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Inhibition of sortase A by chalcone prevents *Listeria monocytogenes* infection

Hongen Li [a,1], Yutao Chen [b,1], Bing Zhang [a,1], Xiaodi Niu [a], Meng Song [a], Zhaoqing Luo [c], Gejin Lu [a], Bowen Liu [a], Xiaoran Zhao [a], Jianfeng Wang [a,*], Xuming Deng [a,*]

[a] Key Laboratory of Zoonosis, Ministry of Education, Department of Food Quality and Safety, College of Veterinary Medicine, Jilin University, Changchun, China
[b] National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
[c] Department of Biological Sciences, Purdue University, West Lafayette, IN, United States

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ABSTRACT

The critical role of sortase A in gram-positive bacterial pathogenicity makes this protein a good potential target for antimicrobial therapy. In this study, we report for the first time the crystal structure of *Listeria monocytogenes* sortase A and identify the active sites that mediate its transpeptidase activity. We also used a sortase A (SrtA) enzyme activity inhibition assay, simulation, and isothermal titration calorimetry analysis to discover that chalcone, an agent with little anti-*L. monocytogenes* activity, could significantly inhibit sortase A activity with an IC_{50} of 28.41 ± 5.34 μM by occupying the active site of SrtA. The addition of chalcone to a co-culture of *L. monocytogenes* and Caco-2 cells significantly inhibited bacterial entry into the cells and *L. monocytogenes*-mediated cytotoxicity. Additionally, chalcone treatment decreased the mortality of infected mice, the bacterial burden in target organs, and the pathological damage to *L. monocytogenes*-infected mice. In conclusion, these findings suggest that chalcone is a promising candidate for the development of treatment against *L. monocytogenes* infection.

1. Introduction

The food-borne pathogen *Listeria monocytogenes* (*L. monocytogenes*), a gram-positive facultative intracellular bacterium, is the causative agent of listeriosis, which is characterized by gastroenteritis, meningitis, encephalitis, bacteremia, soft tissue and parenchymal infections, and mother-to-fetus transmission [1,2]. Upon the ingestion of contaminated foods by the host, the invasion of *L. monocytogenes* into host cells enables the bacteria to evade the humoral immune system, which is a critical step in the establishment of the infection [2]. During infection, *L. monocytogenes* has evolved elaborate molecular strategies to enter, survive, and multiply inside both professional and non-professional phagocytic cells, thus complicating the treatment of *L. monocytogenes* infections [3]. Previous studies have shown that different types of cell wall anchored proteins (internalin A, internalin B, VIP, etc.) and secreted pore-forming cytolysins (listeriolysin) are required for cell entry, suggesting that this process is quite complex [3–5]. Thus, interference with the surface proteins involved in the infection process would be a promising direction for controlling *L. monocytogenes* infections.

Many surface proteins in gram-positive bacteria are anchored to the cell wall envelope by the transpeptidase enzyme sortase A (SrtA, encoded by *srtA*), which recognizes the conserved LPXTG motif, where X is any amino acid [6]. After a protein precursor is targeted for translocation across the membrane by its N-terminal secretion signal, the intermediate is covalently linked to the cell wall by the attack of an amine nucleophile, and then SrtA cleaves the protein precursor on its LPXTG motif between the threonine (T) and glycine (G) residues [7]. The thiolate group of the essential active site Cys in SrtA then attacks the scissile Thr-Gly bond on the precursor protein. Two additional absolutely conserved residues, His127 and Arg197, are also located in the active pocket of the transpeptidase protein [8–10]. Sortases have been well characterized as ideal targets of anti-infective drug development, because many of the surface proteins they display are virulence factors required for infection [11]. Mazmanian's group showed that *Staphylococcus aureus* SrtA mutants are defective in anchoring surface proteins and in the pathogenesis of animal infections [12]. It was also shown recently that the virulence of *Streptococcus pneumoniae* [13] and *Bacillus anthracis* [14] SrtA mutants was reduced by infection of tissue culture cells. In *L. monocytogenes*, the inactivation of

* Corresponding authors.
E-mail addresses: wjf927@jlu.edu.cn (J. Wang), dengxm@jlu.edu.cn (X. Deng).
[1] These authors contributed equally to this work.

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SrtA did not affect the expression of LPXTG-containing proteins, but SrtA mutation inhibited the anchorage of these proteins, which affected bacterial virulence [15]. In their recent study, Zhang et al. [16] and Huang et al. [17] showed that SrtA inhibitors could provide robust protection against *S. aureus* or *Streptococcus mutans* infections. However, to our knowledge, no study examining the implications of *L. monocytogenes* SrtA inhibition has been reported.

Natural products are a major source of chemical and functional diversity and have provided a variety of therapeutic agents with bacteriostatic, bactericidal, or anti-virulence factor activity against many bacterial infections [18]. In this study, we have identified chalcone, a precursor molecule of many flavonoids that is found in traditional Chinese medicine, as a potential inhibitor of *L. monocytogenes* SrtA. Chalcone and its derivatives have been reported to exert a broad spectrum of pharmacological activities including anti-malarial [19], anti-cancer, immunomodulatory, antiviral, and antimicrobial properties [20].

Based on the above description, we systematically evaluated the inhibitory effects of chalcone on *L. monocytogenes* virulence in vitro and in vivo. Furthermore, we simulated the interaction between the SrtA active site and its peptide substrate, LPTTG. We verified the binding site residues through which chalcone interacts with SrtA and the mechanism by which chalcone inhibits sortase A. Taken together, the data in the present study suggest that chalcone provides robust protection against *L. monocytogenes* infection by blocking the active site of SrtA and that chalcone is a promising candidate for the development of an anti-infective drug against listeriosis.

2. Materials and methods

2.1. Bacterial growth and reagents

Chalcone was purchased from company of Tianjin Yifang S&T Co., Ltd (Tianjin, China). *L. monocytogenes* BUG 1600 and its SrtA mutant BUG 1777 were kindly provided by Dr. Pascale Cossart (Institut Pasteur, Paris, France). Bacteria were cultured at 37 °C in Trypticase Soy Broth (TSB, Qingdao Hope Biol-Technology Co., Ltd) with or without the indicated concentrations of chalcone.

2.2. SrtA cloning, expression, and purification

The DNA sequence encoding SrtA residues Ala71 to Lys221 ($SrtA_{\Delta N70}$) was amplified from *L. monocytogenes* BUG1600 genomic DNA using the primers 5′-CTTAGGATCCGGAGCGGCAAATTACGA CAAAG-3′ (forward) and 5′-ATGTTCTCGAGTCTTTTACTGGGAAA TATTT-3′ (reverse). The amplified fragment was digested with *BamH*I and *Xho*I and cloned into a pGEX-6P-1 expression vector. The recombinant vector with an N-terminal GST was transformed into *Escherichia coli* BL21 (DE3) cells for the overexpression of the recombinant protein.

Cultures of BL21 (DE3) cells harboring the $SrtA_{\Delta N70}$-pGEX-6P-1 vector were grown in 1 L of Luria–Bertani (LB) media supplemented with ampicillin (100 mg/L) (Sigma) at 37 °C with shaking until the absorbance reached an A_{600} between 0.6 and 0.8. IPTG (Sigma) was then added to a final concentration of 0.5 mM, and the culture was grown for a further 4 h at 37 °C prior to harvesting. Cell pellets were resuspended in phosphate buffered saline (PBS) and lysed by sonication. The lysed cells were centrifuged at 40,555*g* at 4 °C for 30 min. The cell lysate was mixed for 1 h with glutathione Sepharose beads (GE Healthcare) that were pre-equilibrated with PBS. The unbound proteins were removed with PBS after loading onto the column. The eluted GST-$SrtA_{\Delta N70}$ was mixed with PreScission protease at a ratio of 50:1 (w:w) in buffer A (25 mM HEPES, 100 mM NaCl, pH 7.0) and incubated at 4 °C overnight to cleave off the GST tag. The *L. monocytogenes* $SrtA_{\Delta N70}$ was further purified by ion exchange chromatography with a Resource Q column (GE Healthcare). The hybrid protein that was bound to the column and the aim protein were collected, and both were shown by SDS–PAGE to contain $SrtA_{\Delta N70}$. The target protein was concentrated before being loaded onto a Superdex 75 gel filtration column (GE Healthcare) pre-equilibrated with buffer A. The fractions containing only $SrtA_{\Delta N70}$ were pooled and concentrated to a final concentration of 12 mg/mL and stored at −20 °C.

The expression and purification protocols for SrtA H127A, SrtA R197A, and SrtA C188A were identical to that of the wild type protein ($SrtA_{\Delta N70}$).

2.3. Protein crystallization and structure determination

Crystals of $SrtA_{\Delta N70}$ were grown at 16 °C using the hanging drop vapor diffusion method over a reservoir of 22% w/v PEG 2000, 0.1 mol/L Tris–HCl (pH 8.5), and 0.01 mol/L $Ni_2SO_4{\cdot}6H_2O$. Crystals were grown to full size within 5 days.

Cryo-cooling was performed by soaking the crystals in a reservoir solution containing 20% (v/v) glycerol prior to flash-cooling with liquid nitrogen. All of the diffraction data sets were collected with an in-house X-ray MM007HF detector CCD 944HG with a maximum resolution of ~2.3 Å. The space group of $SrtA_{\Delta N70}$ crystals is $P2_12_12_1$, with one protein molecule per asymmetric unit (ASU). The initial phases were obtained by the molecular replacement method with the Phaser v2.1 program [21], and the *Streptococcus pyogenes* sortase $A_{\Delta N81}$ crystal structure was used as the search model (Protein Data Bank (PDB) ID: 3FN5). Manual model building and refinement were performed with COOT [22] and PHENIX [23] using rigid body refinement, TLS parameters, and individual B-factor refinement. Solvent molecules were located based on stereochemically reasonable peaks in the σ_A-weighted 2Fo–Fc difference electron density map. The quality of the final refined model

Table 1
Data collection and refinement statistics.

Name	LM-$SrtA_{\Delta N70}$ (PDB: 5HU4)
Data collection	
Resolution (Å)	50.0–2.30 (2.38–2.30)
Space group	$P2_12_12_1$
Cell dimensions	
a (Å)	37.0
b (Å)	56.6
c (Å)	60.5
Redundancy	6.7 (6.5)
Completeness (%)	99.8 (99.7)
R_{merge}[a]	0.088 (0.304)
$I/\sigma(I)$	48.9 (4.3)
Refinement	18.45 (6.89)
Resolution (Å)	22.475–2.289
No. reflections	6010
R_{work}/R_{free}[b,c]	0.234/0.275
B-factors (Å^2)	
Protein	27.3
Water	28.9
Ramachandran statistics (%)	
Most favored	97.2
Allowed	2.8
Outliers	0
R.M.S. deviations	
Bond lengths (Å)	0.002
Bond angles (°)	0.642

Values in parentheses are for highest-resolution shell.
[a] $R_{merge} = \Sigma_{hkl}\Sigma_i|I(hkl)_i - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I(hkl)_i$.
[b] $R_{work} = \Sigma_{hkl}|Fo(hkl) - Fc(hkl)|/\Sigma_{hkl}Fo(hkl)$.
[c] R_{free} was calculated for a test set of reflections (5%) omitted from the refinement.