

Biological Activities of Scyphostatin, a Neutral Sphingomyelinase Inhibitor from a Discomycete, *Trichopeziza mollissima*

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Scyphostatin is a specific inhibitor for mammalian neutral magnesium-dependent sphingomyelinase with a fifty percent inhibition concentration (IC₅₀) value of 1.0 μM. When used to inhibit lysosomal acid sphingomyelinase, an approximately 50-fold greater concentration is required. In human peripheral monocytes, the compound inhibits bacterial lipopolysaccharide (LPS)-induced prostaglandin E₂ production and LPS-induced interleukin-1β production with IC₅₀ values of 0.8 μM and 0.1 μM, respectively. In rat, p.o. administration of the compound has also been shown to inhibit carrageenin-induced paw edema. Thus, it is hoped that utility of scyphostatin as a pharmacological tool will contribute to our understanding of the role of ceramide in the cellular inflammation process.

Inflammatory diseases are characterized by edema, cellular infiltration, tissue destruction, and release of arachidonate metabolites. Tumor necrosis factor (TNF)α, interleukin 1β (IL-1β), IL-6, IL-8, and a number of other prototypic inflammatory cytokines have been implicated as central contributors to inflammatory responses. These cytokines share the ability to induce neutrophil degranulation, bone resorption, prostaglandin E₂ (PGE₂) production, procoagulant activity, and collagenase secretion. In immune cells, bacterial lipopolysaccharide (LPS), the molecule responsible for the induction of septic or endotoxic shock, stimulates the secretion of these biologically active cytokines such as TNFα, IL-1β, IL-6, and IL-8. In this regard, some studies have linked the LPS-induced activation mechanism to the sphingomyelin (SM) cycle, a newly described signal transduction pathway^{1~4}.

The SM cycle is initiated by the hydrolysis of membrane SM to ceramide and phosphocholine *via* activation of neutral pH-optimum and magnesium-dependent sphingomyelinase (N-SMase). Ceramide has emerged as a second messenger molecule thought to mimic most of the cellular effects of TNFα, IL-1β, and

LPS in terminal differentiation, apoptosis, and cell cycle arrest^{5~8}). For example, some of the biological effects of ceramide in immune cells include PGE₂ production through cyclooxygenase II activation, IL-6 production, nuclear factor-κB, and inducible nitric-oxide synthase^{9~12}).

As reported in a preceding paper, scyphostatin, a new inhibitor of N-SMase, has been isolated from the culture broth of *Trichopeziza mollissima*. This paper describes the biological activities of scyphostatin with a primary focus on the inflammation processes stimulated by LPS.

Materials and Methods

Materials

Unless otherwise specified, all reagents were obtained from Sigma. All the cell lines were purchased from the American Type Culture Collection. [*N*-methyl-¹⁴C] sphingomyelin (bovine) was purchased from Amersham Co., Ltd.; bacterial lipopolysaccharide (LPS) from Difco; ELISA kits for PGE₂ and IL-1β from Cayman; ELISA kits for TNFα and IL-6 from Genzyme; ELISA kit for

IL-8 from R & D; and AlmarBlue from Bio Source.

Tissue Culture

All the cells were maintained at a density of $1 \times 10^5 \sim 5 \times 10^6$ cells/ml at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The medium used in this study was either RPMI1640 (GibcoBRL) or Dulbecco's modified Eagle's medium (GibcoBRL) supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin G, and 100 µg/ml streptomycin sulfate. For enzyme preparations, cells were allowed to grow to 60~80% confluence and then washed, collected, and treated as indicated.

SMase Assay and Other Enzyme Assays

Preparation of the N-SMase fraction from rat brains was performed by the methods described in the previous paper. The other N-SMase fractions in mammalian cells were prepared according to the method of OKAZAKI *et al.*¹³⁾. Other enzymes used in this study were obtained by the following methods: phosphatidylcholine-specific phospholipase C (PC-PLC) by the method of WU *et al.*¹⁴⁾; phosphatidic acid-specific phosphohydrolase (PA-phosphohydrolase) by the method of PANAGIA *et al.*¹⁵⁾; Cathepsin L by the method of MASON *et al.*¹⁶⁾; and IL-1β converting enzyme by the method of THORNBERRY *et al.*¹⁷⁾. The mixed micelle assay system described in the previous paper was used for the measurement of N-SMase activity, with a slight modification in 0.1 mM [*N*-methyl-¹⁴C] sphingomyelin. A-SMase activity was also measured under the same condition used for the N-SMase assay, but in this case the pH was 5.6, magnesium was omitted, and the enzyme source replaced with rat liver lysosomal fraction. The other enzyme assays were carried out as follows: PC-PLC by the method of WU *et al.*¹⁴⁾; PA-phosphohydrolase by the method of PANAGIA *et al.*¹⁵⁾; Cathepsin L by the method of BARRETT *et al.*¹⁸⁾; and IL-1β converting enzyme by the method of HOWARD *et al.*¹⁹⁾.

Productions of the Inflammatory Mediators in Human Monocytes

Adherent monocytes were isolated from human peripheral blood. After incubating the monocytes (1.0×10^5 cells/ml) stimulated by 10 µg/ml LPS for 4 or 24 hours, the PGE₂, IL-1β, TNFα, IL-6, and IL-8 levels in the conditioned medium were measured by ELISA kits. Scyphostatin was added to this system together with the LPS-addition. Cell toxicities of scyphostatin for the monocytes were tested by AlmarBlue reagent.

Table 1. Specificity of scyphostatin on enzyme inhibition.

Enzyme	Origin	IC ₅₀ (µM)
N-SMase	rat brain microsome	1.0
A-SMase	rat liver lysosome	49.3
N-SMase	<i>S. aureus</i>	>1,000
N-SMase	<i>B. cereus</i>	>1,000
PC-PLC	rat liver cytosol	>1,000
PA-PHL	rat liver microsome	>1,000
Cathepsin L	rat liver	>1,000
ICE	human recombinant DNA	>1,000

Abbreviations: N-SMase, neutral sphingomyelinase; A-SMase, acidic sphingomyelinase; PC-PLC, phosphatidylcholine-specific phospholipase C; PA-PHL, phosphatidic acid specific-phosphohydrolase; ICE, Interleukin-1β converting enzyme.

Carrageenin-induced Paw Edema Study

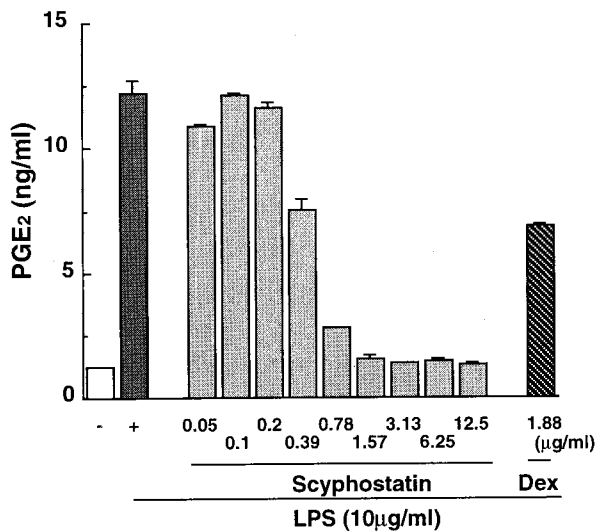
Male Wistar-Imamichi rats used in this study were purchased from Imamichi Institute of Animal Reproduction, Ibaraki, Japan. After an administration of scyphostatin or vehicle to the rat (6 weeks old, weight 120~140 g), an inflammation-inducing solution of 1% carrageenin (Viscarin402) in 0.9% NaCl was subcutaneously injected into the bottom of the paw. After 3 hours, the volume of paw edema was measured by plethysmometer (Ugo Basile) by soaking the paw in a bucket of water, and the results were given as an edema intensity. Acute toxicity of scyphostatin *in vivo* was tested using mice (*ddY*, male, 5 weeks old) purchased from Japan SLC Co., Ltd. through the p.o. administration of 300 mg/kg scyphostatin in corn oil. The animals were observed for 7 days and dissected.

Results and Discussion

Specificity of Scyphostatin in Enzyme Inhibition

Scyphostatin was discovered in the fermentation broth of *Trichopeziza mollissima* during the course of a screening for N-SMase inhibitors. In addition to inhibiting N-SMase activity derived from rat brain microsome with a concentration required for 50% inhibition (IC₅₀) of 1.0 µM (Table 1), scyphostatin also inhibits the N-SMase activities using membrane fractions from the following mammalian cell-lines with IC₅₀s of the same order: human myeloid leukemia U937, human leukemia

Fig. 1. Effect of scyphostatin on PGE₂ production in human monocytes.



Abbreviation: Dex, Dexamethasone.

Molt-4, murine fibrosarcoma L929, and human T cell leukemia Jurkat (data not shown). Thus, to our knowledge, scyphostatin is the most potent inhibitor for N-SMases in the cell-free system.

Our next aim was to assess the specificity of scyphostatin on enzyme inhibition. For this assessment, we examined the effects of the compound on the other SMases, phosphatidylcholine (PC)-phospholipase C, and phosphatidic acid (PA)-phosphohydrolase. Furthermore, since the compound possesses an electrophile epoxy-enone moiety, we also examined the effects on SH-bearing enzymes. As shown in Table 1, scyphostatin inhibits A-SMase activity at an IC₅₀ of 49.3 µM, a value only about one-fiftieth of IC₅₀ for inhibition of N-SMase activity. On the other hand, scyphostatin showed virtually no inhibitory activity towards bacterial SMases, PC-phospholipase C, PA-phosphohydrolase, Cathepsin L, and IL-1β converting enzyme (Table 1). These results indicate that scyphostatin is a specific inhibitor for N-SMase. As one interpretation, we postulate that the structural similarity between scyphostatin and ceramide is the rationale for the inhibition-specificity.

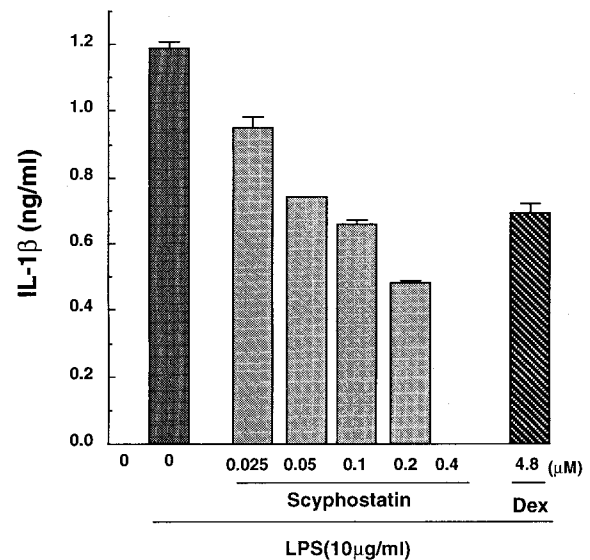
Effects of Scyphostatin on Inflammatory Mediator Production in Human Monocytes

In order to estimate the activity of scyphostatin on the

Table 2. Effect of scyphostatin on production of various cytokines.

Cytokine	IC ₅₀ (µM)
IL-1β	0.1
IL-6	1.4
IL-8	2.1
TNFα	1.3

Fig. 2. Effect of scyphostatin on IL-1β productions in human monocytes.



Abbreviation: Dex, Dexamethasone.

inflammatory process, we initially carried out a representative study on LPS-induced PGE₂ production in human monocytes. As shown in Fig. 1, when scyphostatin was added to the system, LPS-induced PGE₂ production after 24 hours was dose-dependently inhibited at an IC₅₀ value of 0.8 µM. Next, we tested the effects of scyphostatin on inflammatory cytokine productions in the same system. Since both IL-6 and IL-8 productions were secondarily affected by IL-1β and TNFα, we measured these four cytokine productions after 4 hours of LPS stimulation. In the experiments shown in Table 2, scyphostatin inhibited IL-1β production more potently than it did PGE₂ and other cytokines. Thus, scyphostatin dose-dependently inhibited the LPS-induced IL-1β

Table 3. Effect of scyphostatin on carrageenin-induced paw edema.

Dose (mg/kg)	n	Edema intensity (mean \pm S.E.M.)	% Inhibition
0	5	0.515 \pm 0.021	
25	5	0.413 \pm 0.026 *	19.8
100	5	0.263 \pm 0.031 **	48.8

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

production with a 50% inhibition at 0.1 μ M, a concentration with no toxicity to the cells (Fig. 2). Although we are currently unable to offer any speculation about the inhibition mechanism, these results certainly imply that scyphostatin can be classed as a potent inhibitor for IL-1 β production.

Effect of Scyphostatin on *In Vivo* Acute Inflammation

To evaluate the pharmacological effect of scyphostatin on acute inflammation *in vivo*, we performed a carrageenin-induced paw edema study. As shown in Table 3, carrageenin-induced paw edema was dose-dependently inhibited by an oral administration of scyphostatin. Since the generation of the paw edema is largely caused by PGE₂, the inhibition with scyphostatin appeared to be reflected in the PGE₂ production inhibition shown in Fig. 1. In our investigation of the acute toxicity of scyphostatin *in vivo*, no abnormality in mice was observed within at least 7 days following an oral administration with an excess concentration of 300 mg/kg scyphostatin (data not shown).

Although the mechanism is not sufficiently clarified, our results suggest that an endogenous ceramide released through the N-SMase cycle transduces signaling in LPS- and several inflammatory cytokines-activated immune cells. The present study also indicates that scyphostatin may provide us with a useful tool for exploring the molecular mechanism of cellular inflammation processes.

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