Bioorganic & Medicinal Chemistry Letters 27 (2017) 4970–4974

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Antibacterial activities of viriditoxin congeners and synthetic analogues against fish pathogens

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ARTICLE INFO

Article history:
Received 8 September 2017
Revised 6 October 2017
Accepted 7 October 2017
Available online 9 October 2017

Keywords:
Viriditoxin
Antibacterial activity
FtsZ inhibitor
Fish pathogens

ABSTRACT

Viriditoxin is a fungal secondary metabolite of the fungus *Paecilomyces variotii* derived from the inner tissues of the giant jellyfish *Nemopilema nomurai*. Viriditoxin exhibits antibacterial activity against *Streptococcus iniae* and *Streptococcus parauberis*, which are major pathogens of aqua cultured fish. Viriditoxin induced abnormal cell morphologies in the fish pathogens *S. iniae* and *S. parauberis*, presumably by inhibiting FtsZ polymerization as was previously observed in *Escherichia coli*. Synthetic analogues of viriditoxin, designed based on docking simulation results to FtsZ of *Staphylococcus aureus*, were prepared and compared with viriditoxin for antibacterial activity. Reconstitution of free hydroxyl or carboxyl groups of the methoxyl or methyl ester groups of viriditoxin led to significant reduction of antibacterial activity, implying that the natural molecule is optimized for antibacterial activity to deter bacteria potentially harmful to *Paecilomyces*.

According to government statistics, aquaculture production in Korea during the first six months of 2016 was about 43,000 tons, and its economic value was 428 million USD.[1] However, as adoption of high-density aquaculture increased mortality rates attributable to microbial infections. In particular, between May and October in 2011, the total mortality rate of the olive flounder, which is representative of the South Korean aquaculture industry, was 27.18%, and 83% of total mortality was ascribed to infectious diseases. Of the infectious diseases, bacterial infection (mostly streptococcosis, 12.8%) was the second major cause following parasitic infection (scuticociliatosis, 35.9%).[2] Although antibiotics, such as, oxytetracycline, amoxicillin, florfenicol, and oxolinic acid, have been used to treat these bacterial diseases in Korea, antibiotic-resistant bacteria are being continuously reported because the antibiotics have been used repeatedly over long periods of time.[2,3] Accordingly, novel additional antibiotics with new action mechanisms are required to counter this trend.

In our continued search for antibiotics from marine sources, we isolated viriditoxin as an antibacterial component (MIC 0.31 μg/mL against the fish pathogen *Streptococcus iniae* FP5228) from the jellyfish (*Nemopilema nomurai*)-derived fungus *Paecilomyces variotii*.[4] In a subsequent antibacterial evaluation of viriditoxin against major fish pathogens, significant activity was observed against *S. iniae, Streptococcus parauberis* (*vide infra*), several human pathogens, and *Bacillus subtilis*.[4–7] In *Escherichia coli*, viriditoxin was reported to act at the molecular level by inhibiting the polymerization of FtsZ (**F**ilamenting **t**emperature-**s**ensitive mutant **Z**) which participates in bacterial cell division.[5] In view of the high degree of similarity between bacterial FtsZ and eukaryotic β-tubulin, the antimitotic activity of viriditoxin was also investigated in human prostate and human lung cancer cells.[8,9] In the present study, we attempted to modulate the antibacterial activity of viriditoxin by designing analogues based on docking simulation results with FtsZ of Gram (+) *Staphylococcus aureus*. In addition, natural viriditoxin congeners **1** and **2** were purified as minor components from the same fungus and identified by spectroscopic analysis (Fig. 1, Supplementary data).[10–12] The axial chirality (atropisomerism) of congeners **1** and **2** was determined to be *M* (counterclockwise), the same chirality as viriditoxin, on the basis of CD spectral analysis (Supplementary data). The antibacterial activities of these two natural congeners (**1** and **2**) and of three synthetic analogues (**3**–**5**) against fish pathogens were compared with those of viriditoxin.

The antibacterial activities of viriditoxin against typical fish pathogens were evaluated using a MIC test. The human pathogen *Staphylococcus. aureus* was employed as a reference Gram (+) pathogen for docking simulations of viriditoxin analogues to FtsZ, because the structures of the FtsZ of *S. iniae* and *S. parauberis* were not available in the PDB database. Oxytetracycline was used as a positive control for fish pathogens because it is frequently used as a broad-spectrum antibiotic in aquaculture. Viriditoxin showed

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https://doi.org/10.1016/j.bmcl.2017.10.015
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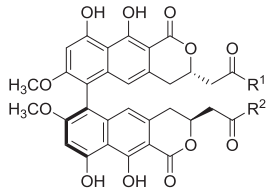

viriditoxin R^1, $R^2 = OCH_3$
1 R^1, $R^2 = CH_3$
2 $R^1 = CH_3$, $R^2 = OCH_3$

Fig. 1. Structures of viriditoxin and its natural congeners.

Table 1
Antibacterial activity of viriditoxin against fish pathogens and *Staphylococcus aureus* (MIC, μg/mL).

Pathogens	Viriditoxin	Oxytetracycline	Tetracycline
Vibrio ichthyoenteri Vi0917-1	>40	0.43	
Vibrio ichthyoenteri FP8487	>40	0.32	
Streptococcus iniae FP 3187	0.21	0.16	
Streptococcus iniae FP 5228 [a]	0.21	26.67	
Streptococcus parauberis FP3287	0.16	0.16	
Streptococcus parauberis KSP28	0.16	0.16	
Streptococcus parauberis SPOF-3K [a]	0.21	20	
Staphylococcus aureus SG 511	0.16		0.16

[a] Oxytetracycline-resistant strains. Oxytetracycline and tetracycline were used as positive controls for fish pathogens and *S. aureus*, respectively. All strains were tested in triplicate, and new inocula were prepared for each test.

significant antibacterial activity against Gram (+) pathogens *S. iniae, S. parauberis*, and *S. aureus* (MIC 0.16 μg/mL, Table 1). In particular, oxytetracycline-resistant *S. iniae* FP 5228 and *S. parauberis* SPOF-3K were well suppressed by viriditoxin whereas oxytetracycline was ineffective (MIC > 20 μg/mL). In fact, viriditoxin was ~ 100 times more potent than oxytetracycline against these drug-resistant fish pathogens. However, two Gram (−) pathogens *Vibrio ichthyoenteri* Vi0917-1 and *V. ichthyoenteri* FP8487 were not responsive to viriditoxin (MIC > 40 μg/mL). These results suggest viriditoxin may be useful for controlling streptococcosis in aquaculture, especially against oxytetracycline-resistant *Streptococci*.

Since viriditoxin showed potent antibacterial activities against oxytetracycline-resistant pathogens, we speculated that its action mechanism differed from that of oxytetracycline in *Streptococcus*. Actually, it has been reported viriditoxin suppressed the growth of *E. coli* by inhibiting FtsZ polymerization,[5] although contradictory results have also been reported.[13] FtsZ is a structural homologue of eukaryotic β-tubulin and a major participant in prokaryotic cell division.[14] In the presence of GTP, FtsZ undergoes polymerization with other cell division proteins to form a Z-ring, which on contraction causes cell division. Thus, the inhibition of FtsZ polymerization disturbs cell division and results in cell death.[15–17] Since FtsZ shows high similarity in most bacteria, we speculated viriditoxin might also inhibit FtsZ polymerization in *Streptococcus* and induce cell death by interrupting mitosis. Therefore, we investigated the effects of viriditoxin on cell division and on the morphologies of Gram (+) *S. iniae, S. parauberis,* and *S. aureus*.

In the case of *S. aureus* SG 511, unlike the non-treated control (Fig. 2A), viriditoxin-treated cells (Fig. 2C) showed many cell clusters and large diameter cocci which might have been caused by cytoskeletal protein disturbance, as was previously reported for *E. coli*.[5] Although no extremely elongated cells, such as, filamentous rod-shaped *Bacillus subtilis* was observed,[17] this observed increase in cell size and heterogeneous cell division supported the involvement of a FtsZ inhibitory mechanism. In contrast, oxytetracycline (a protein synthesis inhibitor) did not induce any morphological changes in *S. aureus* SG 511 (Fig. 2B)

Viriditoxin-treated *S. iniae* FP5228 showed an obvious increase in cell size and uneven cell division (Fig. 3C). Similarly, viriditoxin-treated *S. parauberis* SPOF-3K showed an increase in cell size and uneven cytoplasm separation (Fig. 4C). Oxytetracycline-treated bacteria did not show any morphological changes (Fig. 3B and Fig. 4B). These results support the notion viriditoxin is an inhibitor of FtsZ polymerization in *S. iniae, S. parauberis,* and *S. aureus.*

In view of the presumed distinct antibacterial mechanisms of viriditoxin and oxytetracycline, we expected synergistic or complimentary effects when viriditoxin and oxytetracycline were co-treated. Viriditoxin showed good activity to oxytetracycline-resistant *S. iniae* and *S. parauberis*, while oxytetracycline was effective against Gram (−) strains of *V. ichthyoenteri*, which are resistant to viriditoxin (Table 1). Synergism between viriditoxin and oxytetracycline was examined using fractional inhibitory concentration indices (FICs)[18] as determined by the Epsilometer test (E-test).[19] The FIC index is often employed in assessing *in vitro* combination effect of antibiotics, and it represents the sum of the FICs of each drug tested, where the FIC is determined for each drug by dividing the MIC of each drug when used in combination by the MIC of each drug when used alone (Supplementary data, Table 2). Although no significant synergistic effect was observed after co-treatment, in two *S. parauberis* (KSP28, SPOF-3K) and in *S. iniae* FP5228 additive effects were observed. In particular, MIC against *S. parauberis* SPOF-3K for co-treatment was significantly lower than for oxytetracycline alone. For Gram (−) strains, such as, *Vibrio harveyi*, *V. ichthyoenteri*, *Edwardsiella tarda*, and *Photobacterium damselae*, viriditoxin was inactive even at higher concentrations and FICs were not calculated. These results indicate viriditoxin/oxytetracycline co-treatment could be applied to control general Gram (+) fish pathogens and increase the antibacterial spectrum.

Chemical modifications of viriditoxin were also investigated in an attempt to enhance antibacterial potency and to collect information on structure-activity relationships. Viriditoxin ana-

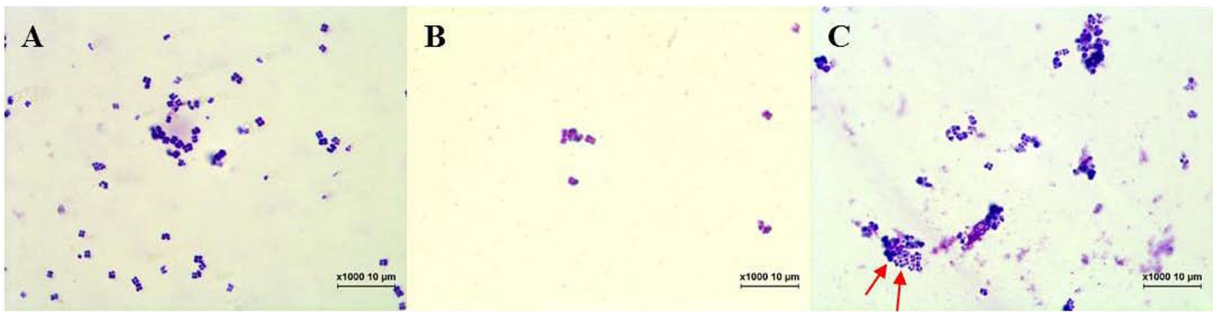

Fig. 2. Effect of viriditoxin on *Staphylococcus aureus* SG511. (A) Treated with 0.5% DMSO as control. (B) Treated with 0.6 μg/ml of oxytetracycline in 0.5% DMSO. (C) Treated with 0.63 μg/ml of viriditoxin in 0.5% DMSO. Unequal cell division and aggregation were observed (indicated by red-arrows).