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Fusaric acid modulates Type Three Secretion System of *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Natural small-molecule products are promising lead compounds for developing a generation of novel antimicrobials agents to meet the challenge of antibiotic-resistant pathogens. To facilitate the search for novel anti-virulence agents, we chose a virulence factor of Type Three Secretion System (T3SS) as a drug target to screen candidates from a small-molecule library in our laboratory. This study demonstrated fusaric acid had dramatically inhibitory effects on secretion of *Salmonella* island 1 (SPI-1) effector proteins and invasion of *Salmonella* into HeLa cells. Moreover, fusaric acid had no inhibitory effects on bacterial growth and viability of host cells. Protein HilA is a key regulator of SPI-1 in *Salmonella*, which affects transcription of SPI-1 effectors and SPI-1 apparatus genes. In this study, fusaric acid (FA) did not affect secretion of SPI-1 effectors in HilA over-expressed strain, suggesting it did not affect the transcription of SPI-1. In addition, fusaric acid did not affect the protein level of apparatus protein PrgH in SPI-1 needle complex. As a result, we proposed fusaric acid had an inhibitory effect on SPI-1 probably depending on its influence on SicA/InvF. In summary, fusaric acid is a novel inhibitor of T3SS with potential for further developing novel anti-virulence agents.

1. Introduction

Salmonella enterica, an important pathogen, has been a serious threat for public health, as antibiotic-resistant strains are constantly emerging in the worldwide [1]. It can cause diseases from mild gastroenteritis to severe systemic infection for human and animals [1]. Therefore, it is urgent to develop alternative therapies to cope with the existing anxiety-provoking situation. The pathogenicity of *S. enterica* mainly depends on two *S*almonella pathogenicity islands, SPI-1 and SPI-2, which encoded two different machineries in T3SS (Type Three Secretion System) [2]. SPI-1 plays a great role on invasion of *Salmonella* into the intestinal epithelium by injecting effector proteins into host cells, while SPI-2 modulated the replication of bacteria in host phagocytic cells [2].

T3SS has a syringe-like secretion apparatus, which is structurally and functionally conserved among different gram-negative pathogens [3]. It is often absent in nonpathogenic bacteria and not essential for bacterial growth [4]. Thus inhibitors of T3SS could have no inhibitory effect on bacterial growth, different from conventional antibiotics with killing effects on pathogens. The applications of T3SS inhibitors as antibiotics in clinic would be dramatically reduced the possibility of occurrence of resistant bacteria [5]. Therefore, T3SS now has increasingly been an attractive drug target for developing novel anti-virulence agents to inhibit gram-negative pathogens.

Previously, we made use of SDS–PAGE and Western blotting to assess the effects of natural products on the secretion of SPI-1 effector proteins in vitro. We found that the fungal polyketide cytosporone B (Csn-B) isolated from the mangrove endophytic fungus *Dothiorella* sp. Strain significantly inhibited the secretion of SPI-1 effectors and the invasion of *Salmonella* into HeLa cells [6]. In this study, mycotoxin fusaric acid (FA) was identified to be a strong inhibitor of SPI-1 and its underlying mechanism of action on SPI-1 was investigated and discussed.

2. Material and methods

2.1. Bacterial strains and growth conditions

S. enterica serovar Typhimurium UK-1 χ8956 was used in this study [7], which was grown in Luria–Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) or on LB agar plates supplemented with 0.2% L-arabinose at 37 °C or 25 °C. *S. enterica* serovar Typhimurium UK-1 χ8956 carrying the vector of

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pBAD-*hilA*-pWSK29 [6] was cultured in LB broth supplemented with L-arabinose (0.02%) and ampicillin (100 μg/ml) to induce the over-expression of HilA.

2.2. Preparation of fusaric acid stock solution

Fusaric acid was separated from the endophytic fungal strain F0182 which collected from the roots of masson pine in Tong-An city of Fujian Province, China. The strain was deposited in the laboratory of Microbial Pharmaceutical Engineering Center, Xiamen University, China. The structure of fusaric acid (FA) was identified by NMR data (Table S1) and confirmed by comparing to literature [8]. FA was dissolved in dimethyl sulfoxide (DMSO, Sigma) at 100 mM as stock solution. FA was used for biological activity at different concentration as indicated. The same volume of DMSO was as a negative control in all experiments.

2.3. Isolation and detection of secreted proteins

Secreted proteins were isolated and detected as previously described by us [6]. In short, SPI-1 effectors were induced by temperature change from 25 °C to 37 °C with agitation with or without FA at the final concentration of 100 μM. The secreted proteins in the supernatant of bacterial culture were precipitated by 10% trichloroacetic acid (TCA) and washed by ice-cold acetone twice. Pellets were dissolved in appropriate volume of sample buffer and heated for 10 min at 95 °C to denature the proteins. The protein samples were subsequently detected by SDS–PAGE or Western blotting.

2.4. Measurement of bacterial growth

S. enterica serovar Typhimurium χ8956 was cultured overnight in LB medium (0.2% L-arabinose) at 37 °C. The culture was diluted 200 times and seeded 100 μl/well in 96 well plates supplemented with FA at the final concentration of 100 μM or the same volume of DMSO. The plates were incubated for 7 h at 37 °C. At the time points indicated, OD_{570} of the culture was measured by a Microplate Reader (Bio-Rad 680, USA). Three replicate samples were measured in each experiment.

2.5. MTT assay

The effect of FA on the growth rate of host cells were assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [9]. 4000–5000 HeLa cells were seeded in 96-well flat-bottomed tissue-culture plates in triplicates and incubated for 12 h in DMEM with 10% FBS, and then FA was added into cell culture medium at the final concentrations ranging from 20 to 100 μM. After 72 h of treatment, cells were washed with PBS and MTT solution (10 μl, 5 mg/ml in PBS, Sigma) was added to each well. Cells were incubated for 3 h at 37 °C, and then the supernatant were discarded. DMSO (200 μl, Sigma) was added to each well and incubated for 10 min at 37 °C. The absorbance at 570 nm was measured using a Microplate Reader (Bio-Rad 680, USA). All the experiments were conducted in triplicates and the data represented percent viability compared to solvent control.

2.6. Invasion assay

Gentamicin protection assay was performed to determine the effect of FA on the invasion of *S. enterica* serovar Typhimurium into host cells [6]. HeLa cells (2×10^4/well) were seeded in 24 wells plate (Costar® 24 well, flat bottom, tissue-culture treated, USA) and incubated for 12 h at 37 °C and 5% CO_2 in DMEM culture medium with 10% FBS. At the same time, *S. enterica* serovar Typhimurium cells were cultured overnight at 25 °C with agitation. The overnight culture was diluted 10-fold with different compounds at the final concentration of 100 μM and cultured for 1 h at 37 °C. HeLa cells were cultured for 30 min with DMEM without FBS, and then infected for 1 h with *S. enterica* serovar Typhimurium at a multiplicity of infection (MOI) of 10. The cells were washed three times with PBS and incubated for another 1 h in DMEM supplemented with 100 μg/ml gentamicin. After incubation, the HeLa cells were washed three times with PBS and lysed with 1% TritonX-100 solution. The colony forming units (CFUs) of the bacteria isolated from HeLa cells were counted by plating the appropriate dilution on LB agar plates with 0.2% L-arabinose. All experiments were carried out in triplicates.

2.7. Bacterial cells fractionation

S. enterica serovar Typhimurium cells were treated with fusaric acid according to the method described in 2.3 fraction. The bacterial cells were harvest by centrifugation for 5 min at 12,000*g*, and the supernatant was collected as the extracellular proteins. The pellet was resuspended and lysed by ultrasonication in the same volume of PBS. The cell lysate was separated into intracellular soluble proteins and cell debris by centrifugation for 5 min at 12,000*g*. When the bacterial culture was directly lysed by ultrasonication, the lysate supernatant was collected as total soluble proteins. The protein samples were analyzed by Western blotting to detect *Salmonella* Flagellar protein FliC, SPI-1 effector SipC or SPI-1 apparatus protein PrgH polyclonal antibodies.

2.8. Preparation of needle complex (NC)

NC were prepared as described [10] with minor modifications. *S. enterica* serovar Typhimurium cells were cultured as described previously. The culture was pelleted and resuspended in Solution I [0.5 M sucrose, 0.15 M Tris–HCl (pH 8.0), 0.5 mg/ml lysozyme and 5 mM EDTA] and then incubated at 4 °C with rocking for 1 h. Triton X-100 was then added into the resuspended culture at the final concentration of 1%, and the culture was incubated for 2 h at 4 °C with rocking. $MgSO_4$ was then added into lysate at 5 mM. Lysates were incubated for 30 min at 25 °C with agitation and centrifuged for 20 min at 10,000*g* to remove non-lysed cells and debris. The supernatants was adjusted to pH 10.5 using NaOH solution and then centrifuged for 1 h at 80,000*g*. The pellet was resuspended in SDS sample buffer and analyzed by Western blotting with SPI-1 apparatus protein PrgH polyclonal antibody.

2.9. Detection of transcriptional level of SicA promoter

To investigate the effect of FA on SicA/InvF transcription regulatory pathway, a plasmid containing the promoter region of sicA was constructed according to the previous report [11]. A 450 bp fragment containing the promoter region of sicA was amplified by PCR. The primer set used in this study were as follows: PsicA-F, 5′-CGCGGATCCCCTGATTGTTGCCAACCCCAC-3′ (BamHI) and PsicA-R, 5′-CCGGAATTCTACTTACTCCTGTTATCTGTCA-3′ (EcoRI). A broad-host-range plasmid pPROBE-AT carrying promoterless gfp [12] and the amplified fragment were digested with EcoRI and BamHI, purified by Gel Extraction Kit (OMEGA, GA, USA) and ligated by DNA Ligation Kit (TAKARA, Dalian, China) to construct plasmid pPROBE-AT-sicA. This plasmid was introduced into *S. enterica* serovar Typhimurium UK-1 χ8956 by electroporation. The bacteria carrying pPROBE-AT-sicA was then incubated overnight at 25 °C in LB (Amp^R) medium. The overnight culture was diluted 1:100 into fresh LB with 100 μM FA or Csn-B and grown for 2 h at 37 °C. The culture was subsequently chilled on ice for 10 min and the bacteria were gathered by centrifugation. The sediment

was resuspended with PBS and analyzed for fluorescence using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.10. Statistical analysis

GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used to calculate the means and standard deviations, and a two-way ANOVA method was used to determine the statistical significance in the study.

3. Results

3.1. Fusaric acid inhibited the secretion of SPI-1 effector proteins in a dose-dependent manner and did not affect the growth of S. enterica serovar Typhimurium

To find novel anti-virulence agents, we initially screened a natural products library in our laboratory for the effect on the secretion of SPI-1 effectors of *S. enterica* serovar Typhimurium at the final concentration of 100 μM. Fusaric acid (FA) (Fig. 1A), a mycotoxin firstly isolated from the species *Fusarium heterosporum* Nees with important effects on pathogenesis of the plant filamentous fungi *Fusarium* species [13], exhibited inhibitory effect on the secretion of SPI-1 effectors SipA/B/C/D compared to solvent control (Fig. 1B). On the other hand, FA at different concentrations did not affect the secretion of flagellar protein FliC flagella (Fig. 1B). Moreover, the inhibition effect of FA was in a dose-dependent manner. The IC_{50} value of FA on SPI-1 was 53.5 μM (Fig. 1C). Next, we investigated whether FA affected the growth of this bacterium. The results showed minor difference between FA and DMSO solvent control (Fig. 1D), which indicated that the inhibition of FA on SPI-1 was not due to affecting the growth of *S. enterica* serovar Typhimurium.

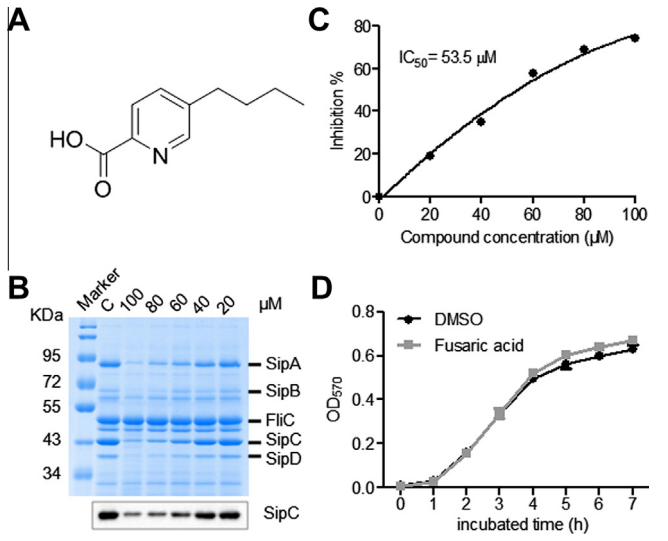

Fig. 1. Fusaric acid inhibited secretion of T3SS effectors in dose dependant manner. (A) The chemical structure of Fusaric acid. (B) Fusaric acid inhibited secretion of SPI-1 effector proteins in dose dependant manner. SipA/B/C/D, SPI-1 effector proteins; FliC, flagellar filament protein; M, Marker; C, DMSO control. (C) Inhibitory effect of fusaric acid at different concentration on secretion of SipC. Inhibition% was calculated according to band intensities of SipC protein in culture supernatants treated by different concentration of fusaric acid compared to DMSO in Western blots assay. Three independent replicates were performed and standard deviations were shown. (D) Effect of fusaric acid on growth curve of *Salmonella Typhimurium* χ8956 at the concentration of 100 μM. Means and standard deviations of three replicates per point are shown.

3.2. Fusaric acid markedly inhibited the invasion of S. enterica serovar Typhimurium into HeLa cells

Since FA did inhibit the secretion of SPI-1 effectors (Fig. 1B), we wanted to know whether this compound had blocking effect on the invasion of *Salmonella* into host cells. Gentamicin protection assay was used to assess the invasion effect. Because the assay is cell-based, the cytotoxicity of FA was measured on HeLa cells by MTT assay. The MTT results showed that FA at different concentrations had nearly no toxic effect on the viability of HeLa cells for 72 h post-treatment (Fig. 2A). In the gentamicin protection assay, FA significantly inhibited the invasion of *Salmonella* into HeLa cells ($P < 0.001$) compared to solvent control (Fig. 2B). Cytosporone B (Csn-B), a known SPI-1 inhibitor found in our laboratory {Li, 2013 #46}, was used as a positive control.

3.3. Fusaric acid may affect T3SS transcriptional regulators SicA/InvF to inhibit the secretion of SPI-1 effectors

The mechanism of action of FA on the secretion SPI-1 effectors was next investigated in this study. Because FA at the final concentration of 100 μM exhibited the strongest inhibition on SPI-1 without affecting the bacterial growth, this concentration was used in further studies. The inhibitory effect of FA on the SPI-1 effector SipC in different bacterial culture fractions (Fig. 3A) was investigated firstly. FA inhibited the protein level of SipC from all the bacterial fractions while it did not affect the protein level of FliC (Fig. 3A), which suggested that FA may affect the expression or translocation of SipC.

Protein HilA is a key transcriptional regulator of SPI-1, which directly activates the transcription of SPI-1 apparatus genes and SPI-1 effector genes by combining with promoter of *invF* [14]. Thus, *S. enterica* serovar Typhimurium χ8956 carrying a vector of pBAD-*hilA*-pWSK29 overexpressing HilA [6] was used to investigate whether FA inhibited the transcription of SPI-1 apparatus. The results indicated that FA inhibited the secretion of SPI-1 effectors SipA/B/C/D when *S. enterica* serovar Typhimurium χ8956 with or without vector pBAD-*hilA*-pWSK29 was cultivated in LB medium without the addition of L-arabinose (Fig. 3B). Conversely, FA did not affect the secretion of SPI-1 effectors when protein HilA was overexpressed with induction of 0.02% L-arabinose (Fig. 3B). In contrast, the positive control compound Csn-B dramatically inhibited the secretion of SPI-1 effectors in both situations (Fig. 3B). Csn-B was found it inhibited the secretion of SPI-1 effectors mainly by Hha/H-NS-HilD–HilC–RtsA–HilA regulatory pathway in the previous report [6]. As a result, the results mirrored that FA did not affect the transcription of SPI-1 regulator HilA.

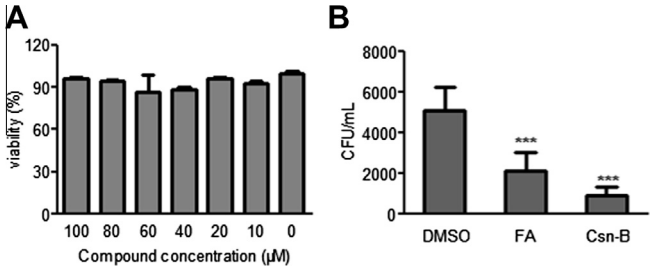

Fig. 2. Fusaric acid inhibited invasion of *S. enterica* serovar Typhimurium into Hela cells. (A) Fusaric acid had no influence on growth of HeLa cells in MTT assay for 72 h. (B) Fusaric acid (100 μM) inhibited the invasion of *S. enterica* serovar Typhimurium into HeLa cells at a MOI of 10. *** Statistical significant difference between DMSO control and compound group ($P < 0.001$). Results presented three independent experiments and error bars indicated standard deviations from the means.

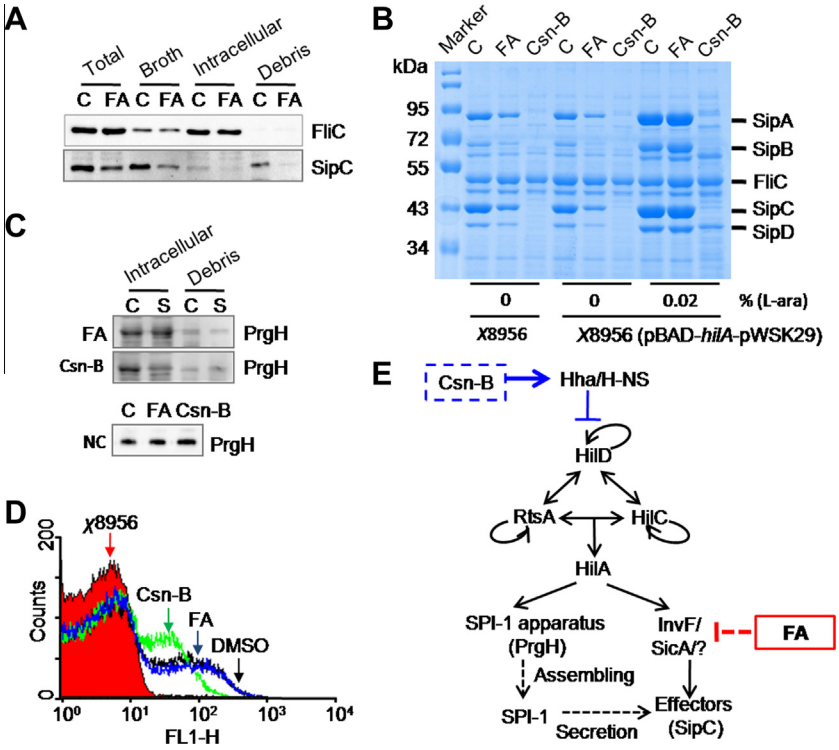

Fig. 3. Fusaric acid inhibited SPI-1 through affecting the assembly of SPI-1 apparatus. (A) Effects of fusaric acid at a final concentration of 100 μM on protein levels of FliC and SipC in different fractions of bacterial culture. Total, total soluble protein, containing soluble protein in supernatant fraction and cytoplasmic fraction; Broth, soluble protein in culture supernatant; Intracellular, soluble protein in intracellular fraction of bacterial culture. Debris, cell debris of bacteria after ultrasonic treatment; C, DMSO control. FA, fusaric acid. (B) Effect of fusaric acid at a final concentration of 100 μM on the secretion of SPI-1 effectors in *S. enterica* serovar Typhimurium χ8956 carrying plasmid pBAD-*hilA*-pWSK29 to over-express HilA. C, DMSO control, FA, fusaric acid, Csn-B, cytosporone B, L-ara, L-arabinose. (C) Effect of fusaric acid or Csn-B at a final concentration of 100 μM on protein level of PrgH in different fraction of bacterial culture. Intracellular, soluble protein in intracellular fraction of bacterial culture; Debris, cell debris of bacteria after ultrasonic treatment; NC, needle complex of Type Three Secretion System. (D) Effect of fusaric acid or Csn-B at a final concentration of 100 μM on SicA/InvF transcription regulatory pathway. The fluorescence of *S. enterica* serovar Typhimurium χ8956 carrying pPROBE-AT-sicA was measured with a FACS flow cytometer. (E) Regulatory pathway of fusaric acid on secretion of SPI-1 effectors. Arrow lines indicated transcriptional activation. Blunt lines indicated transcriptional inhibition. Dotted arrow lines indicated assembly or secretion of proteins. Blunt dotted line indicated blocking the regulators.

SPI-1 apparatus and effector proteins of *S. enterica* serovar Typhimurium were secreted in hierarchy, i.e., the proteins loaded on a cytoplasmic sorting platform in sequence, and then secreted in order to assemble the apparatus and transport effector proteins [3,15]. The translocation of effectors requires the proper assembly of SPI-1 needle complex (NC). Thus, whether fusaric acid could affect assembly of SPI-1 was investigated. SPI-1 apparatus protein PrgH, one of the main components in SPI-1 NC, was chosen as a research target protein. There were no difference on the protein level of PrgH in NC of SPI-1between DMSO control and FA or Csn-B (Fig. 3C), suggesting FA did not affect the assembly of NC. The results were also consistent with the mechanism of action of Csn-B. In addition, FA did not affect the PrgH levels of different fractions of bacterial culture (Fig. 3C), which further illustrated that FA did not influence the transcription of SPI-1 regulators HilA.

SicA is a chaperone of SPI-1 effectors SipB and SipC, which could bind to the SPI-1 effectors, preventing their degradation and premature molecular association [16]. After the two effectors are secreted by the SPI-1, SicA is free to bind InvF, an activator of the SPI-1, to activate the transcription of the sipACDA [17]. We then measured the effect of FA on SicA/InvF transcriptional regulator pathway. The result illustrated that FA did not affect the transcription of the promoter of sicA, the DNA binding region of transcriptional regulator SicA/InvF, while Csn-B dramatically inhibited transcription of the promoter (Fig. 3D). All the results suggested that FA did not affect the transcription of SPI-1 regulators and effectors, as well as, it had no effect on the assembly of SPI-1. Since the protein levels of SPI-1 effector proteins in the supernatant of culture were determined by the expression and translocation of the effectors, therefore, it was inferred that FA may influence the proteins SicA/InvF or others to inhibit the secretion of SPI-1 effectors (Fig. 3E).

4. Discussion

Conventional antibiotics usually directly kill the pathogens or inhibit the growth of pathogens, which is easy to produce the resistance of pathogens to antibiotics under selective pressure for viability [5]. Here we demonstrated the inhibitory effect of FA on the secretion of SPI-1 effector proteins and the invasion of *Salmonella* into HeLa cells without evident toxicities against the growth of *S. enterica* serovar Typhimurium and viability of HeLa cells. Therefore, FA as a novel T3SS inhibitor could not only reduce the virulence in vitro but also be less likely to incur the resistance of *Salmonella* to FA. Additionally, the logP (lipid/water partition coefficient) value of FA calculated by Chemdraw software is 2.42, showing good drug likeness score based on Lipinski's Rule of Five [18]. As a result, the antibacterial activity of FA targeting virulence factor make it well-suited for the development as a novel antimicrobial agent.

Flagella is similar to T3SS genetically and structurally, which all are like syringe [19]. The function of T3SS is injection of effectors to promote the invasion of *Salmonella* [20], but flagella is in charge of the swimming and motility of the bacterium [19]. Interestingly, FA demonstrated different effect on secretion of T3SS effectors and

flagellar protein FliC in this study. As a result, further work to elucidate how fusaric acid affects SPI-1 will be important to understand the different effect between T3SS and flagella, since both of them are important virulence factors in the pathogenicity of gram-negative bacteria.

To date, many T3SS inhibitors have been screened for developing novel anti-virulence agents, such as salicylidene acylhydrazides [21–23], *N*-phenyl-benzamides [24], thiazolidinones [25,26], phenolic acids [11,27] and so on. The T3SS inhibitors inhibited the expression of T3SS effectors or affected the assembly of T3SS needle complex to perform their inhibition on T3SS. Interestingly, fusaric acid showed novel mechanism of action compared to other T3SS inhibitors in this study. But its target proteins are still needed to identify; therefore, additional studies will be required to further investigate mechanism of fusaric acid affecting SicA/InvF or other proteins.

In addition, FA is a mycotoxin firstly isolated from the species *Fusarium Heterosporum* Nees, which has important effects on pathogenesis of plant filamentous fungi *Fusarium* species [13]. It has also been classified as a phytotoxin because it has antibiotic and insecticidal effect [28]. This study firstly identified the inhibitory effect of FA on T3SS of gram-negative bacteria, which may prompt understanding survival competition mechanism between fungi and bacteria [29].

In summary, we have identified fusaric acid with anti-SPI-1 activity in vitro in the μM range. Moreover, the compound appeared to have very low toxicity for *Salmonella* and HeLa cells in vitro at the inhibitory concentrations. Finally, fusaric acid may affect SicA/InvF or other proteins to exert its inhibitory effect on SPI-1. The activities of fusaric acid against *Salmonella* make it potential candidate, likely combined with other agents, to use as a novel microbicide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.044.

References

[1] M.K. Bhan, R. Bahl, S. Bhatnagar, Typhoid and paratyphoid fever, Lancet 366 (2005) 749–762.
[2] M. Hensel, Evolution of pathogenicity islands of *Salmonella enterica*, Int. J. Med. Microbiol. 294 (2004) 95–102.
[3] L.M. Stamm, M.B. Goldberg, Microbiology. Establishing the secretion hierarchy, Science 331 (2011) 1147–1148.
[4] P. Keyser, M. Elofsson, S. Rosell, H. Wolf-Watz, Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against gram-negative bacteria, J. Intern. Med. 264 (2008) 17–29.
[5] B.B.F. Annick, Gauthier, type III secretion system inhibitors are potential antimicrobials, ASM News 68 (2002) 383–387.
[6] J. Li, C. Lv, W. Sun, Z. Li, X. Han, Y. Li, Y. Shen, Cytosporone B, an inhibitor of the type III secretion system of *Salmonella enterica* serovar Typhimurium, Antimicrob. Agents Chemother. 57 (2013) 2191–2198.
[7] R. Curtiss 3rd, S.Y. Wanda, B.M. Gunn, X. Zhang, S.A. Tinge, V. Ananthnarayan, H. Mo, S. Wang, W. Kong, Salmonella enterica serovar typhimurium strains with regulated delayed attenuation in vivo, Infect. Immun. 77 (2009) 1071–1082.
[8] H. Hidaka, T. Nagatsu, K. Takeya, T. Takeuchi, H. Suda, Fusaric acid, a hypotensive agent produced by fungi, J. Antibiot. 22 (1969) 228.
[9] R. Preet, P. Mohapatra, S. Mohanty, S.K. Sahu, T. Choudhuri, M.D. Wyatt, C.N. Kundu, Quinacrine has anticancer activity in breast cancer cells through inhibition of topoisomerase activity, Int. J. Cancer 130 (2012) 1660–1670.
[10] T.G. Kimbrough, S.I. Miller, Contribution of *Salmonella typhimurium* type III secretion components to needle complex formation, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 11008–11013.
[11] A. Yamazaki, J. Li, Q. Zeng, D. Khokhani, W.C. Hutchins, A.C. Yost, E. Biddle, E.J. Toone, X. Chen, C.H. Yang, Derivatives of plant phenolic compound affect the type III secretion system of *Pseudomonas aeruginosa* via a GacS-GacA two-component signal transduction system, Antimicrob. Agents Chemother. 56 (2012) 36–43.
[12] W.G. Miller, J.H. Leveau, S.E. Lindow, Improved gfp and inaZ broad-host-range promoter-probe vectors, Mol. Plant Microbe Interact. 13 (2000) 1243–1250.
[13] H. Wang, T.B. Ng, Pharmacological activities of fusaric acid (5-butylpicolinic acid), Life Sci. 65 (1999) 849–856.
[14] Y.A. Golubeva, A.Y. Sadik, J.R. Ellermeier, J.M. Slauch, Integrating global regulatory input into the *Salmonella* pathogenicity island 1 type III secretion system, Genetics 190 (2012) 79–90.
[15] M. Lara-Tejero, J. Kato, S. Wagner, X. Liu, J.E. Galan, A sorting platform determines the order of protein secretion in bacterial type III systems, Science 331 (2011) 1188–1191.
[16] S.C. Tucker, J.E. Galan, Complex function for SicA, a *Salmonella enterica* serovar Typhimurium type III secretion-associated chaperone, J. Bacteriol. 182 (2000) 2262–2268.
[17] B.H. Kim, H.G. Kim, J.S. Kim, J.I. Jang, Y.K. Park, Analysis of functional domains present in the N-terminus of the SipB protein, Microbiology 153 (2007) 2998–3008.
[18] C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, Adv. Drug Deliv. Rev. 23 (1997) 3–25.
[19] A. Blocker, K. Komoriya, S. Aizawa, Type III secretion systems and bacterial flagella: insights into their function from structural similarities, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 3027–3030.
[20] D. Aiello, J.D. Williams, H. Majgier-Baranowska, I. Patel, N.P. Peet, J. Huang, S. Lory, T.L. Bowlin, D.T. Moir, Discovery and characterization of inhibitors of *Pseudomonas aeruginosa* type III secretion, Antimicrob. Agents Chemother. 54 (2010) 1988–1999.
[21] A.M. Kauppi, R. Nordfelth, H. Uvell, H. Wolf-Watz, M. Elofsson, Targeting bacterial virulence. inhibitors of type III secretion in *Yersinia*, Chem. Biol. 10 (2003) 241–249.
[22] M.K. Dahlgren, C.E. Zetterstrom, S. Gylfe, A. Linusson, M. Elofsson, Statistical molecular design of a focused salicylidene acylhydrazide library and multivariate QSAR of inhibition of type III secretion in the gram-negative bacterium *Yersinia*, Bioorg. Med. Chem. 18 (2010) 2686–2703.
[23] R. Nordfelth, A.M. Kauppi, H.A. Norberg, H. Wolf-Watz, M. Elofsson, Small-molecule inhibitors specifically targeting type III secretion, Infect. Immun. 73 (2005) 3104–3114.
[24] M.K. Dahlgren, A.M. Kauppi, I.-M. Olsson, A. Linusson, M. Elofsson, Design, synthesis, and multivariate quantitative structure-activity relationship of salicylanilides-potent inhibitors of type III secretion in *Yersinia*, J. Med. Chem. 50 (2007) 6177–6188.
[25] C. Baron, Antivirulence drugs to target bacterial secretion systems, Curr. Opin. Microbiol. 13 (2010) 100–105.
[26] H.B. Felise, H.V. Nguyen, R.A. Pfuetzner, K.C. Barry, S.R. Jackson, M.P. Blanc, P.A. Bronstein, T. Kline, S.I. Miller, An inhibitor of gram-negative bacterial virulence protein secretion, Cell Host Microbe 4 (2008) 325–336.
[27] Y. Li, Q. Peng, D. Selimi, Q. Wang, A.O. Charkowski, X. Chen, C.H. Yang, The plant phenolic compound p-coumaric acid represses gene expression in the *Dickeya dadantii* type III secretion system, Appl. Environ. Microbiol. 75 (2009) 1223–1228.
[28] H.V. Swamy, T.K. Smith, E.J. MacDonald, Effects of feeding blends of grains naturally contaminated with Fusarium mycotoxins on brain regional neurochemistry of starter pigs and broiler chickens, J. Anim. Sci. 82 (2004) 2131–2139.
[29] M.J. Wargo, D.A. Hogan, Fungal–bacterial interactions: a mixed bag of mingling microbes, Curr. Opin. Microbiol. 9 (2006) 359–364.