Macquarimicin A Inhibits Membrane-bound Neutral Sphingomyelinase from Rat Brain

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Ceramide, the product of sphingomyelin (SM) hydrolysis by neutral pH-optimum and magnesium-dependent sphingomyelinase (N-SMase), has been reported to increase in response to several inflammatory stimuli including TNFα and IL-1β. Thus, it has been suggested that ceramide generation plays an important role in such stimuli-mediated physiological and pathological processes[1~9]. Moreover, cell permeable ceramide analogues or bacterial SMases cause the generation of pro-inflammatory lipid arachidonic acid in some cell types. Therefore, regulation of ceramide generation by an inhibitor of N-SMase may lead to a new way of therapy for inflammation and autoimmune diseases. Thus, inhibitors of N-SMase were searched for in fermentation products. In our previous studies, scyphostatin[10~12] and F-11334s[13] were found and reported to show inhibitory activity against the enzyme. In addition to their discoveries, one of the strains was found to produce an active metabolite against rat brain N-SMase. The strain was identified to be *Micromonospora* sp. SANK 60294 from its morphological, physiological, and chemotaxonomic charactaristics.

The N-SMase inhibitor-producing organism was incubated as a vegetative culture in a 500 ml Erlenmeyer flask containing 100 ml of vegetative medium (composed of: 2.5% glycerol, 2.5% glucose, 1% pressed yeast, 1% soy been meal, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% $CaCO_3$, 0.005% CB-422) at 28°C on a rotatory shaker (120 rpm) for 5 days. One milliliter of the cultured broth was inoculated into twenty 500 ml Erlenmeyer flasks each containing 100 ml of PY medium (composed of: 2% glucose, 1% soluble starch, 0.9% pressed yeast, 0.5% beef extract, 0.5% polypepton, 0.3% $CaCO_3$, 0.5% NaCl, 0.005% CB-422) at 28°C on a rotatory shaker (200 rpm) for 6 days. The supernatant of the culture broth was extracted three times with ethyl acetate at pH 3.0. The ethyl acetate extract was concentrated and applied on a silica gel column (eluted with CH_2Cl_2 - MeOH, 20 : 1) followed by preparative HPLC (Nacalai tesque, COSMOSIL 5C18-AR, 20i.d.×250 mm) eluted with MeOH - 0.2% triethylamine phosphate buffer (pH 3.3), 1 : 1. The peak showing N-SMase inhibitory activity was collected and 80 mg of the active compound which had characteristic UV absorption at 244 nm and showed optical activity ($[\alpha]_D^{25}$ +285.6° (*c* 0.2, MeOH)) was obtained. The molecular formula was determined to be $C_{19}H_{22}O_5$ based on high-resolution FABMS spectral analyses: $(M+H)^+$, *m/z* 331.1539 (Δ−0.6 mmu). A literature search on the compound based on the molecular formula and the ^1H- and ^{13}C-NMR spectral data confirmed that the active compound was identical to macquarimicin A[14,15] (Fig. 1.), of which very weak anti-anaerobic bacterial activity was reported. In our assay system, macquarimicin A inhibited rat brain N-SMase activity with an IC_{50} value of 48.1 μg/ml.

To analyze the N-SMase inhibition mechanism of macquarimicin A, a kinetic analysis[16] was done. The Lineweaver-Burk plots (Fig. 2. left) showed that only *Km* values were constant against the concentration of SM, revealing the specific pattern for a noncompetitive inhibitor. Also, shown in the Dixon plots (Fig. 2. right), the *Ki* value of macquarimicin A was evaluated to be 70 μM, demonstrating macquarimicin A's weak affinity to rat brain N-SMase.

In order to assess the specificity of macquarimicin A, the

Fig. 1. Structure of macquarimicin A.

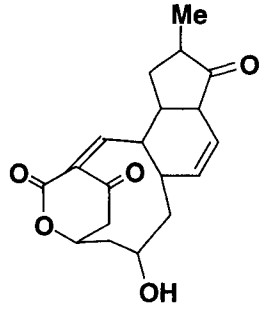


Fig. 2. Kinetic analysis of macquarimicin A.

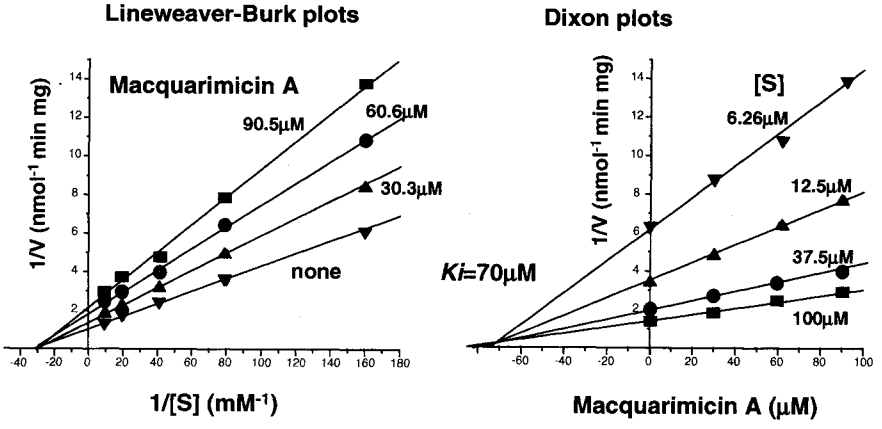


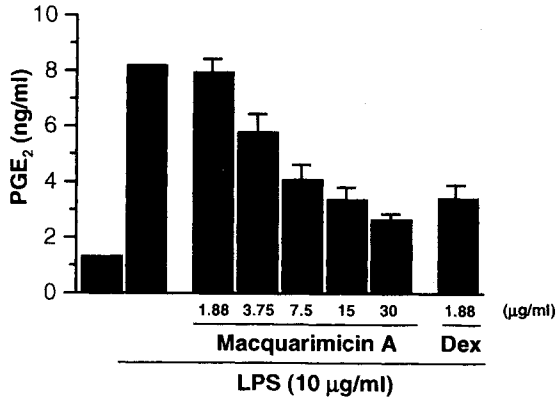
Table 1. Specificity of macquarimicin A on enzyme inhibition.

Enzyme	Origin	$IC_{50}(\mu M)$
N-SMase	Rat brain microsome	145.8
A-SMase	Rat liver lysosome	616.1
N-SMase	*S. aureus*	>1,000
N-SMase	*B. cereus*	>1,000
PC-PLC	Rat liver cytosol	>1,000
PA-PHL	Rat liver microsome	>1,000

Abbreviations: N-SMase, neutral sphingomyelinase; A-SMase, acidic sphingomyelinase; PC-PLC, phosphatidylcholine-specific phospholipase C; PA-PHL, phosphatidic acid specific-phosphohydrolase.

Fig. 3. Effect of macquarimicin A on PGE_2 production in human monocytes.

Dex, dexamethasone; LPS, lipopolysaccharide.



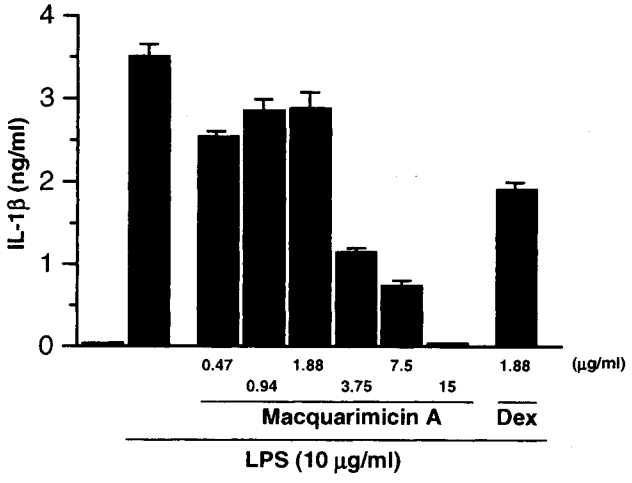
effects of the compound on other SMases or other phosphohydrolases were evaluated. As shown in Table 1, macquarimicin A inhibited acidic SMase activity with an IC_{50} value of 203.0 μg/ml, which is one-fourth the IC_{50} value for N-SMase. On the other hand, macquarimicin A virtually show no inhibitory activity towards any other enzymes listed in Table 1. These results indicate that macquarimicin A is a weak yet specific inhibitor of mammalian N-SMase.

Recently, LPS induced ceramide generation causing inflammatory responses in several cell lines, has been reported[17~19]. To test the anti-inflammatory activity of macquarimicin A in cells, its effect on PGE_2 production in LPS-stimulated human monocytes was observed. Adherent monocytes were isolated from human peripheral blood and stimulated with LPS (10 μg/ml). After incubating the monocytes for 24 hours, an increase in PGE_2 production was detected. When macquarimicin A was added to this system at the same time as LPS, induction of PGE_2 generation was dose-dependently inhibited with an IC_{50} value of 6.14 μg/ml as shown in Fig. 3.

Subsequently, the production of IL-1β, one of the key inflammatory cytokines, was measured after 24 hours of LPS (10 μg/ml) stimulation. In the experiments shown in Fig. 4., macquarimicin A showed a greater, more potent inhibition of IL-1β production compared to that of PGE_2. Thus, macquarimicin A dose-dependently inhibited the LPS-induced IL-1β production and a 50% inhibition occurred at 3.46 μg/ml, which is not toxic to the cells (data not shown). This implies that macquarimicin A can be classified as an inhibitor of IL-1β production, although we are currently unable to offer any speculation on the

Fig. 4. Effect of macquarimicin A on IL-1β production in human monocytes.

Dex, dexamethasone; LPS, lipopolysaccharide.



inhibition mechanism.

To evaluate the pharmacological effect of macquarimicin A on acute inflammation *in vivo*, we performed a carrageenin-induced paw edema study[12]. As shown in Table 2, carrageenin-induced paw edema was dose-dependently inhibited by an oral administration of macquarimicin A. Since the generation of the paw edema is largely caused by PGE_2, the inhibition with macquarimicin A appeared to be reflected in the PGE_2 production inhibition as shown in Fig. 3. As for the acute toxicity of macquarimicin A *in vivo*, on the other hand, no abnormality in mice was observed for at least 7 days after an oral administration of an excess concentration of 300 mg/kg macquarimicin A (data not shown).

From these results and our previous studies regarding anti-inflammatory activities of scyphostatin, it was shown that regulation of ceramide levels by inhibition of N-SMase expands the possibility of therapeutic measures against inflammation.

Table 2. Effect of macquarimicin A on carrageenin induced paw edema.

Dose (mg/kg)	n	Edema intensity (mean±S.E.M.)	% Inhibition
0	5	0.515±0.021	
25	5	0.401±0.039*	22.1
100	5	0.326±0.024**	36.6

*: $p<0.05$, **: $p<0.001$

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