Anti-tumor role of *Bacillus subtilis* fmbJ-derived fengycin on human colon cancer HT29 cell line

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To explore the potential clinical anti-tumor roles of *Bacillus subtilis* fmbJ-derived fengycin on cell growth and apoptosis in colon cancer HT29 cell line.

Fengycin was extracted from *Bacillus subtilis* fmbJ and detected using HPLC. The effects of different concentration of fengycin on colon cell HT29 cell activity at different time points were analyzed using MTT assay. ROS level in colon HT29 cells affected by fengycin was detected using DCFH-DA method, followed by measuring the effects of fengycin on HT29 cell apoptosis and cell cycle by flow cytometry. The effects of fengycin on Bax/Bcl-2, CDK4/cyclin D1, Caspase-6 and Caspase-3 expressions in HT29 cells were analyzed using western blot. Also, mRNA levels of Bax/Bcl-2 and CDK4/cyclin D1 in HT29 cells affected by fengycin were analyzed using qRT-PCR.

Compared with controls, 20 μg/mL of fengycin performed an inhibit role on HT29 cell growth of at 3 day (P<0.05), and high dose of fengycin showed more excellent effect on inhibiting HT29 cell growth with time increasing. Besides, fengycin could induce HT29 cell apoptosis and affect the cell cycle arrest at G1. ROS level in HT29 cells treated by fengycin was significantly increased compared with that in control group (P<0.05). Western blot analysis showed that after being treated with fengycin, Bax, Caspase-3, and Caspase-6 expressions were increased, however, Bcl-2, and CDK4/cyclin D1 expressions were decreased (P<0.05).

Our study suggested that fengycin may play certain inhibit roles in the development and progression of colon cancer through involving in the cell apoptosis and cell cycle processes by targeting the Bax/Bcl-2 pathway.

Key words: colon cancer cell line HT29, fengycin, cell apoptosis, reactive oxygen species, Bax/Bcl-2 pathway

Colon cancer is the second most common digestive tract malignancy that arises from the junction position from den- tate line to sigmoid colon and rectal [1]. The morbidity and mortality of colon cancer are high, and statistics has shown that approximate 96,830 new cases are diagnosed as colon cancer, in which 50,310 are deaths [2]. Studies refer that the main treatment methods for colon cancer are drug therapy, chemotherapy, and surgery, however, the clinical results for colon cure are not satisfactory due to its hard detection and easy metastasis [3, 4]. Therefore, it will be of great significance to investigate several new and effective treatment drugs for colon treatment in clinical.

Recently, the clinical relevance and therapeutic implications of antibacterial peptides, which was derived from bacteria, fungi, and plants, have becoming the hotspot in many diseases [5]. For example, the high expression of plectasin-derived peptide NZ2114 performs an excellent inhibit role against the pyogenic infection bacteria *Staphylococcus aureus* [6]. The *Bacillus subtilis*-derived lipid peptides, are some biosur- factant that composed by hydrophilic peptide chain of fat and hydrocarbon chain of oil, play crucial roles in anti-fungi, anti-bacteria, and anti-tumor [7]. Fengycin is a member of the three kinds of lipid peptides that derived from *Bacillus subtilis*. Increasing evidence has referred that fengycin functions as an important inhibitor in many diseases, such as cancers [8]. Fengycin inhibits the lung cancer cell growth in 95D cells by enhancing reactive oxygen species (ROS) expression [9]. Be- sides, previous study refers that *Bacillus subtilis*-derived fmbJ performed some anti-virus and anti-tumor roles in vitro [10]. The marine bacterium-derived fengycin has cytotoxic activ-
ity against colon cancer cell line [11], and fengycin performs inhibit roles on cell viability and proliferation of breast cancer [12]. Although several researches have devoted to the application of antibacterial peptide fengycin on a variety of cancers in clinical, the anti-tumor role of fengycin on colon cancer still remains largely unknown.

In this present study, we investigated the biological roles of Bacillus subtilis fmbJ –derived antibacterial peptide fengycin on colon cancer cell growth using colon HT29 cell line. Comprehensive biological experimental methods were used to analyze the effects of fengycin on ROS level, cell cycle, apoptosis, and apoptosis-related protein levels in colon cancer HT29 cell line. This study aimed to explore the potential role of fengycin on tumor development of colon cancer and their potential mechanisms. Our experimental research may provide basis for clinical use of antibacterial peptide fengycin on colon tumor treatment.

Materials and methods

Materials and cell culture. Antimicrobial peptide of fengycin that produced by B. subtilis strain fmbJ (CGMCC No.0943) (purchased from Beihai qunlin shengwu LOTD, Guangxi) in an optimized culture medium was extracted using the High Performance Liquid Chromatography (HPLC) analysis, followed by the identification of isolated fengycin by electrospray ionization mass spectrometry (ESI-MS) as previously described [13]. The human colon cancer cell line HT29, breast cancer cell line MCF-7, and normal human colon epithelial cell line PriCells were purchased from Y-Y Chemical reagents (Shanghai, China). Colon HT29 cells and breast MCF-7 cells were cultivated in RPMI 1640 (Gibco, Grand island, NY) supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL penicillin, while colon epithelial cell line PriCells were grown in DMEM (Gibco, Grand island, NY) containing 10% FBS and 100 μg/mL penicillin. Total cells were cultivated in culture flasks at 37°C in a humidified atmosphere with 5% CO₂.

MTT assay. Cell survival rate and sensitivity of the total cultivated tumor cells were detected using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) assay as previously described [14]. Briefly, tumor cells were transferred into 96-well plates containing RPMI 1640 with 10% FBS. After being cultivated for several hours, cells at logarithmic phase were digested with 0.25% trypsin to adjust the cell density at 1×10⁴ cells/well. Then, different concentration of fengycin (from 0 to 200 μg/mL) were added into cells for another 72 h of incubation. Each assay was performed for 6 repeats and repeated for 3 times independently. Consequently, MTT (5 mg/mL) (Sigma, USA) was added into the 96-well plates to mix with cells at 37°C for 4 h. Followed by removing the supernatant and adding into 150 μL dimethyl sulfoxide to dissolve the formazan. The absorbance of cells in each plate was detected using the microplate reader (THERMOMultiskan M3, USA) at 570 nm.

Intracellular ROS level measurement. DCFH-DA (2',7'-dichlorohydrofluorescein) is an unlabeled oxidation sensitive fluorescence probe that can be oxidized into DCF by intracellular ROS, so as to be the marker for detecting the intracellular ROS level [15]. In this study, DCFH-DA (Sigma) was used to determine the production of ROS level in HT29 cells by flow cytometry. Briefly, HT29 cells treated with 200 μg/mL of fengycin at different time points (0, 6, 12, and 24 h) were mixed with 10 μmol/mL of DCFH-DA at 37°C for 30 min in dark place. Consequently, cells were washed with PBS buffer for 3 times, and then the product of DCF fluorescence was detected using the FACScan flow cytometry (BD, California, USA) at an wavelength of 495 nm.

Cell apoptosis. Effect of different concentration of fengycin on tumor cell apoptosis was quantified with a flow cytometry using the annexin-V-FITC Apoptosis Detection Kit (CLONTECH Laboratories, Palo Alto, CA) according to the manufacturer’s protocol [16]. Briefly, cells were treated with fengycin with three concentrations (100, 200, and 400 μg/mL) for 24 h and 48 h respectively. Then the total cells were harvested and was washed with cold PBS buffer (PH 7.4), and then cells were resuspended in 500 μL of annexin-V binding buffer. After that, 5 μL of annexin-V-FITC and 5 μL of propidium iodide (PI) were mixed with the cells for 10 min at room temperature. Then mixtures were analyzed using the FACScan flow cytometry.

Cell cycle. The effect of fengycin on colon HT29 cell cycle was determined using flow cytometry to analyze the total amount of DNA [17]. In a word, cells (at a density of 4×10⁵ cells/well) were transferred into 6-well plates. Then fengycin with various concentrations of 100, 200 and 400 μg/mL were added into the cells for 6 h, 12 h, and 24 h respectively. The cultivated cells were centrifuged at 12,000 for 5 min and were washed with PBS buffer for 3 times, followed by resuspended with cold PBS and 20 mg/mL of RnaseA (Sigma) application for 30 min at 37°C. Then 50 mg/mL of PI mixed in PBS buffer was added into the cells for 30 min at room temperature in dark place. Finally, cell cycle phase was detected using flow cytometry.

Western blotting analysis. Colon cancer HT29 cells treated with 200 μg/mL fengycin at different time points (0, 6, 12 h and 24 h) were centrifuged at 12,000 rpm/min for 5 min at 4°C [15]. The supernatant was collected to determine the concentration of protein using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rochford, IL). Briefly, a total of 100, 200 and 400 μg/mL were added into the cells for 6 h, 12 h, and 24 h respectively. The cultivated cells were centrifuged at 12,000 for 5 min and were washed with PBS buffer for 3 times, followed by resuspended with cold PBS and 20 mg/mL of RnaseA (Sigma) application for 30 min at 37°C. Then 50 mg/mL of PI mixed in PBS buffer was added into the cells for 30 min at room temperature in dark place. Finally, cell cycle phase was detected using flow cytometry.
enic substrate using the enhanced chemiluminescence (ECL) method. In addition, β-actin served as the internal control.

**Quantitative RT-PCR analysis.** Colon HT29 cells treated with 200 μg/mL of fengycin at different time points (0 h, 6 h, 12 h, and 24 h) were centrifuged at 12,000 rpm/min for 5 min at 4°C, followed by washed with PBS buffer (PH 7.4) for 3 times. Total RNA from the harvested HT29 cells was extracted using the Trizol reagent [18] (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Promega Biotech). The concentration and purity for extracted RNA were detected using SMA4000 UV-VIS (Merinton, Shanghai, China). The purified RNA (0.5 μg/μL) mixed with nuclease-free ddH₂O was used for cDNA synthesis using the PrimerScript 1st Strand cDNA Synthesis Kit (Takara, China). Primers for the targets amplification were shown in Table 1. Then RT-PCR was carried out in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). Primers for the targets amplification were shown in Table 1. Then RT-PCR was carried out in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). The reaction system of 20 μL volume containing 1 μL cDNA from the above PCR, 10 μL SYBR Premix EX Taq, 1 μL each of the primers (10 μM), and 7 μL ddH₂O. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control.

**Statistical analysis.** All the experiments were conducted independently 3 times. Data are presented as the mean ± SEM (standard error of mean). Statistically analyses were performed using SPSS 13.0 (Chicago, IL, USA). Statistical comparisons between two groups were conducted using one-way ANOVA (analysis of variance). Student post hoc multiple test was used to calculate the statistical significance among groups. P<0.05 was considered to indicate a significant difference.

**Results**

**Effect of fengycin on colon cell proliferation.** MTT assay was used to assess the effects of fengycin on colon HT29 cell

![Figure 1](image-url)
Colon cancer is the second most common digestive tract malignancy, and with high morbidity and mortality [1, 2]. Clinical relevance and therapeutic implications on antibacte-
rial peptides which is derived from bacteria, fungi, and plants in many diseases have becoming the hotpot [5]. To investigate some new and effective treatment drugs for colon treatment in clinical will be necessary. In this study, we analyzed the potential roles of B. subtilis strain fmbJ-derived antimicrobial peptide of fengycin on development and progression of colon cancer using HT29 cells. Our data showed that fengycin could inhibit the development and progression of colon cancer. High dose of fengycin could inhibit colon cell proliferation, induce cell apoptosis, increase intracellular ROS level, and induced cell cycle arrest at G0/G1 stage with time increasing.

Application of antimicrobial peptides in clinical has provided novel significance for disease treatment and bacteria cure in recent years [20, 21]. Previous paper has proved that

Figure 3. Fengycin induced colon HT29 cell apoptosis. A: Flow cytometry analysis of 200 μg/mL of fengycin on HT29 cell apoptosis at 24 h; B: the effects of different concentration of fengycin on HT29 cell apoptosis at 24 and 48 h; ** stands for P<0.01 compared with the control group (0 μg/mL). C: fluorescence microscope of apoptotic HT29 cells treated with fengycin at 24 h (×400).

Figure 4. Effect of fengycin on HT29 cell cycle arrest. A-C: effects of different concentration of fengycin on HT29 cell cycle at 6 h, 12 h, and 24 h, respectively. * stands for P<0.05 and ** stands for P<0.01 compared with control group (0 μg/mL).
Bacillus circulans DMS-2-derived fengycin shows cytotoxic activity against cancer cells [11]. Also, Duarte et al. proved that fengycin could inhibit breast cancer cell proliferation, which made fengycin to be a potential bio-surfactant for breast cancer treatment [12]. Ma et al. proved that anteiso-C17 fengycin B could inhibit human leukemia-60 cell growth [22]. In agreement with previous studies, our results showed that HT29 cell proliferation was statistically inhibited by fengycin at 72 h of incubation, and this inhibit effect was more apparent with high dose of fengycin (Figure 1), implying that fengycin may play some inhibit roles on colon cancer cell growth.

Meanwhile, our data displayed that fengycin could induce HT29 cell cycle arrest at G0/G1 stage (Figure 4). In previous reports, DNA damage can arrest cell cycle and activate cell apoptosis [23, 24]. CDK4 is an important member of CDK family proteins that function in regulating cell cycle at G1 phase progression [25] while cyclin D1 is a cell cycle-related molecule at G1 phase and is associated with tumor susceptibility [26]. Cell cycle arrest in colon cancer was induced through down-regulation of cyclin D1/CDK4 complex [27]. Our results showed that fengycin application significantly decreased the protein and mRNA levels of CDK4 and cyclin D1, indicating that HT29 cells may be arrested in G1 phase by fengycin through cyclin-CDK4 mediated cell cycle.

Moreover, our results showed that high dose of fengycin statistically increased ROS level in HT29 cells, as well as on cell apoptosis compared to the controls (Figure 2 and 3). Studies have demonstrated that cell apoptosis was accompanied with lots of morphological changes such as DNA fragmentation, cell surface changes and nuclear condensation [28, 29]. ROS is a kind of normal metabolism or harmful xenobiotic exposure product [30], and various ROS induces cell damage or plays key roles in regulating cell apoptosis during its activation [31]. Roles of fengycin in colon cancer cell apoptosis
have been not been fully reported. However, reduction of ROS protected gastrointestinal epithelium from radiation-induced damage [32]. Bufalin induces autophagy-mediated colon cancer cell apoptosis via ROS generation [33]. Also, high dose of fengycin could induce lung cancer 95D cell apoptosis through increasing ROS generation [9]. Based on our results, we speculated that high dose of fengycin may inhibit colon cancer progression by inducing cell apoptosis through ROS generation. On the other hand, Bcl-2 and Bax are cell apoptosis-related proteins that performed verses expressions in cells [34]. Falling up of intracellular CytC is related to cell apoptosis activation [35]. In this study, fengycin decreased Bcl-2 but increased Bax protein and mRNA levels in HT29 cells with time increasing. Also, Caspase-3 and Caspase-6 are two Caspase family proteins that are related to cell apoptosis [36], and Caspase-3 has been considered as the apoptosis protein in the downstream cell apoptosis [37]. Interestingly, we found that expressions of cell apoptosis-related proteins in fengycin-treated HT29 cells could be suppressed by addition of NAC, a ROS inhibitor [19]. Richards et al. proved that accumulation of DNA damage in fibroblasts was caused by ROS generation but can be prevented by NAC [38]. Therefore, we speculated that fengycin could inhibit colon cell progression through inducing cell apoptosis through the Bcl-2/Bax pathway-mediated by Caspase activity.

In conclusion, our study suggests that fengycin may play certain inhibit effects on the development and progression of colon cancer through involving in cell apoptosis process by targeting Bcl-2/Bax pathway. Fengycin may inhibit the development and progression of colon tumor through inhibiting cell proliferation, inducing cell apoptosis, increasing intracellular ROS generation, and inducing cell cycle arrest at G1 phase. Our study may provide theoretical basis for application of fengycin on colon treatment in clinical. However, comprehensive experimental studies are still needed to explore the molecular mechanism of fengycin on colon cancer in the near future.

References


