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Suppressive effects of three diketopiperazines from *marine-derived bacteria* on polyphosphate-mediated septic responses

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ABSTRACT

Diketopiperazine is a natural products found from bacteria, fungi, marine sponges, gorgonian and red algae. They are cyclic dipeptides possessing relatively simple and rigid structures with chiral nature and various side chains. The compounds in this structure class have been known to possess diverse bioactivities including antibiotic activity, anti-cancer activity, neuroprotective activity, and anti-inflammatory activity. Previous studies have reported proinflammatory responses of endothelial cells to the release of polyphosphate (PolyP). In this study, we examined the anti-inflammatory responses and mechanisms of diketopiperazine and its effects on PolyP-induced septic activities in human umbilical vein endothelial cells (HUVECs) and mice. The survival rates, septic biomarker levels, behavior of human neutrophils, and vascular permeability were determined in PolyP-activated HUVECs and mice. Diketopiperazine suppressed the PolyP-mediated vascular barrier permeability, upregulation of inflammatory biomarkers, adhesion/migration of leukocytes, and activation and/or production of nuclear factor-κB, tumor necrosis factor-α, and interleukin-6. Furthermore, diketopiperazine demonstrated protective effects on PolyP-mediated lethal death and the levels of the related septic biomarkers. Therefore, these results indicated the therapeutic potential of diketopiperazine on various systemic inflammatory diseases, such as sepsis or septic shock.

1. Introduction

Inorganic polyphosphate (PolyP), which is a linear polymer that is made up of several orthophosphate residues that are linked by adenosine triphosphate-like phosphoanhydride bonds [1], is present in all bacterial and animal cells [2]. Recent studies that were mostly conducted on microorganisms have reported a number of diverse biological functions of PolyP, including inflammation, apoptosis, proliferation, and blood coagulation, in mammalian systems [3–6]. Our recent studies indicated proinflammatory activities of PolyP, such as mediating vascular hyperpermeability, increasing the adhesion and migration of leukocytes, and upregulating the expression of cell adhesion molecules (CAMs), including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and e-selectin [6–8].

Diketopiperazine is a natural products found from bacteria, fungi, marine sponges, gorgonian and red algae [9]. They are cyclic dipeptides possessing relatively simple and rigid structures with chiral nature and various side chains. The compounds in this structure class have been known to possess diverse bioactivities including antibiotic activity, anti-cancer activity, neuroprotective activity, and anti-inflammatory activity [10]. In the course of screening for the expression and activity of PolyP from natural products, the ethyl acetate extracts of two independently fermented bacterial strains derived from marine sources showed

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potent inhibition on the expression and activity of PolyP in human umbilical vein endothelial cells (HUVECs). Repeated chromatographic separation gave three of diketopiperazines, *cyclo* (L-Pro-D-Val) (**1**), *cyclo* (L-Pro-L-Tyr) (**2**) and *cyclo* (L-Pro-D-Leu) (**3**), and these proline-containing compounds have been reported from the fermented bacterial strains [11], fungal strains [12], and the other macroorganisms [13]. Although several of diketopiperazines have been reported to have diverse biological activities including antibiotic and anti-inflammatory activities [10], the effects of **1**–**3** on the expression and activity of PolyP have not yet been studied. Thus, noting that several of diketopiperazines have a pleiotropic role in anti-inflammatory responses, and that PolyP mediates vascular inflammatory responses and is involved in the pathophysiology of sepsis, we hypothesized that **1**–**3** possesses anti-PolyP-mediated inflammatory responses. Therefore, in the current study, we investigated the effect of **1**–**3** on PolyP-induced vascular inflammatory responses such as the survival rates, septic biomarker levels, behavior of human neutrophils, and vascular permeability in PolyP-activated HUVECs and mice.

2. Methods

2.1. Reagents

Fetal bovine serum and Vybrant® DiD were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). PolyP65, Evans blue, crystal violet, 2-mercaptoethanol, catalase-polyethylene glycol, and antibiotics (penicillin G and streptomycin) were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Antibodies against p21 (rabbit polyclonal), p27 (mouse polyclonal), p53 (mouse monoclonal), and β-actin (goat polyclonal) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D1 antibodies (mouse monoclonal) were purchased from NeoMarkers (Fremont, CA). Antibodies against phospho-retinoblastoma protein (pRb) (rabbit polyclonal) were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Microbial isolation and identification

Bacillus sp. HC001 was obtained from marine sediments from Jeju Island, South Korea, in 2013. The collected sediments were dried under air flow for 1 day on a clean bench. The dried sediments were plated on the SYP SW agar media (soluble starch, 10 g/L; yeast extract, 4 g/L; peptone 2 g/L; agar 16 g/L; sterilized seawater 1 L) and the isolation plate was incubated at 27 °C for two months. A yellow colored bacterial colony (HC001) was picked and a pure bacterial strain was obtained. The bacterial strain HC001 was identified as *Bacillus* sp. based on 16*S* rDNA sequence analysis (99.79%, similarity to *Bacillus amyloliquefaciens* strain Lx-11). Another bacterial strain (12L081) was isolated from a marine sponge collected from the coastal area of Seongsan, Jeju Island in 2012. A collected marine sponge was dried and powdered. The powdered sample was plated on SYP agar media. The plate was incubated for two months and a bright yellow colored bacterial strain (12L081) was isolated. The strain was identified to be *Piscicoccus* sp. based on the 16*S* rDNA sequence similarity (99.42%) to *Piscicoccus intestinalis*.

2.3. Isolation of compounds 1-3

Each bacterial strain was inoculated in 35 L of SYP SW media (soluble starch, 10 g/L; yeast extract, 4 g/L; peptone 2 g/L; sterilized seawater 1 L) at 25 °C for 7 days at 150 rpm. The cultured media of each strain (35 L) was extracted with EtOAc twice (35 L × 2) and the organic layer was concentrated in vacuo to give crude extracts (1.9 g for *Bacillus* sp. HC001 strain; 2.7 g for *Piscicoccus* sp. 12L081 strain). The extract of *Bacillus* sp. HC001 was subjected to VLC for fractionation. A portion of fraction E (eluting with CH_2Cl_2:MeOH = 10:1) was further separated using preparative RP-HPLC (Phenomenex Luna 10μ C18(2) 100 Å, 250 × 21.20 mm, 10 μm, 6 mL/min, 20% CH_3CN in H_2O) to afford compound **1** (2.5 mg) and compound **2** (1.6 mg). The extract of the culture media of *Piscicoccus* sp. 12L081 was also subjected to VLC and the fraction D (eluting with CH_2Cl_2:MeOH = 20:1) was further purified by using preparative RP-HPLC (Phenomenex Luna 10μ C18(2) 100 Å, 250 × 21.20 mm, 10 μm, 6 mL/min, 17% CH_3CN in H_2O) to yield 6.6 mg of compound **3**.

2.4. Structure elucidation of compounds 1-3

The structures of three known diketopiperazines were confirmed by the comparison of spectroscopic data including NMR, MS and optical rotation data of compounds **1**–**3** to the previously reported values [13–16].

2.5. Animals and husbandry

Male mice (C57BL/6 strain; average weight, 27 g; 6–7 weeks old) were obtained from Orient Bio Co., Ltd. (Sungnam, Republic of Korea) and used after a 12-day acclimatization period. The mice were maintained as described previously [17,18]. All of the animals were handled in accordance with the *Guidelines for the Care and Use of Laboratory Animals* that was issued by Kyungpook National University (IRB No. KNU 2016-54).

2.6. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex Corporation (Charles City, IA, USA) and maintained as previously described [17–21]. The neutrophils were freshly isolated from whole blood (15 mL) that was obtained by the venipuncture of five healthy volunteers and maintained as previously described [22,23].

2.7. Immunoblotting

Cells at 60–80% confluence from 6-well plates were washed in ice-cold phosphate-buffered saline (PBS) and scraped into 200 μL of 2 × sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 2% β-mercaptoethanol, 20% glycerol and 1 mg bromophenol blue]. Protein concentration was measured using the RC DC protein assay kit (Bio-Rad, CA) according to the manufacturer's instructions. The lysates were sonicated and then heated at 95 °C for 5 min. From each sample, 50 μg of protein was loaded onto SDS–PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA). The blots were blocked with 2% low-fat milk powder in Tris-buffered saline (TBS) with 1% Triton (TBST) for 1 h at room temperature and washed 3× for 10 min in TBST before overnight incubation at 4 °C with primary antibody in primary antibody buffer (TBST with 0.2% low-fat milk powder). Blots were then washed 3 × 10 min in TBST and incubated for 1 h at room temperature in 1/2000 HRP-conjugated secondary antibody in block buffer. After a final 3 × 10 min wash in TBST, blots were incubated for 5 min in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim).

2.8. Apoptosis assays

Control and compound-treated cells were collected and