Manumycin A and Its Analogues Are Irreversible Inhibitors of Neutral Sphingomyelinase

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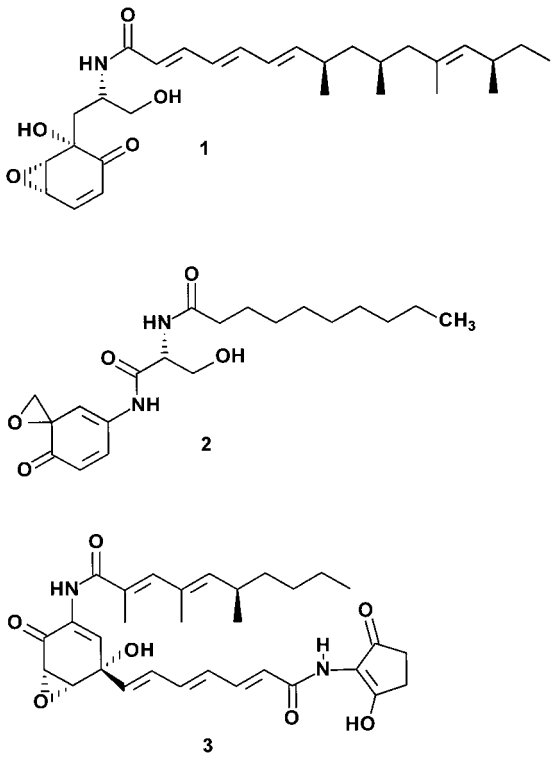
The sphingolipid ceramide can be generated from sphingomyelin—an important constituent of eukaryotic membranes—through the action of various sphingomyelinases. It has achieved much attention because of its possible relevance to signal transduction processes. There is increasing evidence that the sphingomyelinases are activated by various cytokines, or by radiation, heat and oxidative stress, leading to an increased ceramide production. It is suggested that ceramide acts as a second messenger and triggers or modulates a number of fundamental processes like programmed cell death (apoptosis), the cell cycle or inflammatory processes in various tissues and cell lines.[1, 2] Moreover, recent studies strongly suggest a vital role for ceramide as a key mediator in tumor suppression.[3, 4] However, numerous aspects of ceramide-mediated signal transduction, especially those related to apoptosis, remain unclear.[5] Furthermore, the question whether the plasma-membrane-bound Mg^{2+}-dependent neutral sphingomyelinase (N-SMase; sphingomyelin phosphodiesterase, EC 3.1.4.12), the most prominent [6] of several neutral sphingomyelinases described,[7–10] or the lysosomal acid sphingomyelinase (A-SMase) is most important for stimulus-induced ceramide production is discussed controversially.[11]

For the investigation of the biological role of ceramide and of the different sphingomyelinases selective inhibitors of these enzymes would be valuable tools. Recently we reported the synthesis of the spiroepoxide **2**,[12] which was synthesised as an analogue of the natural product scyphostatin (**1**).[13–15] Interestingly, epoxide **2** is an irreversible inhibitior of neutral sphingomyelinase. In contrast, scyphostatin inhibits N-SMase (IC_{50} = 1 μM) and A-SMase (IC_{50} = 49.3 μM) in a competitive manner. Due to the structural similarity of scyphostatin (**1**) and the spiroepoxide **2** to the antibiotic manumycin A (**3**)[16–18] we reasoned that manumycin A might inhibit N-SMase as well.



To test this hypothesis we utilised a raw microsome preparation containing rat brain Mg^{2+}-dependent N-SMase. Indeed, this enzyme was inhibited by manumycin A in an irreversible manner (Figure 1), whereas A-SMase was not affected at a concentration of **3** up to 200 mM (data not shown). Furthermore, we found that increasing concentrations of sphingomyelin in the assay weaken the inhibitory effect of manumycin A (Figure 2) indicating that there is a competition between manumycin A and the substrate for binding in the active site of N-SMase. Our data also indicate that manumycin A is a more potent inhibitor than spiroepoxide **2** and that its affinity for N-SMase is comparable to the affinity of this enzyme for sphingomyelin. Encouraged by these results, we investigated several manumycin A analogues, that is, the recently synthesised[19] compounds **4a**,**b** and **5a**,**b** as possible N-SMase inhibitors.[20] In fact, **4a** and **4b** turned out to be selective irreversible inhibitiors of N-SMase, whereas **5a** as well as **5b** were inactive towards both N-SMase and A-SMase under the same conditions (Table 1).

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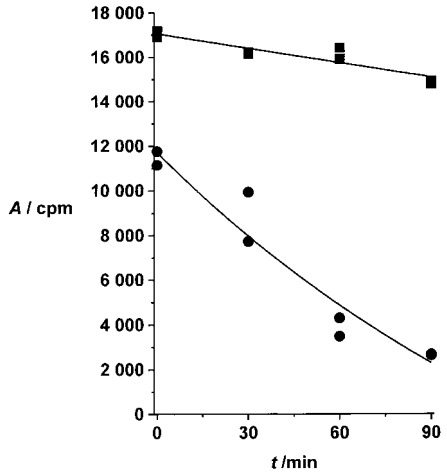


*Figure 1. Time dependence of the inhibition of neutral sphingomyelinase by manumycin A (**3**) (100 μM in pre-incubation buffer). ● = With manumycin A, ■ = control experiment without manumycin A; A = activity, t = pre-incubation time.*

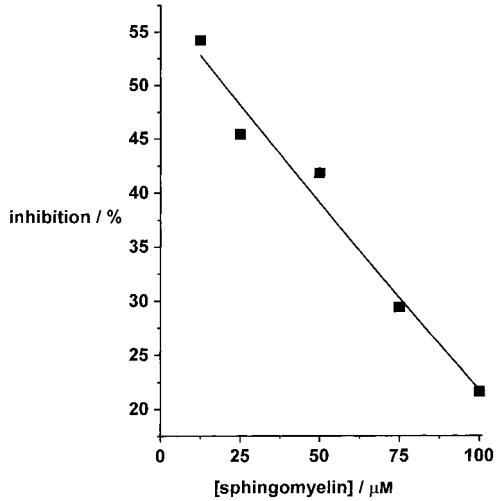


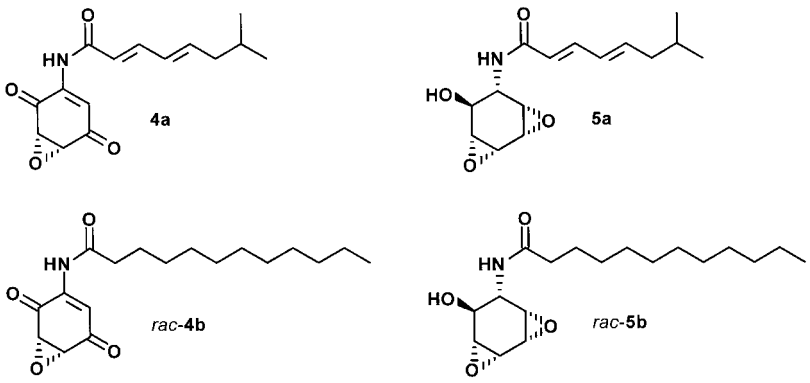
Figure 2. Inhibition of neutral sphingomyelinase at different concentrations of sphingomyelin. The concentration of manumycin A was 50 μM (no pre-incubation).

***Table 1.** Inhibition of neutral sphingomyelinase by manumycin A (**3**) and its analogues **4a,b** without and with pre-incubation.[a]*

Compound	Inhibition (without pre-incubation) [%]	Inhibition (with pre-incubation) [%]
3	34	79
4a	90	98
4b	10	88

[a] The concentration of the inhibitors was 100 μM in the pre-incubation buffer (final concentration 50 μM). The pre-incubation time was 1 h.

The N-SMase inhibition assay was carried out again with and without pre-incubation. The latter likely represents the ability of the inhibitor to compete with the substrate for binding at the active site of the enzyme, whereas the assay with pre-incubation of the inhibitor with the enzyme in the absence of substrate

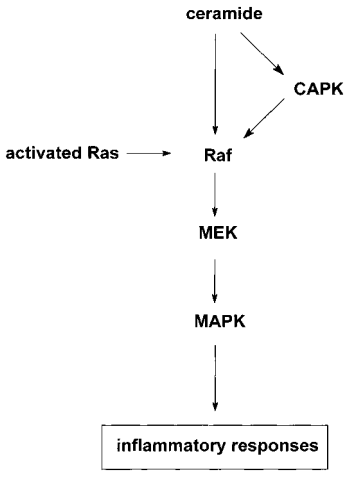


represents the kinetics of the irreversible inhibition. It is worth noting that the nature of the amide side chain strongly influences the inhibitory potency of the compounds. As shown in Table 1 derivative **4a** displayed a higher affinity for N-SMase than compound **4b**, which contains a saturated side chain.

Manumycin A is known as a natural product with antibiotic,[21] antitumour[22] and anti-inflammatory activities.[23] These activities have been correlated with the ability of manumycin A to competitively inhibit Ras farnesyltransferase (FTase; $IC_{50} = 35$ μM (rat)[22]). FTase is essential for processing the Ras protein and is one of the most intensively examined targets for the development of new anticancer drugs.[24] For these reasons we also investigated the ability of compounds **4a**,**b** and **5a**,**b** to inhibit FTase. Compounds **5a** and **5b** proved to be inactive whereas both **4a** and **4b** were competitive inhibitors of Ftase with respect to farnesyl pyrophosphate, with IC_{50} values of 8.4 and 0.85 μM, respectively (for manumycin A: $IC_{50} = 19.4$ μM).

Our results suggest that some of the described biological effects of manumycin A may result from N-SMase inhibition. Furthermore, it is possible that the effects of N-SMase inhibition are amplified by the FTase inhibition, particulary in inflammatory events, since there is evidence for cross talk between Ras- and ceramide-mediated signalling pathways (Scheme 1).[25, 26]

Due to their mode of inhibition compounds **3** and particularly **4a** and **4b** are suitable reagents for affinity labelling of N-SMase.



***Scheme 1.** Proposed signalling pathway of Ras and ceramide in inflammatory events. CAPK = ceramide-activated protein kinase; MAPK = mitogen-activated protein kinase; MEK = MAP/ERK kinase.*

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This in turn may provide some clues to the enzymatic mechanism of N-SMase, which is largely unknown. Furthermore, these new compounds may be useful as efficient molecular tools to gain more insight into the biological role of N-SMase and ceramide in apoptosis and in signal transduction processes. The fact that scyphostatin shows remarkable anti-inflammatory effects justifies the development of further inhibitors of N-SMase and makes N-SMase an interesting target for the experimental therapy of inflammatory diseases. This fact is documented in the establishment of various high-throughput screening assays for a more efficient search for inhibitors of this enzyme.[27, 28]

Experimental Section

Purification of N-SMase: Partial purification of sphingomyelinases was carried out analogous to a method described by Hannun et al.[29] and modified with respect to a more recent publication.[10] Four rats were decapitated. The brains were homogenised and then centrifuged for 1 h at 100 000 *g* in a 25 mM Tris-HCl buffer (pH 7.4) containing leupeptine, chymostatin, antipain, pestatin (each 20 mg L^{-1}), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA and 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA). The supernatant was discarded and the pellet was dissolved in the above-mentioned buffer, containing an additional 1 % (w/v) of Triton X-100. After a 20-fold dilution the solution was loaded onto a strong anion exchange column (POROS 20 HQ, PerSeptive Biosystems, Inc.). After equlibration with 20 mM Tris-HCl (pH 7.4) containing 1 mM each of EDTA, EGTA, PMSF, and a step to 1 M sodium chloride in the same buffer the sphingomyelinases were eluted with a gradient from 0 to 1 % (w/v) Triton X-100 within the equilibration buffer.

Inhibition assay: For the determination of the N-SMase activity the potential inhibitors were dissolved in chloroform. An aliquot of 5 nmol was dried under a nitrogen stream, redissolved in 40 mL buffer (75 mM Tris-HCl, pH 7.4, 0.05 % (w/v) Triton X-100, 5 mM $MgCl_2$) and mixed with 10 mL of the enzyme solution. Together with controls, the probes were pre-incubated for 90, 60, 45, 30 and 15 min at 37 °C. After addition of 10 nmol [^{14}C]sphingomyelin (ca. 40 000 cpm) in 50 mL of the same buffer, the reaction proceeded for another 30 min. The reaction was stopped by adding 750 mL chloroform/methanol (1:1, v/v). After addition of 200 mL water, the lipids were extracted and the radioactivity of the polar upper phase, containing [^{14}C]phosphorylcholine, was determined by scintillation counting. The determination of A-SMase proceeded analogously, but a Mg^{2+}-free sodium acetate buffer (pH 4.5) was used. As manumycin A and its derivatives lose their activity in the presence of thiols (see also ref. [22]) the assays were carried out in a buffer free of dithiothreitol and β-mercaptoethanol.

Ras farnesyltransferase assay: The Ras farnesyltransferase activity was determined as previously described[30, 31] by using a fluorescence-based Ftase assay.[32] As enzyme source we used an *Escherichia coli* strain overexpressing recombinant farnesyltransferase from *Saccharomyces cerevisiae*.[30]

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