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In vitro and *in vivo* anti-*Vibrio vulnificus* activity of psammaplin A, a natural marine compound

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Abstract. *Vibrio vulnificus* is known to induce severely fulminant and fatal septicemia in susceptible hosts. In the present study, the antimicrobial activity of natural marine product-derived compounds against *V. vulnificus*, were investigated *in vitro* and *in vivo*. Twelve pure compounds were isolated from natural marine products and their inhibitory effects on *V. vulnificus*-induced cytotoxicity were determined in INT-407 cells. Among the 12 pure compounds tested, treatment with psammaplin A significantly suppressed *V. vulnificus*-induced cytotoxicity in INT-407 cells. Notably, treatment with psammaplin A (5-50 µg) had improved survival rates compared with that in the untreated mice, when the mice were infected with *V. vulnificus* intraperitoneally. In addition, the bacterial load of *V. vulnificus* in several tissues (spleen, liver and small intestine) was significantly lower in psammaplin A-treated mice than in untreated mice. Furthermore, psammaplin A treatment significantly suppressed the growth of *V. vulnificus*. Taken together, these results indicate that psammaplin A may be a potential agent for the prevention and treatment of *V. vulnificus* infections.

Introduction

Vibrio vulnificus is a gram-negative bacterium, is known to cause primary sepsis and gastroenteritis in humans. Following an infection with *V. vulnificus*, the disease proceeds rapidly, resulting in extensive cellular damage. Additionally, the consumption of contaminated shellfish or wound infection with *V. vulnificus* can induce fatal septicemia in susceptible individuals with chronic liver disease (1). A variety of virulence factors produced by *V. vulnificus* can induce septic shock, which is often fatal. Putative virulence factors, including capsular polysaccharides (2,3), siderophores (4), hemolysin (5), matrix metalloproteinase (6), flagella (7,8) and RtxA toxin (9-11) have been reported *in vivo* and *in vitro*. These virulence factors may induce the persistent production of proinflammatory mediators, such as interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α and nitric oxide in the host (12,13). Therefore, highly active antimicrobial agents are required for the efficient treatment of *V. vulnificus* infections. In this study, the anti-*V. vulnificus* activity of psammaplin A was investigated *in vitro* and *in vivo*.

Psammaplin A is a natural marine product isolated from sponges, such as *Poecillastra* sp., *Jaspis* sp. and *Psammaplysilla* sp. (14,15). Psammaplin A is known to possess antimicrobial (16), antitumor and cytotoxic activities against several cell lines, including the P388 leukemia cell line (14,15), as well as lung, ovarian and colon cancer (17). It was also reported to have inhibitory activities against DNA gyrase, DNA topoisomerase, farnesyl protein transferase and leucine aminopeptidase (16,18-22). Previous studies showed that psammaplin A possesses an antimicrobial effect against methicillin-resistant *Stapylococcus aureus* (MRSA) (16,23,24). However, the effects of psammaplin A on *V. vulnificus* infection *in vitro and in vivo* have not been investigated.

In this study, the antibacterial activity of psammaplin A against *V. vulnificus* as well as its suppressive effects against the cell cytotoxicity induced by *V. vulnificus* were examined *in vitro* and *in vivo*.

Materials and methods

Animal cell culture and chemicals. The INT-407 human epithelial cell-line (ATCC CCL-6) was purchased from the American Type Culture Collection (Manassas, VA, USA), and maintained at 37°C under 5% CO_2 in Minimum Essential Medium (MEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (10 U/ml penicillin G and 10 µg/ml streptomycin) (growth medium). Psammaplin A is a natural marine product that was isolated

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from two sponges, *Jaspis* sp. and *Poecillastra wondoensis* (17). The other compounds were isolated from a sponge-derived fungus *Acremonium* sp. and their configurations were determined by CD spectroscopic data, along with comparison of ^{1}H and ^{13}C spectroscopic data (25). All chemicals used in the study were a gift from Professor Jung (College of Pharmacy, Pusan National University, Busan, Korea). The natural marine products were dissolved in anhydrous ethanol to make a 10 mg/ml stock solution. Subsequent dilutions were made in Dulbecco's modified Eagle's medium.

Bacterial strains and growth conditions. V. vulnificus strain MO6-24/O used in the present study was isolated from patients (9,10) and provided by Professor Sang Ho Choi (Seoul National University, Seoul, Korea). The *V. vulnificus* bacteria were grown to log phase at 30˚C in Luria-Bertani medium (produced in the laboratory) supplemented with 2.0% NaCl LBS medium, after which they were diluted to $6x10^8$ CFU/ml in LBS medium, and then centrifuged for 3 min at 2,500 x g and resuspended in antibiotic-free MEM medium prior to infection of INT-407 cells. The concentration of bacteria was confirmed via viable colony counting conducted on LBS agar.

In vitro broth cultures of V. vulnificus. The *V. vulnificus* inoculum size was $6x10^8$ CFU/ml. Variable concentrations of natural pure compounds 1, 4, 6, 8 and 10 (1, 5, 10, 12.5, 20, 25, 40, 50, 75 and 100 μg/ml) were solubilized in 20 ml of growth medium (2% NaCl LBS) and then tested for their ability to alter bacterial growth by spectrometry (OD_{540}). This was conducted by culturing *V. vulnificus* for 0-13 h in the presence of 50 μg/ml psammaplin A or 0-100 μg/ml psammaplin A for 13 h at 37˚C in 2% NaCl LB medium, and bacterial growth was evaluated by measuring the optical density at 540 nm (OD_{540}). The *V. vulnificus* cultures were then incubated with aeration at 150 rpm using a gyratory shaker for 5 h at 37˚C.

Infection protocol. INT-407 human epithelial cells were infected with *V. vulnificus* as previously described (9,10). Briefly, INT-407 cells were grown in growth medium at 37˚C in a 5% CO_2 incubator. Next, the cells were seeded onto 6-well ($8x10^5$ cells/well) and 96-well ($2x10^4$ cells/well) culture plates and then cultured for 24 h in antibiotic-free growth medium. Prior to infection, the bacteria were centrifuged for 3 min at 2,500 x g, resuspended and adjusted to $6x10^8$ CFU/ml in antibiotic-free MEM medium. The bacterial suspensions were then added to psammaplin A-treated or untreated-epithelial cells at various multiplicities of infection (MOI; the ratio of the number of bacteria to the number of epithelial cells), after which the infected cells were incubated for 1-4 h in antibiotic-free growth medium at 37˚C under 5% CO_2.

Cytotoxicity assay. The bacteria-infected INT-407 cell cultures were aliquoted into a 96-well tissue culture plate (Nunc, Roskilde, Denmark) as previously described (9,10). The cytotoxicity was then determined by measuring the activity of lactate hydrogenase (LDH) in the supernatant using a cytotoxicity detection kit (Roche, Mannheim, Germany). The cytotoxic level was expressed as a percentage relative to the total LDH activity of cells that were completely lysed by 1% Triton X-100 (9,10).

Morphological study. INT-407 ($8x10^5$ cells/well) cells were incubated with bacteria in a 6-well plate for 3 h at an MOI of 10, after which the cells were washed with phosphate-buffered saline (PBS). The cells were then fixed with 4% para-form-aldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature, washed and completely dried. Next, the cells were stained with Giemsa solution (Molecular Probes, Thermo Fisher Scientific, Inc.) for 1 h at room temperature. The cells were then washed twice with distilled water and dried, after which the images of the specimens were acquired using a microscope (Olympus IX 71, Tokyo, Japan).

Survival of V. vulnificus-infected mice. A total of 35 female ICR mice (Samtaco Bio Korea, Gyounggi-do, Korea; age, 8 weeks; weight, 20-22 g) that were housed under specific-pathogen free conditions were used for all experiments. They were maintained at 24˚C with a relative humidity of 50%, under a 12-h light/dark cycle. The mice had access to food and water *ad libitum*. The present study was approved by Korea University (Seoul, Korea). The mice were intraperitoneally infected with 0.1 ml of 250 μg iron dextran (Sigma-Aldrich) 30 min prior to injection with *V. vulnificus*. Next, the mice were intraperitoneally injected with $1x10^3$ CFU/0.1 ml *V. vulnificus*. The use of iron dextran produces a useful model to investigate systemic disease that results from *V. vulnificus* infection. The mice were administered 0.2 ml psammaplin A (DCM 1-9-1) solution (5, 10, 25 or 50 μg per mouse) or a PBS intraperitoneally (control), after which their survival status was assessed every hour for 24 h.

Quantitative analysis of bacteria in tissues. The *V. vulnificus*-inoculated mice were sacrificed by cervical dislocation 7 h after infection. A ventral incision was made to observe the abdomen of the infected mice treated with or without psammaplin A (Nikon D60; Nikon Corporation, Tokyo, Japan), and the spleen, liver and small intestine lesions were then aseptically removed. The removed specimens were homogenized in 2 ml PBS using glass tissue homogenizers, after which the homogenates were diluted in PBS and plated on 2% NaCl HI agar. The samples were then incubated at 37˚C for 12 h and bacterial colonies were counted.

Statistical analysis. The data were analyzed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). Student's t-test and one-way analysis of variance followed by the Bonferroni method were employed to identify statistical differences between the values of the various experimental and control groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Psammaplin A suppresses V. vulnificus-induced cytotoxicity in human epithelial cells. Twelve pure compounds were isolated from natural marine products, and their structures were characterized as previously described (17,25) (Fig. 1). The inhibitory effects of these compounds were determined on *V. vulnificus*-induced cytotoxicity (Fig. 2). INT-407 cells were infected with *V. vulnificus* at an MOI of 10 for 3 h in the presence or absence of the 12 marine product-derived
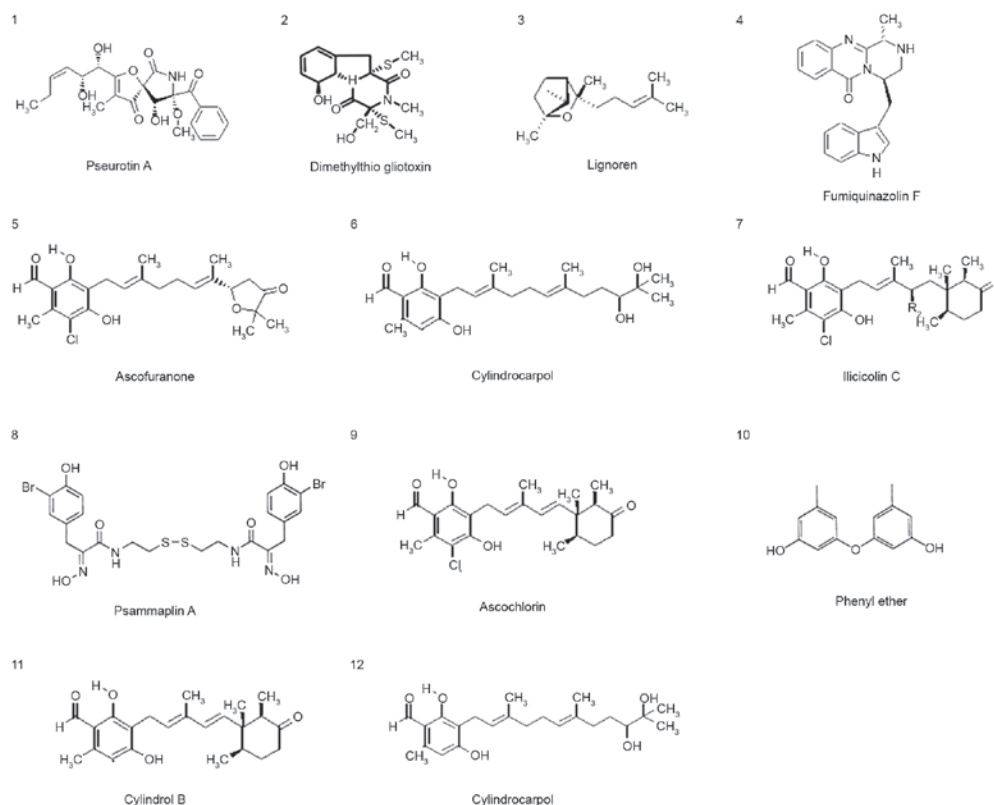
Figure 1. Structures and names of 12 pure compounds isolated from natural marine products.
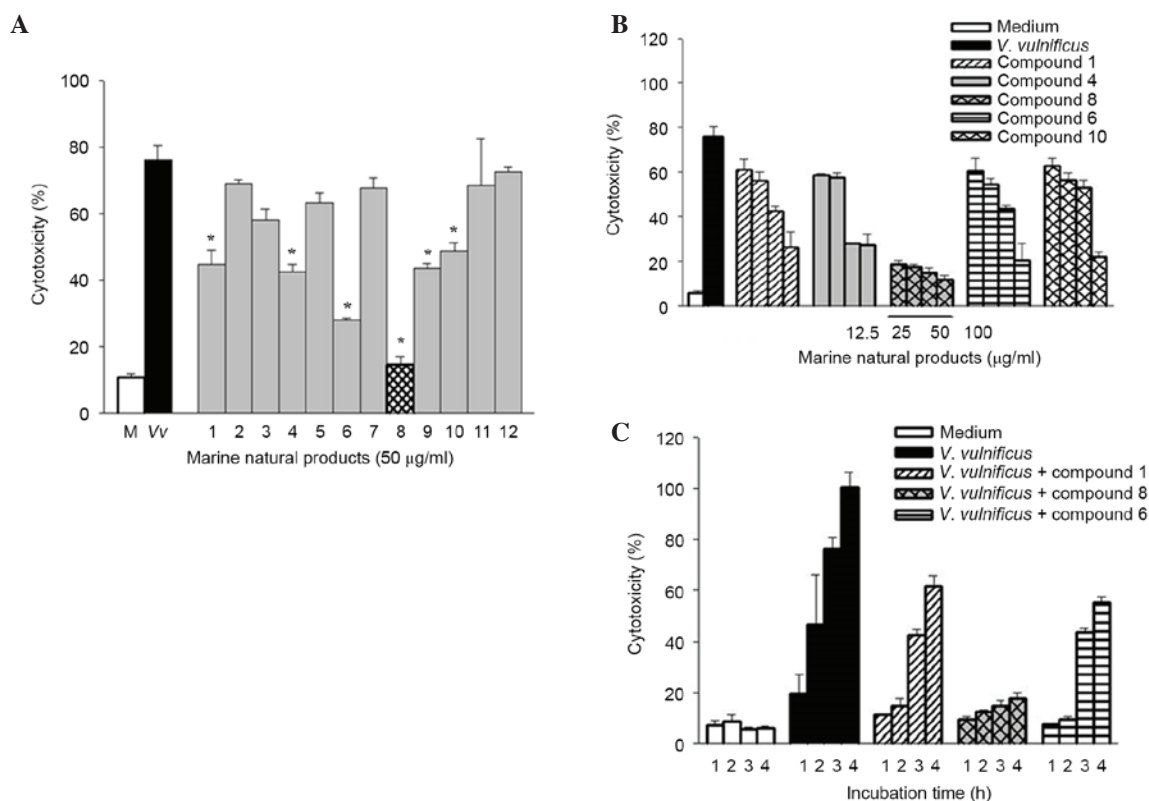

Figure 2. Effects of psammaplin A, a natural marine product on *Vibrio vulnificus*-induced cytotoxicity in human epithelial cells. (A) INT-407 cells were infected with *V. vulnificus* for 3 h at an MOI of 10 in the presence or absence of 12 natural marine products (50 µg/ml), and cytotoxicity was determined using the lactase dehydrogenase release assay. The white bars indicate non-infected cells; black, infected but not treated with natural marine products; grey, infected and treated with natural marine products; checkered, infected, treated with psammaplin A. *P<0.05 vs. infected but untreated group. (B) INT-407 cells were infected with *V. vulnificus* for 3 h at an MOI of 10 in the presence of compounds 1, 4, 6, 8 and 10 (0, 12.5, 25, 50 and 100 µg/ml). (C) INT-407 cells were infected with *V. vulnificus* at an MOI of 10 for varying times (1, 2, 3 and 4 h) in the presence of compounds 1, 6 and 8. Data are presented as the mean ± standard error (n=3) for all experiments. MOI, multiplicity of infection.
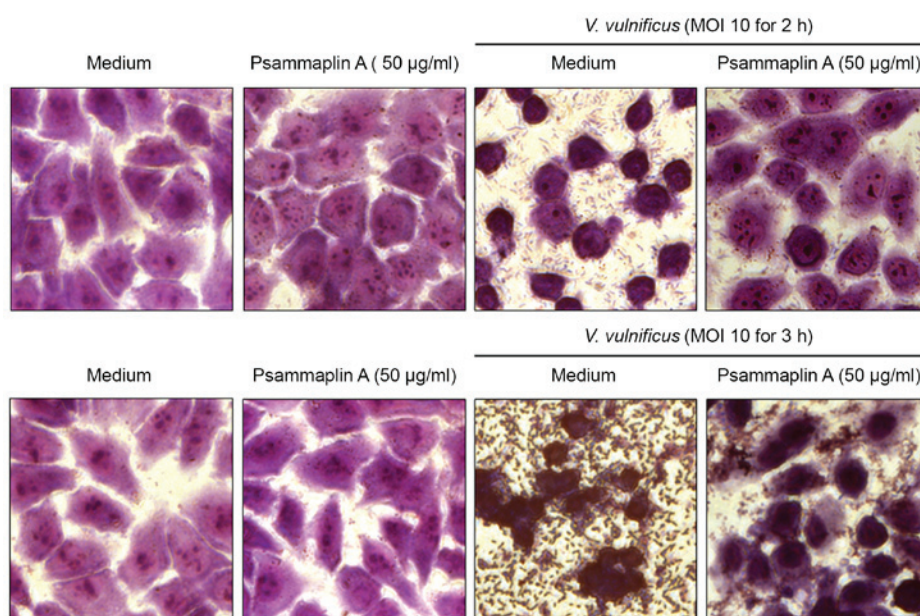
Figure 3. Psammaplin A protects human epithelial cells against *Vibrio vulnificus*-induced cellular damage. INT-407 cells ($8x10^5$ cells/well) were incubated with *V. vulnificus* for 2 or 3 h at MOI 10 in the absence or presence of psammaplin A (50 μg/ml) in six-well plates. Culture plates were then centrifuged, washed twice with pre-warmed PBS (pH 7.4), and fixed with 4% paraformaldehyde. Next, cells were washed twice with PBS and then stained with Giemsa solution. Images of the specimens were acquired using a microscope (magnification, x400). PBS, phosphate-buffered saline; MOI, multiplicity of infection.
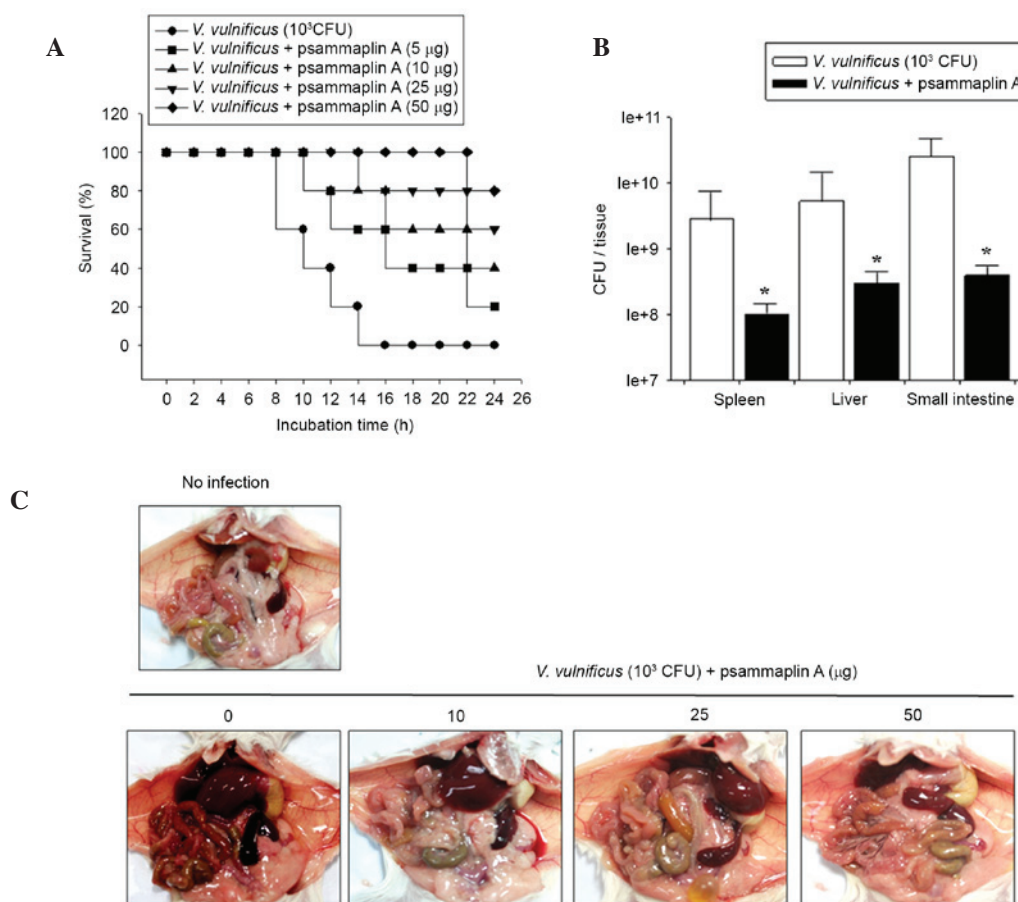

Figure 4. Psammaplin A treatment prolongs the survival of *Vibrio vulnificus*-inoculated mice. (A) Survival of *V. vulnificus*-inoculated mice. ICR female mice (n=5 per group) were intraperitoneally inoculated with 10^3 CFU *V. vulnificus* and then treated with psammaplin A (5-50 μg per mouse). Survival of the mice was recorded every hour for 24 h. (B) Determination of the number of *V. vulnificus* colonies recovered from various tissues. ICR female mice (n=5 per group) were intraperitoneally inoculated with 10^3 CFU *V. vulnificus*, and treated with or without psammaplin A (5-50 μg per mouse). At 7 h after *V. vulnificus* infection, the spleen, liver and small intestine were excised. Specimens were homogenized, diluted, plated on 2% NaCl HI agar plates, incubated at 37˚C for 12 h, and then the number of bacterial colonies was counted. Data are presented as the mean ± standard error. *P<0.05 vs. infected but untreated group. (C) Opened abdomen of *V. vulnificus*-infected mice treated with or without psammaplin A treatment observed using a digital camera (Nikon D60).
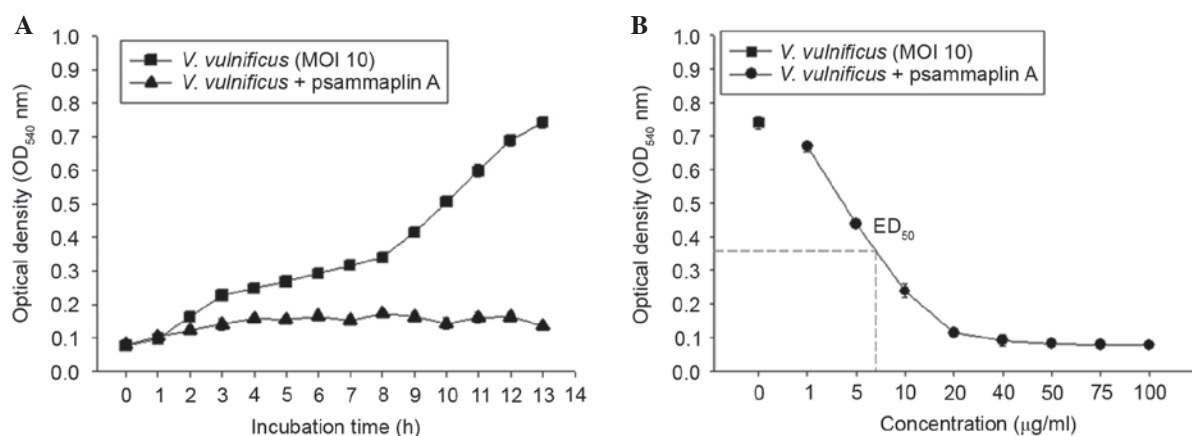
Figure 5. Inhibition of *Vibrio vulnificus* growth by psammaplin A. (A) *V. vulnificus* was cultured for 0-13 h with 50 μg/ml psammaplin A and (B) 0-100 μg/ml psammaplin A for 13 h at 37°C in 2% NaCl Luria-Bertani medium, and bacterial growth was evaluated by measuring the optical density at 540 nm (OD$_{540}$). MOI, multiplicty of infection; OD$_{540}$, optical density at 540 nm.

compounds. Then, the cytotoxicities of the compounds were evaluated in cells using LDH assays. As shown in Fig. 2A, there was significantly decreased cytotoxicity in cells treated with compounds 1, 4, 6, 8, 9 and 10 compared with the untreated cells infected with *V. vulnificus*, indicating that these compounds have inhibitory effects on *V. vulnificus*-induced cytotoxicity. Treatment with these compounds significantly inhibited the cytotoxicity of *V. vulnificus* in a concentration- and time-dependent manner (Fig. 2B and C). Of these compounds, psammaplin A (compound 8) had the strongest inhibitory effect on the *V. vulnificus*-induced cytotoxicity.

To confirm the inhibitory effects of psammaplin A on the *V. vulnificus*-induced cytotoxicity of INT-407 cells, the size and morphology of nuclei were assessed using a microscope (Fig. 3). INT-407 cells infected with *V. vulnificus* at an MOI of 10 for 2-3 h showed typical phenotypic features of cell death, such as cytoplasmic loss and cellular damage, while treatment with psammaplin A reversed that phenotype. Psammaplin A ameliorated the significant cellular damage at 3 h after infection with *V. vulnificus*. These results suggest that psammaplin A inhibits the cytotoxicity against host cells induced by *V. vulnificus* infection.

Psammaplin A treatment prolongs the survival of V. vulnificus-infected mice. To investigate whether psammaplin A prolonged survival, mice were infected with *V. vulnificus* and administered psammaplin A (0-50 μg per mouse). Mice inoculated intraperitoneally with 1×10^3 CFU *V. vulnificus* all died within 16 h. However, psammaplin A treatment of *V. vulnificus*-infected mice increased the survival rate. Following psammaplin A treatment, four out of five mice infected with *V. vulnificus* (50 μg per mouse) survived for 24 h (Fig. 4A).

To investigate the effects of psammaplin A treatment on the growth of *V. vulnificus in vivo*, mice were intraperitoneally infected with 1×10^3 CFU *V. vulnificus* and administered psammaplin A (0, 10, 25 and 50 μg per mouse). After 7 h, several tissue samples, including from the spleen, liver and small intestine were excised from the mice, and the number of *V. vulnificus* colonies in each tissue was evaluated. Fig. 4B shows that the number of *V. vulnificus* colonies was significantly reduced in all tissue samples isolated from psammaplin A-treated mice

compared with the number of *V. vulnificus* colonies isolated from untreated controls. In addition, the necropsy results of *V. vulnificus*-infected mice at 7 h post infection showed edema, hemorrhage, vasodilation and necrosis in the intestines, livers and spleens isolated from the untreated mice. However, the tissue samples from the psammaplin A-treated mice did not show any of the symptoms observed in the tissues of untreated mice (Fig. 4C). These results suggest that psammaplin A significantly suppresses the growth of *V. vulnificus* and the associated pathology *in vitro* and *in vivo*.

Psammaplin A strongly inhibits V. vulnificus growth in vitro. To investigate the antibacterial activities of psammaplin A against *V. vulnificus*, *V. vulnificus* was incubated in the presence or absence of psammaplin A (0-100 μg/ml) for 0-13 h. As shown in Fig. 5, the bacterial numbers of *V. vulnificus* increased in an incubation time-dependent manner. However, psammaplin A treatment inhibited the growth of *V. vulnificus* in a concentration-dependent manner. These findings suggest that psammaplin A significantly inhibited the growth of *V. vulnificus*.

Discussion

V. vulnificus, which is a gram-negative bacterium, causes fatal septicemia in individuals with liver cirrhosis, diabetes, hemochromatosis or immunocompromised conditions (26,27). Infection with *V. vulnificus* causes extensive cellular damage and >50% of patients with *V. vulnificus*-induced septicemia die. Recent studies revealed that hemolysin produced by *V. vulnificus* (VvhA) induces nuclear factor κ-light-chain-enhancer of activated B cells-dependent mitochondrial cell death via lipid raft-mediated reactive oxygen species production in human epithelial cells (28). Therefore, there is an increasing requirement for effective antimicrobial agents for the treatment of *V. vulnificus* infections. Psammaplin A was first isolated from the *Psammaplinaplysilla* sponge and it was known to impede bacterial growth by inhibiting the activities of several key enzyme-mediated processes in prokaryotic systems including DNA replication, microbial detoxification and epigenetic control of gene expression. The results of this study proved

that the marine sponge-derived psammaplin A exerted strong inhibitory activity against *V. vulnificus* in epithelial cells and mice.

The 12 pure compounds isolated from natural marine products were incubated with *V. vulnificus*-infected epithelial cells. Among the compounds, psammaplin A exhibited lower cytotoxicity than the other 11 compounds. In addition, psammaplin A treatment exerted inhibitory effects on *V. vulnificus*-induced cytotoxicity in a concentration- and time-dependent manner, indicating that it prevented the *V. vulnificus*-induced epithelial cell death. Moreover, cytoplasmic loss and cellular damage were not observed in *V. vulnificus*-infected epithelial cells treated with psammaplin A. Furthermore, administration of psammaplin A to *V. vulnificus*-infected mice improved their survival rate compared with that of untreated mice. The number of *V. vulnificus* colonies in the spleens, livers and small intestines of psammaplin A-treated mice was significantly lower than the number of *V. vulnificus* colonies in the untreated mice. Unlike the untreated mice, there was no edema, hemorrhage, vasodilation or necrosis in the intestine, liver and spleen isolated from the psammaplin A-treated mice. Treatment with psammaplin A effectively suppressed the growth of *V. vulnificus* throughout the incubation time in a dose-dependent manner.

The underlying mechanism of the potent anti-*V. vulnificus* activity of psammaplin A remains unclear. Previously, psammaplin A was reported to possess antibacterial activity against gram-positive bacteria, including MRSA, possibly by inhibiting DNA synthesis and gyrase activity. The anti-*V. vulnificus* activity of psammaplin A warrants further investigation to determine the specific underlying mechanism.

In conclusion, the results of this study clearly demonstrated that psammaplin A exerted strong inhibitory activity against *V. vulnificus in vitro* and *in vivo*. These findings suggest that psammaplin A may be a candidate therapeutic agent for the treatment of *V. vulnificus*-related diseases.

Acknowledgements

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