Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;34:197-212
DOI: 10.1159/000362995
Published online: June 16, 2014
Accepted: April 24, 2014

1421-9778/14/0341-0197$39.50/0

Review

Small Molecule Inhibitors of Ceramidases

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Key Words
Sphingolipids • Lipid signalling • Enzyme inhibitors • Enzyme assays • Lipid probes • Ceramidases • Ceramide

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

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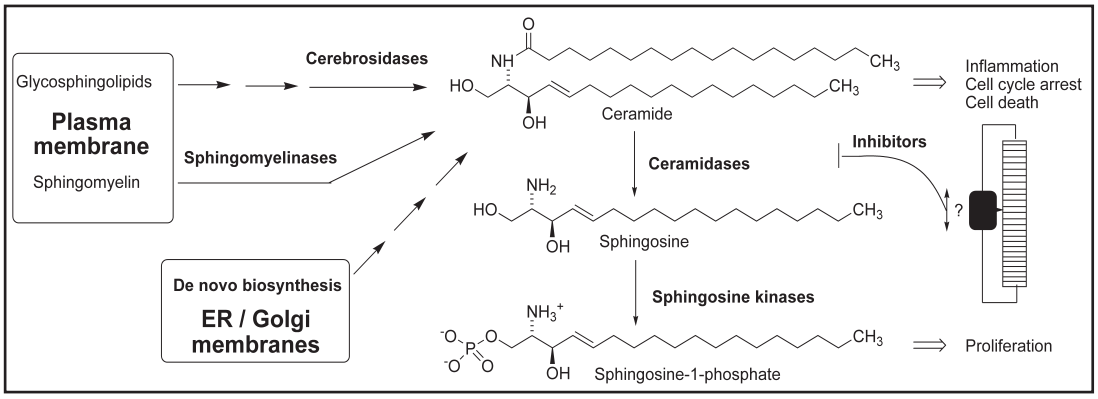

Fig. 1. The 'sphingolipid rheostat' and key enzymes that influence cellular ceramide levels (simplified).

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 C_6-ceramide [31]. Recently it was demonstrated that in prostate tumors, over-expressed 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.

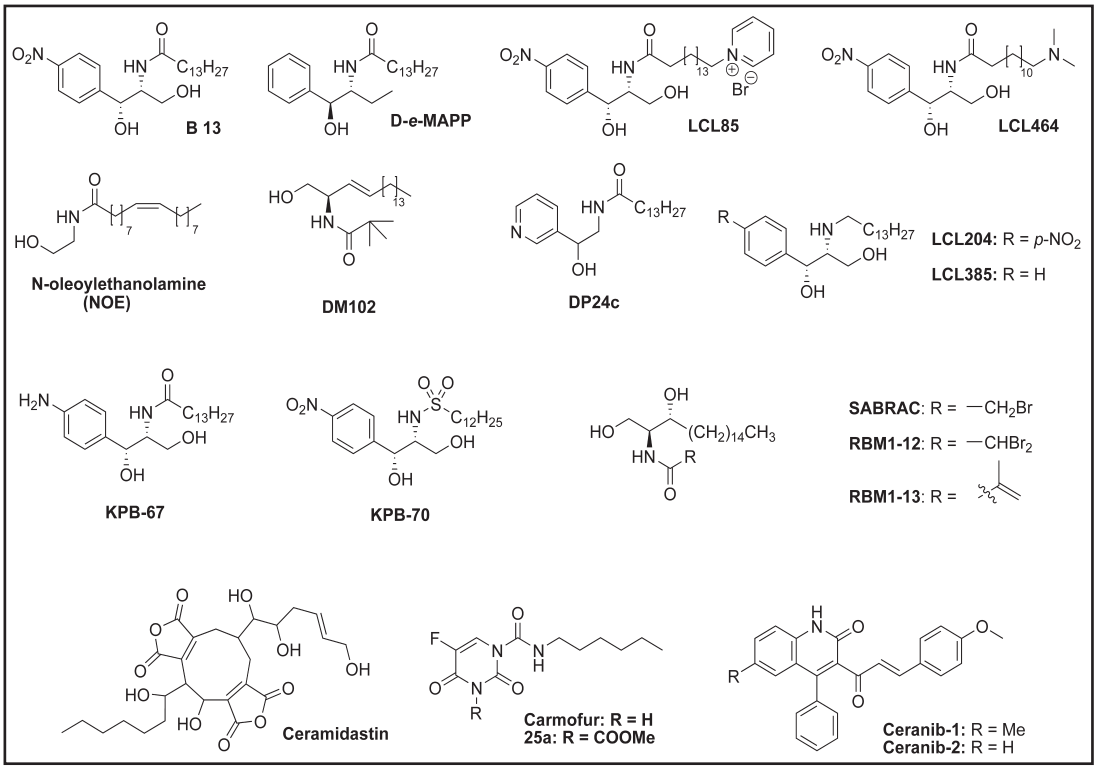

Fig. 2. Selection of previously described ceramidase inhibitors. Bottom row: compounds un-related to ceramide.

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,

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-myristoylamino)-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 IC_{50} 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 C_{16}-ceramide via *N*-deacylation [75]. *D-MAPP* was also reported to function as a moderate inhibitor of acid ceramidase (IC_{50} 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 IC_{50} 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 mitochondrium-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 (IC_{50} = 2.3 μM), more promising anticancer activity (GI_{50} -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 C_{14}-, C_{16}-, and C_{18}-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 vivo* 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 lysosomal 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 lysosomal 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 analog, was found to inhibit aCDase activity *in vitro* ($IC_{50} \approx 50$ 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 lysosomal 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 C_{14}- and C_{16}-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 group 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 IC_{50} 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_{16}-urea-Cer) and ceramine (C_{18}-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 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-carboxiamide 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_5-C_6 double bond of the uracil ring and the unsubstituted nitrogen in the 1-carboxiamide 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

aCDase. 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 ceramidase 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_{10}-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_{10}-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

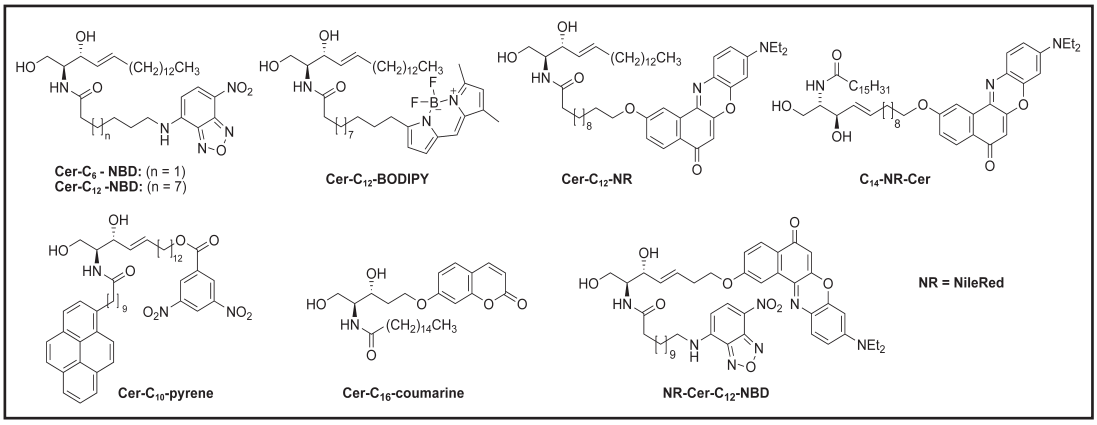

Fig. 3. Modified ceramides as probes for measuring ceramidase activities.

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

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 *in vitro* and *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.

Acknowledgements

Both authors would like to thank the Deutsche Forschungsgemeinschft (DFG) for generous funding (SPP 1267 Sphingolipids – Signal and Disease). E.M.S. is very grateful for a Yousef Jameel Scholarship.

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