Enzyme Inhibitors

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Potent and Selective Inhibition of Acid Sphingomyelinase by Bisphosphonates**

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Dedicated to Professor Konrad Sandhoff on the occasion of his 70th birthday

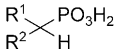
The acid sphingomyelinase (aSMase) is emerging as an important drug target for a variety of diseases.[1–3] Inhibition of aSMase prevents bacterial infections in a rat model of cystic fibrosis[4] and formation of acute lung injury (ALI) elicited by endotoxin, acid instillation, or platelet-activating factor (PAF).[5] Moreover, aSMase is essential for infection of non-phagocytotic cells with *Neisseria gonorrhoeae*[6] and formation of pulmonary emphysema.[7] Pharmacological or genetic inhibition of aSMase prevents apoptosis and degeneration of liver cells in a mouse model for Wilson's disease.[8] In addition, there are several reports that aSMase significantly contributes to the formation of atherosclerotic plugs.[9]

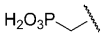
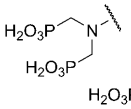
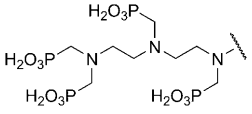
This promising progress in aSMase research, based on sophisticated animal models and cultured cells from patients, is thwarted, however, by the lack of potent and selective inhibitors of this enzyme. Phosphatidylinositol-3,5-bisphosphate (PtdIns3,5P_2), to date the most potent inhibitor (K_M = 0.53 μM),[10] is not suitable for cell culture studies, because of its fivefold negative charge and its two long fatty acid chains which cause it to stack in cellular membranes. Last but not least, this inhibitor is labile towards phospholipases A_1, A_2, C, and D and phosphoinositide phosphatases.

The aSMase is a soluble lysosomal sphingolipid hydrolase, which constitutively degrades sphingomyelin from internalized membrane fragments.[11] Upon stimulation, however, a portion of this enzyme can be found on the outer side of the plasma membrane.[12] This membrane-associated enzyme shows biochemical activity in serum and urine and has been termed secretory sphingomyelinase (sSMase), although it is virtually identical to the lysosomal variant. Its activity is elevated in several diseases. The secretory form of aSMase is believed to play an important role in signal transduction, since it alters the composition of the plasma membrane within putative sphingolipid- and cholesterol-rich membrane microdomains. These so-called "lipid rafts" have been suggested to act as "signaling platforms",[13] and there is significant evidence that the cleavage of sphingomyelin to ceramide can dramatically alter the biophysical properties of the putative rafts.[14] In addition, it is well established that ceramide is a potent inductor of apoptosis, which is the main reason for cell degeneration in many of the diseases mentioned above. However, it is unknown whether ceramide acts by remodeling the plasma membrane or by interacting with proteins like cathepsin B, which is involved in cellular signaling. Beside aSMase, two cytosolic, magnesium-dependent and membrane-bound neutral sphingomyelinases (nSMase1 and nSMase2)[15] and an alkaline sphingomyelinase[16] are known, whose cellular function is rather unclear. Recently nSMase has been shown to be essential for the formation of exosomes,[17] lipid vesicles that play a key role in the infection by retroviruses.[18] In contrast to aSMase, there are some potent small-molecule inhibitors for nSMase.[19]

Our attempts at synthesizing phosphonate analogues of PtdIns3,5P_2 as potential inhibitors of aSMase yielded only moderately active substances.[27] However, we also gained access to a collection of (bis)phosphonates that had been synthesized in the GDR Academy of Sciences and that contained some compounds that are structurally related to our phosphoinositide analogues. When we initially tested these substances at a concentration of 20 μM, we were surprised that some of them were potent inhibitors of aSMase (Tables 1 and 2). Among these substances, α-amino-

Table 1: Inhibition of aSMase by the initial phosphonate collection.



Cpd	R^1	R^2	Inhibition [%][a]
1		H	16
2		H	2
3		NH_2	47
4		H	0
5		H	−5

[a] The inhibition values were determined in a single experiment at 20 μM.

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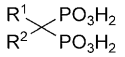
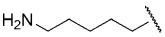
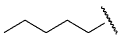
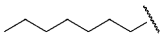
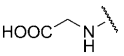
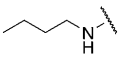
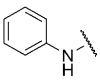
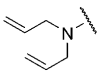
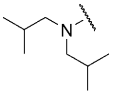
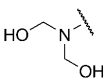
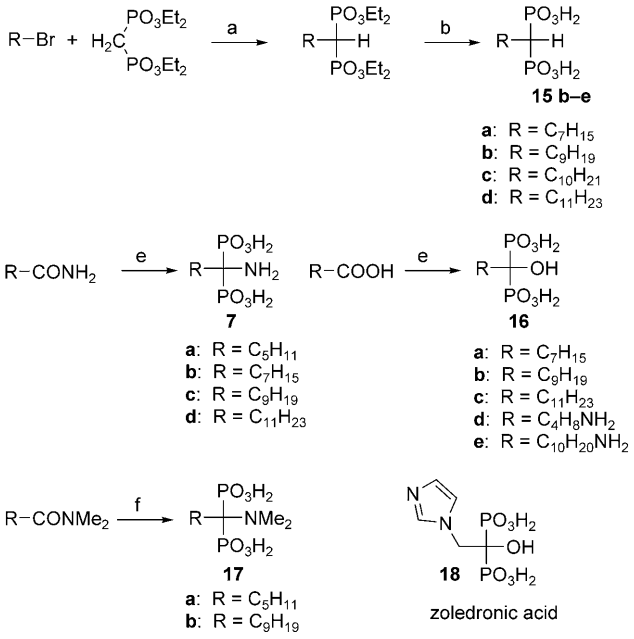
Table 2: Inhibition of aSMase by the initial bisphosphonate library.



Cpd	R^1	R^2	Inhibition [%][a]
6		OH	54
7a		NH_2	92
7b		NH_2	93
8		CH_3	62
9		H	76
10		H	8
11		H	32
12		H	2
13		H	36
14		H	24

[a] The inhibition values were determined in a single experiment at 20 μM.



Scheme 1. Synthesis of bisphosphonates. Reagents and conditions: a) NaH, toluene, 60°C, 16 h; b) HCl, reflux, 16 h; c) $P(OMe)_3$, 0°C, 2 h; d) $HP(OMe)_2$, nBu_2NH, 0°C, 16 h; e) H_3PO_3, MsOH; then PCl_3, 90°C, 16 h; f) PCl_3/H_3PO_3, 70°C, 12 h, then H_2O, 2 h.

bisphosphonate **7b** turned out to be about one order of magnitude more potent than PtdIns3,5P_2. Furthermore, **7b** contains two more methylene units than **7a**, which leads to a dramatic increase in inhibitory potency.

To gain a deeper insight into the structure–activity relationship, we decided to synthesize 15 additional bisphosphonates harboring different functional groups at the α position and displaying lipid tails of different lengths, according to well-established protocols (Scheme 1).[20] On the basis of this new collection of compounds, we could show that inhibition correlates with the length of the lipid tail (this correlation is true as long as the substances are readily soluble) and that a functional group with lone electron pairs at the α position (-NH_2 more strongly than -OH) increases the inhibition of acid sphingomyelinase relative to the activity of H-bisphosphonates **15a–d**.

Bisphosphonates are known to form bidentate complexes with divalent metal ions like Ca^{2+}, Zn^{2+}, and Mg^{2+}.[21] If the bisphosphonate contains an additional hydroxy or amino group, even more stable tridentate complexes can be formed. In fact, α-amino substitution leads to more stable complexes than α-hydroxy substitution, suggesting that aSMase inhibition also correlates with the tendency of the compounds to form complexes in which the Zn^{2+} ion resides at the reactive center of the aSMase. It is noteworthy that aSMase, both in its lysosomal and its secreted form, is a Zn^{2+}-dependent enzyme. However, the lysosomal variant is not inhibited by EDTA and not stimulated by Zn^{2+}; this can be explained by abundance of Zn^{2+} ions in the lysosomes. In contrast, the secreted variant is stimulated by Zn^{2+} ions.[22] In order to characterize the aSMase-inhibiting bisphosphonates with regard to their metal-binding properties, we tested **7c** in the presence of millimolar concentrations of Ca^{2+}, Mg^{2+}, and Zn^{2+} ions, respectively. The inhibitory activity was not significantly diminished by the metal ions. However, the poor inhibition of aSMase by *N*-phenylaminobisphosphonate **10** and by (ω-aminoalkyl)hydroxybisphosphonate **16e** clearly shows that complex-forming propensity and the presence of a hydrophobic moiety alone are not sufficient for aSMase inhibition.

In addition, we tested nearly all substances for inhibition of the Mg^{2+}-dependent nSMase, without observing any substantial inhibition of this isoenzyme at concentrations up to 100 μM (see the Supporting Information for details). This clearly indicates that inhibition of aSMase is not only very potent but highly selective for only this enzyme. Moreover, we tested the best aSMase inhibitor, **7c**, for inhibition of Ser/Thr phosphatase 1 (PP1), which—like the phosphodiesterase domain of aSMase—belongs to a family of dimetal-containing phosphoesterases.[23] The latter enzyme was not inhibited by **7c**, even at a concentration of 2 μM (see the Supporting Information for details), which shows that this aSMase inhibitor is ineffective against PP1.

Bisphosphonates are well-established drugs against osteoporosis, bone cancer, and several other bone diseases.[21] The key for bisphosphonate specificity is their capacity to bind rapidly and with high affinity to unknown structures on bone surfaces. The high affinity to the bone surface leads to the fast clearance of bisphosphonates from blood and soft tissues.[24] Interestingly, zoledronic acid (**18**), a widely used drug against osteoporosis, showed a marked inhibition of aSMase with an IC_{50} value of approximately 5 μM (Table 3). This finding emphasizes that the pharmacological relevance of aSMase

Table 3: Inhibition of aSMase by the synthesized bisphosphonates.[a]

Cpd	IC_{50} [μM]	Cpd	IC_{50} [μM]	Cpd	IC_{50} [μM]
7a	4.66 ± 1.07	**15c**	0.30 ± 0.05	**16e**	1.95 ± 0.22
7b	0.04 ± 0.01	**15d**	0.17 ± 0.04	**17a**	9.50 ± 4.00
7c	0.02 ± 0.00	**16a**	0.16 ± 0.04	**17b**	0.18 ± 0.03
7d	0.29 ± 0.09	**16b**	0.08 ± 0.01	**18**	5.08 ± 0.74
15a	0.35 ± 0.08	**16c**	0.07 ± 0.01		
15b	0.31 ± 0.12	**16d**	6.80 ± 2.40		

[a] The IC_{50} value for inhibition of nSMase was >100 μM for all compounds.

inhibition by bisphosphonate drugs cannot completely be ruled out.

Since aSMase produces the proapoptotic ceramide, inhibition of this enzyme by RNAi or unspecific inhibitors reportedly protects cells from dexamethasone-[25] and Cu^{2+}-induced[8] apoptosis. When we treated HepG2 liver cells with dexamethasone (10^{-8} M) in order to induce apoptosis, aSMase inhibitor **7c** at a concentration of 0.1 μM efficiently inhibited apoptosis, as measured by DNA-fragmentation ELISA (Figure 1).

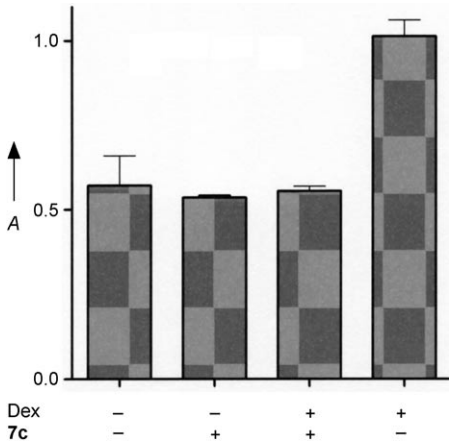


Figure 1. The aSMase inhibitor **7c** (0.1 μM) inhibits dexamethasone-(Dex)-induced apoptosis in HepG2 cells. The absorbance in a DNA-fragmentation ELISA is plotted.

Encouraged by the high biological activity in cultured cells and because of the evident pharmacological interest in potent aSMase inhibitors for the treatment of lung diseases, we examined whether the inhibitor would also be able to reduce PAF-induced pulmonary edema, similar to the unspecific and indirect aSMase inhibitor imipramine.[5] Indeed, addition of **7c** to the perfusate reduced edema formation in a concentration-dependent fashion in isolated, ventilated, and perfused rat lungs (IPL, Figure 2). Like imipramine (10 μM), the inhibitor **7c** attenuated but not completely prevented edema formation in this model.

The simple bisphosphonate **7c** is the most potent aSMase inhibitor found to date. Its selectivity for aSMase is more than 5000 times greater than that for the Mg^{2+}-dependent isoenzyme nSMase, and it is also nonselective for the dimetal-containing remote aSMase homologue Ser/Thr protein phosphatase 1. The compound, which can be synthesized easily on gram scale, is also active in cell culture and efficiently protects HepG2 cells from dexamethasone-induced apoptosis. Thus, we describe a powerful tool for aSMase research which is very likely to replace the tricyclic antidepressants imipramine and desipramine which have been widely used despite their indirect and unspecific mode of action. Moreover, the fact that bisphosphonates are potent aSMase inhibitors brings up the exciting notion that this property may be relevant to their well-established clinical usage in the treatment of osteoporosis, a very common disease with a lifetime risk of 40–50% for women in North America.[26] And finally, the finding that the aSMase inhibitor **7c** significantly reduces edema formation in isolated rat lungs suggests that parenteral administration of bisphosphonates may be useful for the treatment of acute lung disorders and other inflammatory ailments.

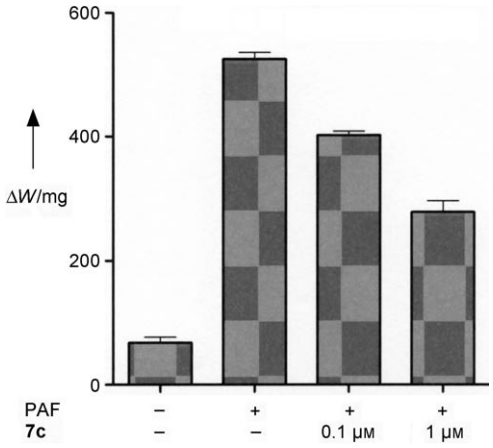


Figure 2. The aSMase inhibitor **7c** reduces PAF-induced pulmonary edema in isolated, ventilated, and perfused rat lungs (IPL). Weight gain (ΔW) was measured 10 min after addition of PAF (5 nM).

Experimental Section

Enzyme assays: Crude preparations containing aSMase or nSMase were made from stripped rat brains.[19d] The micellar nSMase assays using ^{14}C-labeled sphingomyelin as the substrate were performed as described previously.[19d] The fluorescent aSMase assay was performed in a 384-well plate using HMU-PC (6-hexadecanoylamino-4-methylumbelliferylphosphorylcholine) as the substrate. Reaction mixtures consisted of 13.3 μL HMU-PC, 13.3 μL reaction buffer (100 mM NaOAc, pH 5.2, 0.2% (w/v) Na-TC, 0.02% (w/v) NaN_3, 0.2% (v/v) Triton X-100), and 13.3 μL enzyme preparation. Inhibitors were added in various concentrations, and the reaction mixtures were incubated for 3 h at 37 °C in a plate reader (FLUOstar OPTIMA, BMG labtech). The fluorescence of 6-hexadecanoylamino-4-methylumbelliferone (HMU) was measured (excitation 380 nm, emission 460 nm) in real time. Assays using the ^{14}C-labeled sphingomyelin gave the same results.

Compound libraries and syntheses: All described compounds were characterized by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and mass spectrometry. The syntheses were performed as described previously.[20]

Apoptosis assay: First, the kinetics of DNA fragmentation after dexamethasone donation was measured in the lysate and in the supernatant. Between 6 h and 8 h, there was a steep increase in absorbance in the probes from the supernatant, which is typical for apoptosis (data not shown). The apoptosis assay was performed

according to the manufacturer's protocol (Roche catalogue number 11585045). Briefly, cells were harvested and suspended in culture medium (2×10^5 cells mL^{-1}) containing BrdU labeling solution (10 μM final concentration) and plated in a 96-well cell-culture dish at $\approx 1 \times 10^4$ cells per well. After 16 h, the cells were washed and new media was added. Then, the cells were treated with 10^{-8} M dexamethasone and 0.1 μM **7**. After the cells had been incubated for 7 h, 100 μL of the supernatant was collected and added to a 96-well plate containing immobilized anti-BrdU antibody. After incubation, removal of the supernatant, and extensive washing, the secondary antibody and the TMB substrate were added and absorbance was measured at 370 nm (FLUOstar OPTIMA, BMG labtech). The experiment was performed in five times.

PAF-induced pulmonary edema: Female Wistar rats (weight 220 to 250 g) were kept under standard laboratory conditions (rat food and water ad libitum). Rat lungs were prepared, perfused, and ventilated essentially as described.[5] Briefly, lungs were perfused through the pulmonary artery at a constant hydrostatic pressure (12 cm H_2O) with Krebs–Henseleit buffer containing 2% albumin, 0.1% glucose, and 0.3% HEPES. Edema formation was assessed by continuously measuring the weight gain of the lung. In this model, platelet-activating factor causes rapid edema formation that is in part dependent on acid sphingomyelinase. Inhibitor **7c** was dissolved in buffer and added to the buffer reservoir 10 min prior to administration of PAF (5 nmol). Isolated perfused rat lungs were perfused for 30 min before **7c** was added to the perfusate; 10 min later 5 nmol PAF was added as a bolus and weight gain was monitored for 10 min. Data are shown as mean (with standard deviation) from four independent experiments in each group. Statistics: 0.1 μM **7c**: $p < 0.01$ vs. PAF alone; 1 μM **7c**: $p < 0.01$ vs. PAF alone and vs. 0.1 μM **7c**/PAF (Tukey's test).

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