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Shikonin alleviates the biotoxicity produced by pneumococcal pneumolysin

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ABSTRACT

Aims: Streptococcus pneumoniae (*S. pneumoniae*) is a common pathogen that can cause severe infections in humans. Pneumolysin (PLY) is an important virulence trait of *S. pneumoniae* and has cytotoxicity, genotoxicity and pro-inflammatory activity; it is essential for the pathogenesis of *S. pneumoniae* pneumonia and is an anti-virulence target of small molecule drug development. The treatment options for this microbe were limit due to the ubiquitous antibiotic resistance; therefore, new drugs and treatment strategies are needed.
Methods: Shikonin was selected by drug screening based on haemolysis assays, and its mechanism of suppressing PLY toxicity was determined by oligomerization assay. Meanwhile, the *in vitro* cell viability assays and *in vivo* experiments were performed to explore the capability of shikonin to protect cells and tissue from *S. pneumoniae*-mediated damage.
Key findings: Shikonin was found to significantly decrease PLY-induced haemolytic activity, cytotoxicity and genotoxicity *via* lessening the formation of oligomers; moreover, the agent can reduce the mortality of mice caused by lethal pneumonia and mitigate the injury of target organs as well.
Significance: We suggest that shikonin could be a potent candidate for a novel therapeutic or auxiliary substance in the treatment of infections encountering insufficient vaccines and antimicrobial resistance to traditional antibiotics.

1. Introduction

S. pneumoniae is a commensal of the human nasopharynx and a common aetiological agent of several diseases, such as bacterial meningitis, otitis media, and conjunctivitis [1,2], and it constitutes the most frequently detected pathogen in cases of community-acquired pneumonia (CAP) [3]. Bacterial pneumonia is known to be a major worldwide cause of death for children under the age of 5 years [4]. The development of penicillin resistance in pneumococcus was variably reported, from 14.9% to 25.7% [5], and turned the antibacterial drug therapy of pneumococcus related respiratory infections and pneumonia to macrolides [6]. However, the universal use of macrolide antibiotics is closely correlated with expanded macrolide resistance in *S. pneumoniae* [7]. Meanwhile, in Iran, the emergence and identification of *S. pneumoniae* strains that are resistant to ceftazidime with suppressed vancomycin susceptibility prompted caution from health authorities globally [8]. The extensive use of antibiotics artificially enhanced the effect of multidrug-resistant bacteria screening.

Many virulence traits of *S. pneumoniae* contribute to its pathogenicity. This pathogenic bacteria create haemolysin, which belongs to the family of cholesterol-dependent cytolysin (CDC) and is commonly referred to as pneumolysin (PLY) [9]. All currently known *S. pneumoniae* clinical isolates produce PLY, a 53-kDa protein without a signal peptide, and cytolysin, which is known to be released during enzyme-mediated autolysis [10], competence-induced lysis [11] and antibiotic-mediated lysis of pneumococci [12]. Indeed, purified PLY or an *S. pneumoniae* strain can induce apoptosis, edema and lung injury [13,14]. PLY causes the release of pro-inflammatory mediators [15], resulting in the discharging of reactive oxygen and nitrogen species that induce host tissue damage [16]. Furthermore, in a previous study, PLY was found to damage DNA and induce cell cycle arrest [17]. PLY is a crucial virulence factor of *S. pneumoniae*, plays an important role in the pathogenesis and is a potential therapeutic target candidate.

The exploration of an anti-virulence strategy could reduce or even disarm the pathogenicity of essential virulence traits in pathogens rather than kill them directly and brutally [18]. Although the traditional approach of using antibiotics is highly effective in treating bacterial

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infections, it exerts substantial pressure on the target bacterium and rapidly selects resistant subpopulations [19].

Shikonin, a naphthoquinone pigment, is one of the active components isolated from the roots of the traditional Chinese herb *Lithospermum erythrorhizon* or from the medicinal plant *Arnebia euchroma* (Royle) Johnst, also known as Zicao in China [20]. In the Chinese medicine system, Zicao has a long history of use as a traditional Chinese herbal medicine used to clinically treat purpura, dehydration, erysipelas, constipation, burns, haematuria, and more [21]. The active ingredient of Zicao, shikonin, has been shown to possess significant anti-inflammatory [22], anti-tumour [23] and anti-allergic activities [24]; additionally, shikonin has also been used in wound healing applications with fairly good results [20].

Our previous studies have shown certain compounds competitively bind to the PLY cholesterol binding site or to the PLY monomer to inhibit oligomer formation, thereby reducing protein toxicity and alleviating tissue damage caused by streptococcal pneumonia [14,25]. Here, for the first time, we have used shikonin as an inhibitor of *S. pneumoniae* PLY to prevent its cytotoxicity and genotoxicity and confirmed the mitigation effect of *S. pneumoniae*-infected pneumonia.

2. Materials and methods

2.1. Regents

Shikonin (98% purity) was bought from Herbpurify (CAS number 517–89-5, Chengdu, China). Phosphate buffer saline (PBS) powder was purchased from Boster (Wuhan, China). Dimethyl sulfoxide (DMSO) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Alveolar epidermal cells (A549) were obtained from ATCC (Manassas, VA). Dulbecco's modified Eagle medium (DMEM) and foetal calf serum (Invitrogen, CA, USA) were used for the procedures carried out in this study.

2.2. Microorganism and cell culture

S. pneumoniae strain D39 serotype 2 (NCTC 7466) was obtained from a prior study [26] and cultured in a 37 °C shaker using Todd-Hewitt broth (THB). A549 human lung epithelial cells were propagated in DMEM/high glucose containing 10% foetal calf serum at 37 °C in a condition of 5% CO_2. The cell culture fluid replacement was performed every day.

2.3. Recombinant pneumolysin

Construction of a pET28a vector containing the *ply* sequence and protein purification methods were based on previous depictions [14]. Briefly, the harvested cells were lysed by sonication, and the supernatant of centrifuged cell lysate was loaded onto a Ni-NTA agarose column. The recombinant protein was then rinsed with washing buffer (PBS consisting of 20 mM imidazole, pH 7.4). The target protein was flushed with elution buffer (PBS containing 200 mM imidazole, pH 7.4). Then the recombinant protein was concentrated using a Millipore Amicon filter (30 kDa cutoff) for desalting.

2.4. Haemolysis assay

In our study, the methods of evaluating haemolytic activity were referred to in a previous article [27]. In short, 1.0 μl of purified PLY (0.2 mg/ml) was mixed with 974 μl of PBS. The reaction mixtures were incubated at 37 °C for 10 min, with or without the addition of shikonin. Then, defibrinated rabbit erythrocytes were added to a final concentration of 2.5% to each tube, followed by another incubation at 37 °C for 10 min. After centrifugation (10,000 g; 1 min), the supernatants were placed into a quartz colorimetric utensil, and the absorption at 543 nm was measured for each sample by a spectrophotometer. Only PBS was added to the negative group, and 0.2% Triton X-100 was used for 100% lysis as the positive control.

2.5. Minimal inhibitory concentrations (MICs) determination

The MIC of shikonin for the *S. pneumoniae* strain D39 was measured using the broth microdilution method. Refer to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The method was previously described [28]. In short, shikonin was diluted over the concentrations range of 1–1024 μg/ml using double dilution method, and then 5×10^5 cfu were inoculated in each tube. The co-cultured samples were placed at 37 °C for 12 h. The MIC was defined as the lowest concentration at which the growth of *S. pneumoniae* was suppressed.

2.6. Oligomerization analysis

Shikonin was dissolved in DMSO, and the recombinant PLY (rPLY, in PBS) incubated with a series of shikonin concentrations (0.125–1 μg/ml) were cultured at 37 °C for 1 h in an incubator. A group without shikonin served as a control. Each specimen included 1 μl of rPLY, 1 μl of shikonin solution or pure DMSO, and 28 μl of PBS. Equal volume samples were incubated at 50 °C for 10 min in Laemmli sample buffer. Afterwards, the processed specimens were loaded onto a 6% SDS-PAGE gel and transferred to the polyvinylidene fluoride (PVDF) membrane through a half-dry carrier cell. The membrane was then clipped and blocked with 5% bovine serum albumin (BSA) at 25 °C for 2 h. The PVDF membrane was incubated with an anti-pneumolysin antibody (Abcam #ab71810) at a dilution ratio of 1:500 overnight at 4 °C. Rabbit Anti-Mouse IgG H&L (HRP) (Abcam #ab97046) was used at a 1:1000 dilution for 2 h at 25 °C. A chemiluminescence (ECL) plus kit (Beyotime # P0018) was employed to develop the blots. The quantitative analysis of the band shifts were estimated by software ImageJ.

2.7. Live/dead testing

A549 cells were digested with trypsin, transferred into a 96-well plate at 2.0×10^4 cells per well and incubated overnight for approximately 12 h. The A549 cells were combined with different concentrations of shikonin and PLY (3.0 μl per well) and treated in isolation. All the samples and the positive control containing DMEM were placed in a temperature-controlled incubator for 6 h at 37 °C. Cell viability was observed using live/dead (green/red) reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's user guide. Sample visualization and image acquisition were performed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

2.8. Lactate dehydrogenase (LDH) release analysis

The cells were delivered into a 96-well plate, wherein each well can deliver 2.0×10^4 cells. The A549 cell was subjected to isolated incubation under assistance of PLY and increasing concentrations of shikonin. The test samples, negative controls (DMEM) as well as positive controls (0.3% Triton X-100) were placed in a temperature-controlled box for 6 h at 37 °C. The 96-well plate was centrifuged (1000 rpm, 10 min), and the supernatants were mixed with the reagents (Cytotoxicity Detection Kit, Roche, Mannheim, Germany). After 30 min dark reaction, the values of optical density (OD) were measured with a microplate reader at 490 nm (TECAN, Salzburg, Austria). LDH release % = $(OD_{sample} - OD_{Neg.}) / (OD_{Pos.} - OD_{Neg.}) \times 100\%$.

2.9. Immunofluorescence

After the cells were digested, one cell climbing piece was added to each well and 2×10^5 cells were seeded in each well; the cells were allowed to proliferate for 24 h. Culture medium, DMSO, PLY (0.5 μg/ml) and shikonin (4 μg/ml or 8 μg/ml) were added respectively to the