2013;32:249-257.

41 Nilsson A: The presence of spingomyelin- and ceramide-degrading enzymes in the small intestinal tract. Biochim Biophys Acta 1969;176:339-347.

42 Okino N, Ikeda R, Ito M: Expression, purification, and characterization of a recombinant neutral ceramidase from Mycobacterium tuberculosis. Biosci Biotechnol Biochem 2010;74:316-321.

43 Yoshimura Y, Okino N, Tani M, Ito M: Molecular cloning and characterization of a secretory neutral ceramidase of Drosophila melanogaster. J Biochem 2002;132:229-236.

44 El Bawab S, Roddy P, Qian T, Bielawska A, Lemasters JJ, Hannun YA: Molecular cloning and characterization of a human mitochondrial ceramidase. J Biol Chem 2000;275:21508-21513.

45 Tani M, Okino N, Mori K, Tanigawa T, Izu H, Ito M: Molecular cloning of the full-length cDNA encoding mouse neutral ceramidase. A novel but highly conserved gene family of neutral/alkaline ceramidases. J Biol Chem 2000;275:11229-11234.

46 Mitsutake S, Tani M, Okino N, Mori K, Ichinose S, Omori A, Iida H, Nakamura T, Ito M: Purification, characterization, molecular cloning, and subcellular distribution of neutral ceramidase of rat kidney. J Biol Chem 2001;276:26249-26259.

47 Yoshimura Y, Tani M, Okino N, Ito M: Molecular cloning and functional analysis of zebrafish neutral ceramidase. J Biol Chem 2004;279:44012-44022.

48 Ito M, Okino N, Tani M: New insight into the structure, reaction mechanism, and biological functions of neutral ceramidase. Biochim Biophys Acta 2014;1841:682-691.

52 Hwang YH, Tani M, Nakagawa T, Okino N, Ito M: Subcellular localization of human neutral ceramidase expressed in HEK293 cells. Biochem Biophys Res Commun 2005;331:37-42.

53 Olsson M, Duan RD, Ohlsson L, Nilsson A: Rat intestinal ceramidase: purification, properties, and physiological relevance. Am J Physiol Gastrointest Liver Physiol 2004;287:G929-937.
54 Choi MS, Anderson MA, Zhang Z, Zimonjic DB, Popescu N, Mulherjee AB: Neutral ceramidase gene: role in regulating ceramide-induced apoptosis. Gene 2003;315:113-122.

55 Duan RD, Nilsson A: Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development. Prog Lipid Res 2009;48:62-72.

56 Galadari S, Wu BX, Mao C, Roddy P, El Bawab S, Hannun YA: Identification of a novel amidase motif in neutral ceramidase. Biochem J 2000;363:93:687-695.

57 O’Neill SM, Yun JK, Fox TE, Kester M: Transcriptional regulation of the human neutral ceramidase gene. Arch Biochem Biophys 2011;511:21-30.

58 Osaka Y, Uchinami H, Bielawski J, Schwabe RF, Hannun YA, Brenner DA: Roles for C16-ceramide and sphingosine 1-phosphate in regulating hepatic apoptosis in response to tumor necrosis factor-alpha. J Biol Chem 2005;280:27879-27887.

59 Zhu Q, Jin JF, Shan XH, Liu CP, Mao XD, Xu KF, Liu C: Chronic activation of neutral ceramidase protects beta-cells against cytokine-induced apoptosis. Acta Pharmacol Sin 2008;29:593-599.

60 Franzen R, Pautz A, Brautigam L, Geisslinger G, Pfeilschifter J, Huwiler A: Interleukin-1beta induces chronic activation and de novo synthesis of neutral ceramidase in renal mesangial cells. J Biol Chem 2001;276:35382-35391.

61 Canals D, Jenkins RW, Roddy P, Hernandez-Corbacho MJ, Obeid LM, Hannun YA: Differential effects of ceramide and sphingosine 1-phosphate on ERK phosphorylation: probing sphingolipid signaling at the outer plasma membrane. J Biol Chem 2010;285:32476-32485.

62 Tani M, Igarashi Y, Ito M: Involvement of neutral ceramidase in ceramide metabolism at the plasma membrane and in extracellular milieu. J Biol Chem 2005;280:36592-36600.

63 Kono M, Dreier JL, Ellis JM, Allende ML, Kalkolien DN, Sanders KM, Bielawski J, Bielawska A, Hannun YA, Proia RL: Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. J Biol Chem 2006;281:7324-7331.

64 Snider AJ, Wu BX, Jenkins RW, Sticca JA, Kawamori T, Hannun YA, Obeid LM: Loss of neutral ceramidase increases inflammation in a mouse model of inflammatory bowel disease. Prostaglandins Other Lipid Mediat 2012;99:124-130.

65 Tanaka K, Tamiya-Koizumi K, Hagiwara K, Ito H, Takagi A, Kojima T, Suzuki M, Iwaki S, Fujii S, Nakamura M, Banno Y, Kannagi R, Tsurumi T, Kyogashima M, Murate T: Role of down-regulated neutral ceramidase during all-trans retinoic acid-induced neuronal differentiation in SH-SYSY neuroblastoma cells. J Biochem 2012;151:611-620.

66 Wu BX, Zeidan YH, Hannun YA: Downregulation of neutral ceramidase by gemcitabine: Implications for cell cycle regulations and cell cycle regulation. Biochim Biophys Acta 2009;1791:730-739.

67 Sugita M, Williams M, Dulaney JT, Moser HW: Ceramidase and ceramide synthesis in human kidney and cerebellum. Description of a new alkaline ceramidase. Biochim Biophys Acta 1975;398:125-131.

68 Spinedi A, Di Bartolomeo S, Piacentini M: N-Oleoyl ethanolamine inhibits glucosylation of natural ceramides in CHP-100 neuroepithelium cells: possible implications for apoptosis. Biochem Biophys Res Commun 1999;255:456-459.

69 Houben E, Uchida Y, Nieuwenhuizen WF, De Paepe K, Vanhaecke T, Holleran WM, Rogiers V: Kinetic characteristics of acidic and alkaline ceramidase in human epidermis. Skin Pharmacol Physiol 2007;20:187-194.

70 Grijalvo S, Bedia C, Triola G, Casas J, Llebaria A, Teisido J, Rabal O, Levade T, Delgado A, Fabrias G: Design, synthesis and activity as acid ceramidase inhibitors of 2-oxooctanoyl and N-oleylethanolamine analogues. Chem Phys Lipids 2006;144:69-84.

71 Bedia C, Triola G, Casas J, Llebaria A, Fabrias G: Analogs of the dihydroceramide desaturase inhibitor GT11 modified at the amide function: synthesis and biological activities. Org Biomol Chem 2005;3:3707-3712.

72 Bedia C, Canals D, Matabosch X, Harrak Y, Casas J, Llebaria A, Delgado A, Fabrias G: Cytotoxicity and acid ceramidase inhibitory activity of 2-substituted aminoethanol amides. Chem Phys Lipids 2008;156:33-40.

73 Gouaze-Andersson V, Flowers M, Karimi R, Fabrias G, Delgado A, Casas J, Cabot MC: Inhibition of acid ceramidase by a 2-substituted aminoethanol amide synergistically sensitizes prostate cancer cells to N-(4-hydroxyphenyl) retinamide. Prostate 2011;71:1064-1073.

74 Bielawski A, Linardic CM, Hannun YA: Ceramide-mediated biology: Determination of structural and stereospecific requirements through the use of N-acyl-phenylaminool alcohol analogues. J Biol Chem 1992;267:18493-18497.
Saied/Arenz: Ceramidase Inhibitors

75 Bielawska A, Greenberg MS, Perry D, Jayadev S, Shayman JA, McKay C, Hannun YA: (1S,2R)-D-erythro-2-(N-
myristoylamino)-1-phenyl-1-propanol as an inhibitor of ceramidase. J Biol Chem 1996;271:12646-12654.

76 Raisova M, Goltz G, Bektas M, Bielawska A, Rieberling C, Hossini AM, Eberle J, Hannun YA, Orfanos CE, Geilen
CC: Bcl-2 overexpression prevents apoptosis induced by ceramidase inhibitors in malignant melanoma and HaCaT keratinocytes. FEBS Letters 2002;516:47-52.

77 Bielawska A, Bielawski J, Szulc ZM, Mayroo N, Liu X, Bai A, Elojeimy S, Rembiesa B, Pierce J, Norris JS,
Hannun YA: Novel analogs of D-e-MAPP and B13. Part 2: signature effects on bioactive sphingolipids. Bioorg Med Chem 2008;16:1032-1045.

78 Bhabak KP, Arenz C: Novel amide- and sulfonamide-based aromatic ethanolamines: effects of various
substituents on the inhibition of acid and neutral ceramidases. Bioorg Med Chem 2012;20:6162-6170.

79 Bai A, Szulc ZM, Bielawski J, Mayroo N, Liu X, Norris J, Hannun YA, Bielawska A: Synthesis and
bioevaluation of omega-N-amino analogs of B13. Bioorg Med Chem 2009;17:1840-1848.

80 Selzner M, Bielawska A, Morse MA, Rudiger HA, Sindram D, Hannun YA, Clavien PA: Induction of apoptotic
cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer:
Cancer Res 2001;61:1233-1240.

81 Samsel L, Zaidel G, Drumgoole Honesty M, Jelovac D, Drachenberg C, Rhee Juong G, Brodie Angela MH,
Bielawska A, Smyth Miriam J: The ceramide analog, B13, induces apoptosis in prostate cancer cell lines and
inhibitors tumor growth in prostate cancer xenografts. Prostate 2004;58:382--393.

82 Usta J, El Bawab S, Roddy P, Szulc ZM, Yusuf, Hannun A, Bielawska A: Structural requirements of ceramide
and sphingosine based inhibitors of mitochondrial ceramidase. Biochemistry 2001;40:9657-9668.

83 Szulc ZM, Mayroo N, Bai A, Bielawski J, Liu X, Norris JS, Hannun YA, Bielawska A: Novel analogs of D-e-
MAPP and B13. Part 1: synthesis and evaluation as potential anticancer agents. Bioorg Med Chem 2008;16:1015-1031.

84 Dindo D, Dahm F, Szulc Z, Bielawska A, Obeid LM, Hannun YA, Graf R, Clavien PA: Cationic long-chain
ceramide LCL-30 induces cell death by mitochondrial targeting in SW403 cells. Mol Cancer Ther 2006;5:1520-1529.

85 Senkal CE, Ponnusamy S, Rossi MJ, Sundararaj K, Szulc Z, Bielawski J, Bielawska A, Meyer M, Cohanoglu B,
Koybasi S, Sinha D, Day TA, Obeid LM, Hannun YA, Ogretmen B: Potent antitumor activity of a novel cationic
pyridinium-ceramide alone or in combination with gemcitabine against human head and neck squamous
child carcinomas in vitro and in vivo. J Pharmacol Exp Ther 2006;317:1188-1199.

86 Szulc ZM, Bielawski J, Gracz H, Gustilo M, Mayroo N, Hannun YA, Obeid LM, Bielawska A: Tailoring
structure-function and targeting properties of ceramides by site-specific cationization. Bioorg Med Chem 2006;14:7083-7104.

87 Dagan A, Wang C, Fibach E, Gatt S: Synthetic, non-natural sphingolipid analogs inhibit the biosynthesis
of cellular sphingolipids, elevate ceramide and induce apoptotic cell death. Biochim Biophys Acta 2003;1633:161-169.

88 Separovic D, Joseph N, Breen P, Bielawski J, Pierce JS, Buren EV, Bhatti G, Saad ZH, Bai A, Bielawska A: Combining anticancer agents photodynamic therapy and LCL85 leads to distinct changes in the
sphingolipid profile, autophagy, caspase-3 activation in the absence of cell death, and long-term
sensitization. Biochim Biophys Acta Commun 2011;409:372-377.

89 Paschall AV, Zimmerman MA, Torres CM, Yang D, Chen MR, Li X, Bieberich E, Bai A, Bielawski J, Bielawska A,
Liu K: Ceramide targets xIAP and cIAP1 to sensitize metastatic colon and breast cancer cells to apoptosis
induction to suppress tumor progression. BMC Cancer 2014;14:24.

90 Elojeimy S, Liu X, McKillop JC, El-Zawahry AM, Holman DH, Cheng JY, Meacham WD, Mahdy AE, Saad AF,
Turner LS, Cheng J, T AD, Dong JY, Bielawska A, Hannun YA, Norris JS: Role of acid ceramidase in resistance
to FasL: therapeutic approaches based on acid ceramidase inhibitors and FasL gene therapy. Mol Ther 2007;15:1259-1263.

91 Holman DH, Turner LS, El-Zawahry A, Elojeimy S, Liu X, Bielawski J, Szulc ZM, Norris K, Zeidan YH, Hannun
YA, Bielawska A, Norris JS: Lysoosomotropic acid ceramidase inhibitor induces apoptosis in prostate cancer
cells. Cancer Chemother Pharmacol 2008;61:231-242.

92 Granot T, Milhas D, Carpentier S, Dagan A, Segui B, Gatt S, Levade T: Caspase-dependent and -independent
cell death of Jurkat human leukemia cells induced by novel synthetic ceramide analogs. Leukemia 2006;20:392-399.
93 Mahdy AE, Cheng JC, Li J, Elojeimy S, Meacham WD, Turner LS, Bai A, Gault CR, McPherson AS, Garcia N, Beckham TH, Saad A, Bielawska A, Bielawski J, Hannun YA, Keane TE, Taha MI, Hammouda HM, Norris JS, Liu X: Acid ceramidase upregulation in prostate cancer cells confers resistance to radiation: AC inhibition, a potential radiosensitizer. Mol Ther 2009;17:430-438.

94 Proksch D, Klein J, Arenz C: Potent inhibition of Acid ceramidase by novel B-13 analogues. J Lipids 2011;97:1618.

95 Bhabak KP, Kleuser B, Huwiler A, Arenz C: Effective inhibition of acid and neutral ceramidas by novel B-13 and LCL-464 analogues. J Lipids 2013;21:874-882.

96 Camacho L, Meca-Cortes O, Abad JL, Garcia S, Rubio N, Diaz A, Celia-Terrassa T, Cingolani F, Bermudo R, Fernandez PL, Blanco J, Delgado A, Casas J, Fabrias G, Thomson TM: Acid ceramidase as a therapeutic target in metastatic prostate cancer. J Lipid Res 2013;54:1207-1220.

97 Inoue H, Someno T, Kato T, Kumagai H, Kawada M, Ikeda D: Ceramidastin, a novel bacterial ceramidase inhibitor, produced by Pencillium sp. Mer-f17067. J Antibiot (Tokyo) 2009;62:63-67.

98 Draper JM, Xia Z, Smith RA, Zhuang Y, Wang W, Smith CD: Discovery and evaluation of inhibitors of human ceramidase. Mol Cancer Ther 2011;10:2052-2061.

99 Realini N, Solorzano C, Pagliuca C, Pizzirani D, Armirrotti A, Luciani R, Costi MP, Bandiera T, Piomelli D: Discovery of highly potent acid ceramidase inhibitors with in vitro tumor chemosensitizing activity. Sci Rep 2013;3:1035.

100 Pizzirani D, Pagliuca C, Realini N, Branduardi D, Bottegoni G, Mor M, Bertozi F, Scarpelli R, Piomelli D, Bandiera T: Discovery of a new class of highly potent inhibitors of acid ceramidase: synthesis and structure-activity relationship (SAR). J Med Chem 2013;56:3518-3530.

101 Eliyahu E, Shtraizent N, He X, Chen D, Shalgi R, Schuchman EH: Identification of cystatin SA as a novel inhibitor of acid ceramidase. J Biol Chem 2011;286:35624-35633.

102 Yavin E, Gatt S: Enzymatic hydrolysis of sphingolipids. 8. Further purification and properties of rat brain ceramidase. Biochemistry 1969;8:1692-1698.

103 Mitsutake S, Kita K, Okino N, Ito M: [14C]ceramide synthesis by sphingolipid ceramide N-deacylase: new assay for ceramidase activity detection. Anal Biochem 1997;247:52-57.

104 Tani M, Kita K, Komori H, Nakagawa T, Ito M: Enzymatic synthesis of omega-amino-ceramide: preparation of a sensitive fluorescent substrate for ceramidase. Anal Biochem 1998;263:183-188.

105 Tani M, Okino N, Mitsutake S, Ito M: Specific and sensitive assay for alkaline and neutral ceramidas involving C12-NBD-ceramide. J Biochem 1999;125:746-749.

106 He X, Li CM, Park JH, Dagan A, Gatt S, Schuchman EH: A fluorescence-based high-performance liquid chromatographic assay to determine acid ceramidase activity. Anal Biochem 1999;274:264-269.

107 He X, Dagan A, Gatt S, Schuchman EH: Simultaneous quantitative analysis of ceramide and sphingosine in mouse blood by naphthalene-2,3-dicarboxyaldehyde derivatization after hydrolysis with ceramidase. Anal Biochem 2005;340:113-122.

108 Nieuwenhuizen WF, van Leeuwen S, Gotz F, Egmond MR: Synthesis of a novel fluorescent ceramide analogue and its use in the characterization of recombinant ceramidase from Pseudomonas aeruginosa PAO1. Chem Phys Lipids 2002;114:181-191.

109 Bedia C, Casas J, Garcia V, Levade T, Fabrias G: Synthesis of a novel ceramide analogue and its use in a high-throughput fluorogenic assay for ceramidas. Chembiochem 2007;8:642-648.

110 Bedia C, Canals D, Matabosch X, Harrak Y, Casas J, Llebaria A, Delgado A, Fabrias G: Cytotoxicity and acid ceramidase inhibitory activity of 2-substituted aminothanol amides. Chem Phys Lipids 2008;156:33-40.

111 Bedia C, Camacho L, Abad JL, Fabrias G, Levade T: A simple fluorogenic method for determination of acid ceramidase activity and diagnosis of Farber disease. J Lipid Res 2010;51:3542-3547.

112 Bhabak KP, Proksch D, Redmer S, Arenz C: Novel fluorescent ceramide derivatives for probing ceramidase substrate specificity. Bioorg Med Chem 2012;20:6154-6161.

113 Bhabak KP, Hauser A, Redmer S, Banhart S, Heuer D, Arenz C: Development of a novel FRET probe for the real-time determination of ceramidase activity. Chembiochem 2013;14:1049-1052.

114 Schwarzmann G, Arenz C, Sandhoff K: Labeled chemical biology tools for investigating sphingolipid metabolism, trafficking and interaction with lipids and proteins. Biochim Biophys Acta 2013; DOI 10.1016/j.bbalip.2013.12.011.