Small Molecule Inhibitors of Acid Sphingomyelinase

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Key Words

Sphingolipids • Sphingomyelinase • Enzyme Inhibitors
• Drug Targets

Abstract

Despite of the importance of the acid sphingomyelinase for sphingomyelin homeostasis and sphingolipid signalling, potent and selective inhibitors for this enzyme are rare. An increasing set of data on the inhibition of acid sphingomyelinase in different disease models using indirect inhibitors has been generated and strongly implies acid sphingomyelinase as an emerging drug target. Very recently, some new and promising inhibitors from different substance classes have been developed. In this review, previous and current developments in the field are summarized.

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Introduction

Acid sphingomyelinase (aSMase) is a lysosomal enzyme that digests sphingomyelin from internalized mem-

brane fragments [1]. Ceramide, the product of the cleavage reaction catalyzed by aSMase is known to induce apoptosis in a variety of cell lines and is emerging as an important mediator of inflammation in various pathological backgrounds [2, 3]. Although ceramide can also be produced by *de novo* biosynthesis or by lipolytic cleavage of glycosphingolipids, obviously only the cleavage of the highly abundant lipid sphingomyelin by sphingomyelinases is fast enough to quickly produce significant amounts of ceramide as a reaction to internal and external stimuli [4]. Beside the aSMase an alkaline isoenzyme (alkaline sphingomyelinase) [5] and two isoenzymes with neutral pH optima (neutral sphingomyelinases 1 & 2, nSMase1 and nSMase2) [6, 7] have been described. Recently, the nSMase has been implicated in the formation of endosomes [8], a lipid compartment important for cell-cell contacts and antigen presentation that is also essential for infection of cells by retroviruses [9]. In contrast to the relatively few clear results for the nSMases in mediation of important cellular events, a whole plethora of recent data implies an unexpectedly important role for aSMase in the regulation of physiological and pathological events. As a matter of fact, aSMase is now regarded as an important potential drug target for a variety of diseases [10-12]. The enzyme plays an im-

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portant role in acute lung injury [13], lung emphysema [14], cystic fibrosis [2], Wilson disease [15] and atherosclerosis [16]. While a number of inhibitors for the nSMase are available [17-23], potent and selective inhibitors of acid sphingomyelinase are very rare. However, for dissecting the patho-physiological roles of aSMase in cell culture and *in vivo* studies potent and specific inhibitors of this enzyme are urgently needed.

The acid Sphingomyelinase

The coding region of the aSMase mRNA spans 1896 nucleotides and leads to the synthesis of a 78 kDa prepro-form which is glycosylated and proteolytically cleaved to a 72 kDa pre-form and subsequently to a 70 kDa mature enzyme [24]. One portion of this enzyme is transported to the lysosomes and a second portion is secreted to extracytosolic side of the plasma membrane. The latter enzyme activity also has been designated as the secreted sphingomyelinase (sSMase), although it stems from the mRNA as the aSMase [25]. It is noteworthy that the sSMase is activated by Zn^{2+} whilst the lysosomal aSMase is (although also dependent on Zn^{2+}) is not further stimulated. This difference very likely can be explained by the abundance of Zn^{2+} in the lysosomes and the absence of these ions during trafficking of the secreted variant.

An inborn deficiency in aSMase activity leads to a lysosomal storage of sphingomyelin and a fatal pathology that has been designated as the type A and type B of Niemann-Pick-Disease (NPD) [26]. An early hint for a role of the secreted form of aSMase in disease was provided by Sandhoff and colleagues, who reported on a 200fold increased level of aSMase in the urine from patients with acute peritonitis [27]. Indeed, in most cases the secreted form rather than the lysosomal portion of the aSMase plays a key role in pathological events. However, the mechanism by which the ceramide produced at the outer leaflet of the plasma membrane affects cellular signalling is still elusive. One scenario suggests ceramide to alter the biophysical properties of putative sphingolipid- and cholesterol-enriched lipid microdomains within the plasma membrane, also known as “lipid rafts” [28]. Such changes in lipid raft composition could facilitate receptor clustering and signalling across the plasma membrane [29]. However, the existence and at least the properties of lipid microdomains or rafts within the plasma membrane have been doubted and are subject of ongoing controversy [30]. A recent work describing the observation of the nanoscale dynamics of membrane lipids

in living cells however provides new evidence for the existence of raft-like microdomains [31].

In an alternative model, ceramide enters the cell via endocytosis and binds to intracellular receptors like protein kinase C (PKC) α/δ [32] or the inhibitor 2 of protein phosphatase 2A (I2PP2A) [33] that induce cellular responses to the elevated ceramide levels.

It is noteworthy that neither genetic knock-outs or cells from NPD patients nor the silencing of aSMase activity using RNA interference are suited to address the issue of aSMase topology in disease development. In contrast, small molecule inhibitors of the aSMase which are unable to enter cells directly or via endocytosis might lead to a concluding answer to this question.

Substrate Analogues

The most rational way to develop enzyme inhibitors is the synthesis of substrate analogues (Fig. 1) in which the scissile bond is replaced by an uncleavable isostere. Such an approach was conducted by Yokumatsu et al. who envisioned synthesizing inhibitors of nSMase. The compound *ent*-12 **1** however (IC_{50} vs. nSMase = 3.3 μ M) is completely unselective between nSMase and aSMase (48% inhibition at 3.3 μ M) [34]. Nonetheless, for a substrate analogue it is remarkably active since the K_M for sphingomyelin is ~ 60 μ M (aSMase). In contrast, L-carnitine **2**, which is physiologically abundant in the cytosol and in mitochondria was reported to moderately inhibit aSMase only. In cells, L-carnitine is probably transformed to the more active L-acylcarnitine [35].

Recently Schuchman and colleagues characterized a thiourea analogue of sphingomyelin (AD2765, **3**) as an *in vitro* inhibitor of aSMase (K_i = 70 μ M). In cell culture, this compound surprisingly led to an increase in cellular ceramide at concentrations >10 μ M. More detailed investigation revealed that sphingomyelin synthase is also inhibited by this compound [36].

Indirect Inhibitors

A typical example for an indirect inhibition of aSMase is the calcium channel inhibitor SR33557 (**4**, Fig. 2), that inhibits aSMase by an unknown mechanism only in cell culture and without directly interacting with the enzyme [37]. Because of the activity profile, this substance has no impact on current aSMase research. A special class of indirect aSMase inhibitors however has become a very

Fig. 1. Substrate analogs that are moderate inhibitors of aSMase.

Fig. 2. Indirect Inhibitors of aSMase.

important tool for a wide range of cellular and animal studies: The indirect ‘functional’ inhibition of aSMase by tricyclic antidepressants like imipramine **6**, desipramine **5** and amitriptyline **7** (Fig. 2) has been known since the mid eighties [38]. These substances efficiently reduce aSMase activity in cell culture, but not in micellar assays using the purified enzyme [39]. Several detailed studies have led to a rather detailed understanding of how the functional inhibition of aSMase takes place: Upon administration, these agents are able to enter the lysosomes partially by diffusion and in part by endocytosis. During maturation and acidification of the endosomes, these basic agents get fully protonated and thus are enriched in the endosomal and lysosomal compartments [40]. After accumulation in the lysosomes, the protonated compounds stimulate the proteolytic degradation of the aSMase [41]. It is very likely, that the positively charged molecules shield the negatively charged bis(monoacylglycerol)phosphate (BMP), which is specific for lysosomes [42] and has been shown to be essential for the function of aSMase, acid

Ceramidase and other lysosomal sphingolipid hydrolases [43]. Once aSMase has lost its negatively charged binding partner, it readily detaches from the intralysosomal membrane fragments (‘coated pits’), thereby making the enzyme susceptible to fast proteolytic degradation [44].

This putative mechanism of action, which is based on a significant amount of data provides two important clues: First, it can be expected that the mechanism described above also holds true for other lysosomal sphingolipid hydrolases. In fact, Hannun and coworkers showed that the acid Ceramidase is inhibited by the tricyclic antidepressants, as well [45]. Another important notion is that the antidepressants do not inhibit the purified aSMase in a micellar *in vitro* assay [39]. These *in vitro* assays and the proposed BMP-dependent mechanism of action strongly implicate that these agents aren’t able to inhibit the aSMase in its secreted form. This is an important notion, since most investigators clearly consider the secreted form of aSMase to be decisive for their observations from disease models. Due to the fact that many

Fig. 3. Adenosine 3,5 bisphosphate **8** and Phosphatidylinositol 3,5 bisphosphate **9**.

of the effects of aSMase can be suppressed by the tricyclic antidepressants, it is very likely that the aSMase readily translocates from the acidic compartments of the cell to the plasma membrane upon stimulation. A treatment with tricyclic antidepressants depletes the lysosomes from aSMase, thus probably making translocation of this enzyme to the plasma membrane less efficient. Although there are other hints that additionally support this idea [46], this issue has not been conclusively clarified yet.

During the past several years, the tricyclic antidepressants have been proven as invaluable tools for pharmacological and biological studies in cell culture and in animal models, unravelling the role of aSMase in human disease. However, because of the indirect mechanism and the known unselectivity towards other sphingolipid metabolizing enzymes, the use of these drugs has to be regarded with reservation. The value of the tricyclic antidepressants for acid sphingomyelinase research however cannot be denied, since these agents are clinically approved drugs and because they are very-well characterized and available.

Recently, a systematic investigation of the inhibitory properties of a collection of similar weak basic molecules has been conducted. A detailed structure-property-activity relationship (SPAR) model clearly showed that pKa and log p values of the compounds are necessary but not sufficient preconditions for functional inhibition of aSMase. In contrast, the SPAR model supports the requirement of a factor, which is related to steric hindrance of the basic nitrogen and thus presumably to the ability of the compounds to present a positive charge at the surface of the intra-lysosomal vesicles ('coated pits') [47]. A total of 26

new functional inhibitors of aSMase were identified.

Phosphate-Containing Inhibitors

In an initial work Sandhoff et al. observed a moderate inhibition of aSMase by the nucleoside-3,5-bisphosphates (Fig. 3) and phosphatidylinositol-4,5-bisphosphate [48]. A more detailed investigation led to the identification of further phosphoinositides as inhibitors of aSMase, with phosphatidyl inositol-3,5-bisphosphate **9** (Fig. 3) being the most potent [49]. Interestingly, the stereochemical orientation and the number of phosphate esters cannot be changed without a dramatic loss in inhibitory activity. It is noteworthy that phosphoinositides are ill-suited for cell culture and *in vivo* applications for several reasons:

Chemical and biological instability

Since phosphoinositides are biologically active molecules they probably have a minimum of chemical stability in biological systems. The preparation and storage of these molecules however can be very difficult because migration of esters and phosphoesters can easily occur. On the other hand, phosphoinositides are substrates for a variety of enzymes, including phospholipases A₁, A₂, C and D, as well as other inositol-specific or unspecific phosphatases.

Penetration of cellular membranes

Phosphoinositides are highly charged molecules and are thus unable to penetrate biological membranes.

Fig. 4. One-tailed Phosphate-Containing aSMase Inhibitors.

However, due to their lipophilicity, phosphoinositides will readily insert into the outer leaflet of the cellular plasma membrane and may reach the endosomal and lysosomal compartment via endocytosis.

Poor off-rates from biomembranes

Like other phospholipids, phosphoinositols are important structural components of biological membranes. According to the fluid mosaic model of biological membranes, these lipids can laterally move within the membrane, but a transversal movement (flip-flop) or the translocation from the lipid into the water phase is very unlikely. Poor off rates from biological membranes strongly reduce intracellular distribution of small molecules. So far, there are no reports on the successful use of phosphatidyl inositols as inhibitors of aSMase in cell culture or in living animals.

In lipid-transfer experiments employing biotinylated liposomes we recently observed that but in contrast to two-tailed lipids, lysolipids like sphingosine and sphingosine-1-phosphate readily migrate between different liposomal membranes [50]. Accordingly, we started efforts to synthesizing one-tailed analogues of PtdIns3,5P₂, in order to improve the biophysical properties of the aSMase inhibitor. First of all, we did a more in-depth SAR study clearly showing that not only the inositol hydroxyl groups are important for inhibition, but also the lipophilic residue, in this case diacylglycerol (DAG) [51]. When the latter was replaced by a one-tailed alkyl dodecyl sulfone ester the inhibitory properties of the resulting analogue

10 (Fig. 4) were virtually identical to the natural PtdIns3,5P₂. In contrast to the natural PtdIns3,5P₂ however, the new derivative is inert against phospholipases. Indeed, the sulfo-analogue **10** is active in cell culture and inhibits apoptosis induced by dexamethasone in HEK 293 cells at a 2 μ M concentration.

Very recently we also synthesized a carbohydrate-derived PtdIns3,5P₂ analogue **11** (Fig. 4) [52]. Surprisingly, this compound was even slightly more active versus aSMase than the inositol-based analogue **10**. More importantly however, this analogue offers new possibilities for further structural modifications and thus possibly further improvement of the inhibitory properties.

In an effort obtain molecules in which the biologically labile phosphate groups are replaced by uncleavable phosphonates we synthesized a collection of isosteric phosphonates. These compounds however did not exert significant inhibitory activity on aSMase. In contrast, a small collection of geminal bisphosphonates surprisingly showed remarkable inhibition of aSMase. A subsequent more detailed SAR study led to the development of the aSMase-inhibiting bisphosphonates **12-14** [53]. The geminal aminobisphosphonate **12** (ARC 39) is the most potent inhibitor of aSMase (IC₅₀ = 20 nM), so far. It is active in cell culture and completely suppresses dexamethasone-induced cell death in HEK 293 cells at a 0.1 μ M concentration. Furthermore, this compound showed a dose-dependent inhibition of platelet activating factor (PAF)-induced pulmonary oedema formation in perfused rat lungs. The structure-activity relationship of the

Fig. 5. Natural Products and Natural-Product-Like Compounds Inhibiting aSMase.

bisphosphonates implies that these molecules are able to form complexes with the Zn^{2+} ions in the active center of aSMase. However, milimolar concentrations of Zn^{2+} and other bivalent cations have no significant influence on the inhibitory properties of this compound and serine/threonine protein phosphatase 1 (PP1), a remote homologue of aSMase [54] is not affected by ARC 39. While the substance is remarkably active in cell culture and *ex vivo*, the known affinity of bisphosphonates to bone surfaces may limit its application in animal *in vivo* studies. In general, bisphosphonates are known to bind to the surface of osteoclasts leading to a fast clearance from the blood and soft tissues [55]. After endocytosis bisphosphonates can inhibit farnesyl pyrophosphate synthase in the μ M to nM range. Whether other metal containing enzymes are inhibited by the bisphosphonates is not known.

In addition, the questions whether ARC 39 is able to enter cells or whether it exhibits its function at the cell surface or if it affects intracellular sphingomyelin metabolism are still open and need further clarification.

Natural Products as Sphingomyelinase Inhibitors

In the course of a screening for acid sphingomyelinase inhibitors, Okudaira et al. identified three inhibiting

xanthone compounds α -mangostin, covanin and covanol from the bark of *Garcinia speciosa*, inhibiting aSMase with IC_{50} values of 14.1, 19.2 and 10.9 μ M, respectively [56]. Among these substances α -Mangostin (**15**, Fig. 5) showed the greatest selectivity against the nSMase (IC_{50} = 113 μ M). In a subsequent SAR-study, a handful of derivatives were synthesized. The synthetic substances however were less active and/or less selective against nSMase [57].

Very recently, we have identified some natural product-derived compounds as inhibitors for several enzymes, including aSMase [58]. Following a chemo-informatics approach a collection of ~700 compounds was assembled and screened in various enzyme assays. The most potent compounds **18**, **19** and **20** (Fig. 5) inhibit aSMase with IC_{50} values of 3.1, 9.5 and 21 μ M, respectively. These substances fulfil the Lipinski 'rule-of-five' for oral availability [59] of small molecules and are selective towards nSMase (tested at concentrations up to 50 μ M), which makes them attractive leads for further optimization.

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