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Comparative analysis of methicillin-sensitive and resistant *Staphylococcus aureus* exposed to emodin based on proteomic profiling

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ABSTRACT

Emodin has a strong antibacterial activity, including methicillin-resistant *Staphylococcus aureus* (MRSA). However, the mechanism by which emodin induces growth inhibition against MRSA remains unclear. In this study, the isobaric tags for relative and absolute quantitation (iTRAQ) proteomics approach was used to investigate the modes of action of emodin on a MRSA isolate and methicillin-sensitive *S. aureus* ATCC29213(MSSA). Proteomic analysis showed that expression levels of 145 and 122 proteins were changed significantly in MRSA and MSSA, respectively, after emodin treatment. Comparative analysis of the functions of differentially expressed proteins between the two strains was performed via bioinformatics tools blast2go and STRING database. Proteins related to pyruvate pathway imbalance induction, protein synthesis inhibition, and DNA synthesis suppression were found in both methicillin-sensitive and resistant strains. Moreover, Interference proteins related to membrane damage mechanism were also observed in MRSA. Our findings indicate that emodin is a potential antibacterial agent targeting MRSA via multiple mechanisms.

1. Introduction

*Staphylococcus aureus*is an important pathogen responsible for a broad range of diseases [1]. Unfortunately, *S. aureus* has developed resistance to methicillin [2,3]. Moreover, vancomycin which had once been called the last line of defense against infection by *S. aureus* also fail to control some *S. aureus* strains [4]. With high virulence and antibiotics-resistance *S. aureus* is a serious health threat. Therefore, it is urgent to develop novel drugs against methicillin-resistant *S. aureus* (MRSA).

Emodin, 3-methyl-1,6,8-trihydroxy-anthraquinone, synthesized by a wide variety of plants has antimicrobial [5], anti-cancer [6], and anti-inflammatory [7] activity. Antibacterial activity of emodin against MRSA has been reported recently and antibacterial mechanisms interfering with cell wall and membrane synthesis have been proposed [8–10]. A comprehensive proteomic analysis of methicillin sensitive and resistant *S. aureus* exposed to emodin will facilliate the understanding of its antibacterial mechanism.

In the present study, we used minimal inhibitory approach to study the antibacterial activity of emodin against *S. aureus* and we found that emodin has a remarkable effect on the growth of MSSA and MRSA. We compared the functions of the significant changes in two proteomics after stimulated by emodin. Interestingly, the proteins associated with antibacterial mechanisms of protein synthesis inhibition, interference on pyruvate pathway, the DNA synthesis, and membrane structure were revealed. Multiple target mechanism in MRSA may be involved after emodin treatment.

2. Materials and methods

2.1. Bacterial strains and cultivation

The methicillin-resistant *S. aureus* strain (numbered as

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MRSA133630) was isolated from a patient at Sun Yat-sen Memorial Hospital, Sun Yat-sen University, China and identified by using VITEK®2 Compact (bioMérieux, France). The methicillin-sensitive strain *S. aureus* ATCC 29213 from American type culture collection was included as reference strain. The strains were cultured in Mueller Hinton (MH) medium (Sigma-Aldrich, America) with or without antibacterial agents at 37 °C, 200 rpm. Cell suspension (about 1×10^6 cfu/mL) of *S. aureus* in mid-exponential phase was used as standard inoculum material.

2.2. In vitro susceptibility testing

The inhibitory activity of emodin against *S. aureus* strains were determined by standard microdilution method as described by Chinese Medicine [11]. Serial two-fold dilutions of emodin ranging from 150 μg/mL to 0.5 μg/mL were prepared by diluting the stock solution (in 100% DMSO) with broth MH medium in 96-well microtitre plate (100 μL per well). An equal volume of *S. aureus* cell suspension was added and incubated for 18 h at 37 °C. Cultures treated with 1% DMSO and vancomycin were used as negative and positive control, respectively. The minimal inhibitory concentrations (MICs) were determined as the lowest concentration that completely inhibit the bacterial growth. All the experiments were done in triplicate.

2.3. Growth curve of MRSA and MSSA exposed to emodin

To obtain the growth curve of *S. aureus* at the presence of emodin, 100 mL of MH medium supplemented with emodin at 0.25 × MIC were inoculated with 1% volume of cell suspension mentioned above and cultured at 37 °C with constant shaking. After the incubation for 5 h, the Optical Density (OD) was measured every 30 min at 595 nm using a spectrophotometer GeneQuant pro (Amersham, UK) and the growth curve was drawn. *S. aureus* grown in MH broth without emodin were used as controls. The inhibition of emodin was calculated as the percentage of maximum growth under two conditions using the following formula: (OD_{595} at 18 h with emodin/OD_{595} at 18 h without emodin) × 100%. All experiments were done in triplicate.

2.4. Protein sample preparation and labeling

Cell suspensions of MRSA and MSSA were inoculated into fresh MH medium at 1% volume, respectively, and allowed to grow to the exponential phase (corresponding to an OD_{595} of approximately 0.5). Then the cultures were added with emodin at the concentrations of 0.25 × MIC and grew further for 5 h. Cultures treated with 1% DMSO were used as the control group. Each group was in duplicate. For convenience, the abbreviations MRSA-T and MRSA-C refer to the groups of MRSA with and without treatment of emodin, respectively, while MSSA-T and MSSA-C stand for those of MSSA in the subsequent text.

Cells were harvested by centrifugation at 12,500 *g* for 5 min and washed twice with phosphate buffer saline (PBS, pH 7.3). The pellets were stored at −80 °C until further analysis. Every experiment was done twice. The cell pellets were lysed with lysis buffer 8 M urea, 4% CHAPS, 40 mM Tris-HCL, 5 mM EDTA containing protein inhibitor cocktail (Sigma-Aldrich, USA) and nuclease mix (Roche, Germany). Then pellets were sonicated three times on ice using a high-intensity ultrasonic processor. The debris and unbroken cells were removed by centrifugation at 25,000 *g* at 4 °C for 15 min. The supernatant was transferred to a new tube, reduced with 10 mM dithiothreitol for 1 h at 37 °C followed by alkylation with 55 mM iodoacetamide for 45 min at room temperature in darkness. The proteins were precipitated with 4-vol of pre-chilled acetone for 30 min at −20 °C. After centrifugation, the pellet was dissolved in 0.5 M TEAB and sonicated for 5 min. Protein concentration was determined with 2-DE Quant kit (Bio-Rad, USA) according to the manufacturer's instruction. Approximately 100 μg protein of each sample was digested with mass spectrometry grade trypsin (Promega, Madison, WI) for overnight at 37 °C. Peptides were labeled with 8-plex iTRAQ kit (Applied Biosystems Instruments, USA) according to the manufacturer's instruction. Briefly, one unit of iTRAQ reagent (defined as the amount of reagent required to label 100 μg of protein) was reconstituted in 70 μl isopropanol and used to label one sample by incubation at room temperature for 2 h. Peptides from treatment groups and control groups were labeled with different iTRAQ tags. The labeled peptide mixtures from all samples were pooled and dried under vacuum.

2.5. Fractionation by strong cationic exchange (SCX) chromatography

The iTRAQ-labeled peptide mixtures were reconstituted with buffer A (25 mM NaH_2PO_4 in 25% acetonitrile, pH3.0) and fractionated using a LC-20AB HPLC Pump system (Shimadzu, Japan) coupled with a PolySULFOETHYL ATM SCX column (200 × 4.6 mm, 5 μm, Phenomenex). The flow rate was set at 1.0 mL/min and a gradient was used: buffer A for 10 min, 5–35% buffer B (25 mM NaH_2PO_4, 1 M KCl in 25% acetonitrile, pH 3.0) for 11 min, 35–80% buffer B for 1 min. The system was then preserved at 80% buffer B for 3 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was detected by UV at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled as 10–12 fractions, desalted with Strata X C18 column (Phenomenex) and vacuum-dried.

2.6. LC-MS/MS analysis

Each fraction was reconstituted with 20 μl of buffer A (5% acetonitrile, 0.1% formic acid) and centrifuged at 20,000*g* for 10 min. 8 μl of supernatant was loaded on an Eksigent Nano-Ultra System by the autosampler into a C_{18} trap column and the peptides were eluted onto an analytical C18 column (75 μm × 150 mm, Eksigent). Chromatographic separation was achieved using the mobile phases consisting of A (5% acetonitrile, 0.1% fomic acid in water) and B (95% acetonitrile, 0.1% fomic acid) at a flow rate of 0.3 μL/min in a gradient elution: 1 min, 2% B; 45 min, 35% B; 50 min, 80% B; 54min, 80% B; 55 min 5% B.

The mass spectroscopy analysis was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Mass spectroscopy data was acquired using an ion spray voltage of 2.2 kV, curtain gas of 20 psi, nebulizer gas of 6 psi, and an interface heater temperature of 150 °C. The MS was operated with a resolution greater than or equal to 30,000 FWHM for TOF MS scans. Smart information-dependent acquisition (IDA) was activated with survey scans in 250 ms and as many as 8, 20, or 50 product ion scans were collected if exceeding a threshold of 125 counts per second (counts/s) and with a 2 + to 5+ charge-state. Total cycle time was 3.3 s. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. Sweeping collision energy set at 35 ± 15 eV was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set at 8s for 1/2 of peak width and the *m*/*z* scan range was 350–2000 Da.