Veterinary Microbiology 239 (2019) 108463

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Cinnamaldehyde inhibits type three secretion system in *Salmonella* enterica serovar Typhimurium by affecting the expression of key effector proteins

Yan Liu[a,c,1], Yong Zhang[a,c,1], Yonglin Zhou[c], Tingting Wang[c], Xuming Deng[c], Xiao Chu[a,*], Tiezhong Zhou[b,*]

[a] Department of Respiratory Medicine, The First Hospital of Jilin University, 71 Xinmin Street, Changchun, China
[b] Institute of Animal Husbandry and Veterinary Medicine, Jinzhou Medical Uniersity, No. 48 People's Street, Liaoning, China
[c] Key Laboratory of Zoonosis, Ministry of Education, Institute of Zoonosis, College of Veterinary Medicine, Jilin University, Changchun, 130062, China

ARTICLE INFO

Keywords:
Type III secretion
Salmonella
Anti-virulence
Natural compounds

ABSTRACT

The increasing understanding of bacterial pathogenesis has revealed many new targets for the development of non-traditional antibacterial drugs. Interference with bacterial virulence has become a new strategy to treat bacteria-mediated diseases. As an important food-borne pathogen, *Salmonella* enterica serovar Typhimurium uses type III secretion system (T3SS) to facilitate invasion of host cells. In this study, we identified cinnamaldehyde as a *Salmonella* pathogenicity island 1 (SPI-1) inhibitor which blocks the secretion of several SPI-1 associated effector proteins and consequently exhibits a strong inhibitory effect on SPI-1-mediated invasion of HeLa cells. Further study revealed that cinnamaldehyde significantly reduced the transcription of some SPI-1 genes, such as *sipA* and *sipB,* in *S.* Typhimurium by affecting multiple SPI-1 regulator genes. In an animal infection model, cinnamaldehyde effectively protected infected mice against *S.* Typhimurium-induced mortality and pathological damages. In summary, this study presented an effective SPI-1 inhibitor, cinnamaldehyde, which reduces the expression of SPI-1 effector proteins by regulating the transcription of main regulator genes.

1. Introduction

Various gram-negative pathogens use novel secretion systems to traverse host barriers delivering virulence proteins into the cytoplasm of eukaryotic cells (Thanassi and Hultgren, 2000). These machineries belong to such systems as type III, type IV and type VI secretion apparatus (Koraimann, 2003), among which type III secretion system plays an indispensable role in the pathogenicity of Yersinia spp, *Shigella flexneri*, *Salmonella* Typhimurium, enteropathogenic *Escherichia coli* (EPEC) (Du et al., 2016), *Pseudomonas aeruginosa* and *Chlamydia* spp (Aiello et al., 2010). Without T3SSs, these pathogens cannot overcome host defenses, find niches, and interfere signal transduction pathways in host cells (Galán, 2009). Thus, this system has become an ideal target for the development of anti-microbial agents (Charro and Mota, 2015).

Salmonella enterica serovars Typhimurium causes a variety of diseases ranging from mild gastroenteritis to life threatening systemic infections in a wide range of hosts (Coburn et al., 2007). It has resulted in considerable medical costs to society and economic losses in poultry industries around the world (Forshell and Wierup, 2006; Sockett, 1995). The clinical treatment of infectious diseases is compromised by the spread of antibiotic resistance in bacterial pathogens (Piddock et al., 2000). Therefore, there is an urgent need to develop new antibacterial drugs to mitigate these challenges (Threlfall, 2002). It has been well implicated that *Salmonella enterica* harbors a type three secretion system which is pivotal for its survival within eukaryotic cells (Galán and Wolf-Watz, 2006). It functions by encoding two main protein secretion machineries. The *Salmonella* pathogenicity island 1 (SPI-1) forms a needle-like structure that injects effector proteins which induce actin rearrangement and promote invasion of the bacteria into host cells (Galán and Bliska, 1996). The SPI-2 exports effector proteins that maintain the intracellular survival and replication of the bacteria within the *Salmonella*-containing vacuole (SCV) (Waterman and Holden, 2003).

Given the fact that T3SS is the main virulence factor of the bacteria, inhibitors of T3SS have been developed to disarm these pathogens. For example, a set of seven analogues of 2-(benzo[1,2,5]thiadiazole-4-sulfonylamino)-5-chloro-N-(3,4-dichloro-phenyl)-benzamide was identified as putative type III secretion inhibitors in Yersinia (Kauppi et al., 2007). A series of salicylaldehyde derivatives reduced the production of several type III apparatus components in EPEC potentially by regulating

* Corresponding authors.
E-mail addresses: 421591993@qq.com (X. Chu), ztz1818@126.com (T. Zhou).
[1] Yan Liu and Yong Zhang contributed equally to this study.

https://doi.org/10.1016/j.vetmic.2019.108463
Received 27 June 2019; Received in revised form 15 October 2019; Accepted 16 October 2019

the transcription of type III system-associated genes (Gauthier et al., 2005). The thiazolidinone dipeptide derivatives were also reported as inhibitors of the type III secretion system (Kline et al., 2008). In addition to synthetic chemicals, natural product extracts such as caminosides and guadinomines have been identified as potent anti-T3SS agents (Linington et al., 2002; Iwatsuki et al., 2008). Traditional Chinese Medicines (TCMs) have long been used to treat infectious diseases but the mechanism of actions of these herbal medicines mostly remain elusive (Yuan and Lin, 2000). A recent study showed that baicalin and some related compounds targeted T3SS-1 of *S*. Typhimurium by covalently inactivating some of its effectors (Tsou et al., 2016). In this study, we identified cinnamaldehyde as a strong inhibitor of T3SS-1 in *S*. Typhimurium. Further studies revealed that cinnamaldehyde strongly inhibited the production of SPI-1 related effector proteins by repressing transcription of SPI-1 associated genes.

2. Results

2.1. Cinnamaldehyde affected the stability of multiple SPI-1 virulence proteins without inhibiting bacterial growth

S. Typhimurium employs many virulence factors to facilitate invasion of epithelial cells. Effector proteins encoded by SPI-1, such as SipA (Zhou et al., 1999) and SipB (Hayward et al., 2000), play indispensable role in promoting bacteria internalization into HeLa cells. Based on this fact, we screened a chemical library that has 50 tradition Chinese medicines with previously suggested anti-infective activity but unknown mechanisms. To determine the inhibitory effect of these natural compounds on the level of SipA and SipB in *S*. Typhimurium, *S*. Typhimurium SipA-3 × FLAG and *S*. Typhimurium SipB-3 × FLAG mutant strains were constructed by placing a 3 × FLAG at the C-termini of *sipA* and *sipB* respectively. Chromosomal insertions of 3 × FLAG strains were used to identify chemicals that inhibit the production of SipA and SipB by detecting the amount of expressed Flag-SipA and Flag-SipB fusions using Western blot. We found that several compounds showed inhibitory effects against the production of SPI-1 effectors (data not shown). Most interestingly, cinnamaldehyde exhibited significant inhibitory effect on the level of Flag-SipA and Flag-SipB (Fig. 1**A–B**). The inhibitory rates of cinnamaldehyde against Flag-SipA and Flag-SipB at concentrations of 0.2 and 0.4 mM were all higher than 90 %, while that of 0.1 mM was substantially lower, only 78 % and 65 %, respectively (Fig. 1**A–B**). The down regulation of SipA was confirmed by Western blot analysis of wild type strain SL1344 incubated with or without cinnamaldehyde for detection of endogenous SipA (Fig. 1C).

Given the fact that cinnamaldehyde greatly affected the production of SipA and SipB, we further examined the expression of other important SPI-1 components. Similarly, 0.2 mM of cinnamaldehyde treatment significantly reduced the level of SipC, which is responsible for the translocation of effector proteins and modulation of actin (Hayward and Koronakis, 1999) (Fig. 1D). Further, probing of endogenous proteins with a HilA-specific antibody revealed that 0.1 mM of cinnamaldehyde reduced the level of HilA about 10-fold (Fig. 1E). 0.2 mM of cinnamaldehyde made the protein completely undetectable under our experimental conditions (Fig. 1E). These results indicated that cinnamaldehyde inhibited the production of several SPI-1 virulence proteins in a dose-dependent manner.

To confirm that the suppression activity of cinnamaldehyde on SPI-1 effector proteins was not caused by the antibiotic effect of the drug, the growth curve of *S*. Typhimurium in LB broth was measured at indicated concentrations. No detectable inhibitory effect on bacterial growth was observed at all time points (Fig. 2B). The minimum detectable growth inhibition concentration is 1.0 mM (Fig. 2B). 0.1 mM of cinnamaldehyde reduced the level of SPI-1 effector proteins dramatically, therefore, this concentration was adopted for further investigation to study the mechanism of action of cinnamaldehyde on SPI-1.

2.2. Cinnamaldehyde inhibited the invasion of Salmonella enterica serovar Typhimurium into epithelial cells

To test the ability of cinnamaldehyde inhibiting *S*. Typhimurium invasion of epithelial cells, a phenotype predominantly mediated by SPI-1 (Zhou and Galán, 2001), we conducted the gentamicin protection assay. In this assay, the non-invasive *S*. Typhimurium *inv*A mutant (Ginocchio and Galan, 1995), a strain lacks an essential component of the SPI-1, was taken as a negative control. Compared to the DMSO control, bacteria grown in the presence of cinnamaldehyde at indicated concentrations showed significant reduction in invasion of HeLa cells. At the concentration of 0.2 mM, the uptake of cinnamaldehyde-treated bacteria was almost completely reduced, which is comparable to that of the *inv*A mutant group (Fig. 3A).

This inhibition of *S*. Typhimurium invasion was also observed through confocal laser scanning microscopy and image analysis of intracellular bacteria (Fig. 3B). In this assay, bacteria were stained using the double-immunolabeling fluorescence microscopy method, by which

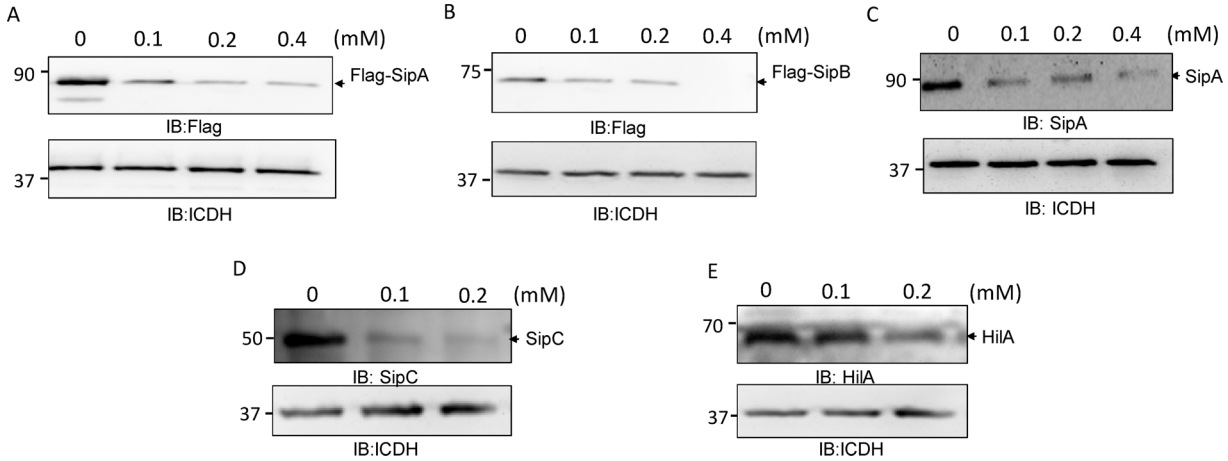

Fig. 1. Cinnamaldehyde inhibits the production of several SPI-1 virulence effector proteins in *S*. Typhimurium.
SipA-3xFlag, SipB-3xFlag knock-in strains or wild type *S*. Typhimurium were treated with the indicated concentrations of cinnamaldehyde for 4 h. Cleared cell lysates resolved by SDS-PAGE were probed with anti-flag antibody or antibodies specific for SipA, SipC or HilA. Isocitrate dehydrogenase (ICDH), which catalyzes the conversion of isocitrate to a-ketoglutarate in the tricarboxylic acid (TCA) cycle, was probed as a loading control. **A.** Dose-dependent effect of cinnamaldehyde on the level of SipA **B.** Dose-dependent effect of cinnamaldehyde on the level of SipB. **C–E** Decreased level of endogenous SipA, SipC and HilA upon wild type *S*. Typhimurium infection. Results shown were from three independent experiments done in triplicates.

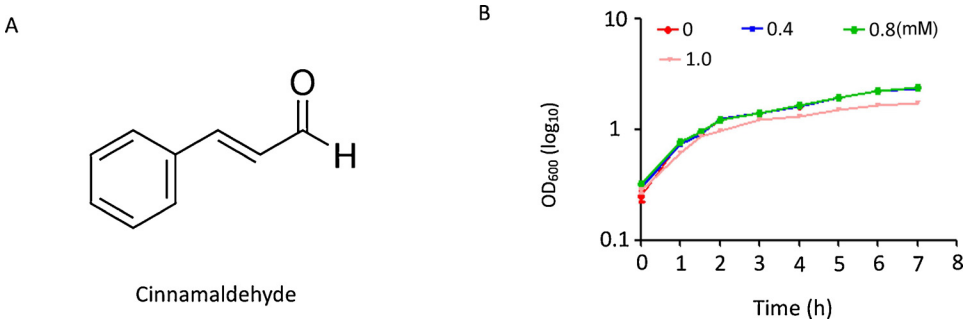

Fig. 2. The effect of cinnamaldehyde on the growth of S. Typhimurium.
A. The chemical structure of cinnamaldehyde. **B**. Cinnamaldehyde was added to bacterial cultures at indicated concentrations and OD_{600} of the cultures were measured at indicated time points. Note that 0.8 mM of cinnamaldehyde did not detectably affect bacterial growth. Results shown were from three independent experiments done in triplicates. Bar, s.e.m. (n = 3) *p < 0.05.

bacteria inside and outside of HeLa cells were distinguished by incubating them with secondary antibodies conjugated with different fluorochromes. Our results showed that the internalized bacteria were reduced significantly in cinnamaldehyde-treated group (Fig. 3B). These results demonstrated that cinnamaldehyde was a strong inhibitor of SPI-1 T3SS-1 mediated invasion of host cells.

2.3. Cinnamaldehyde exerted protective effect against S. Typhimurium induced mice mortality

The strong inhibitory effect of cinnamaldehyde against invasion of *S*. Typhimurium of HeLa cells prompted us to evaluate its protective affect against *S*. Typhimurium induced infection in animals. Streptomycin mouse model for *Salmonella* diarrhea (Suar et al., 2006) was used to determine the effectiveness of cinnamaldehyde in improving the survival rate of mice infected by *S*. Typhimurium. Mice were infected with wild type or the *InvA* mutant strain. The body weight of the infected animals by low doses (5×10^6/mouse) was examined. Animals infected with wild type strain without cinnamaldehyde treatment exhibited prolonged body weight loss as the experiments proceeded. As expected, animals pretreated with cinnamaldehyde or infected with the Δ*invA* mutant gained weight through the experimental duration (Fig. 4A).

The mortality of mice challenged by high doses bacteria (1×10^8) was measured in the following experiment. From the 5th day to the 9th day post infection, the mortality of mice treated with wild type strain was up to 80 %; while no death was observed in the 7th day after *S*. Typhimurium challenge in mice administered with cinnamaldehyde (50 mg/kg) prior and post bacteria challenge (Fig. 4B). About 70 % of the animals survived at the 8th day and 30 % of them survived to the 10th day post infection (Fig. 4B).

Simultaneously, the clinical protective effect of cinnamaldehyde was validated by examining the histological changes of the cecum using hematoxylin and eosin (H&E) staining. Pathological changes were scored as described before (Suar et al., 2006). Compared with severe submucosal edema, loss of goblet cells along with epithelial integrity damage and polymorphonuclear granulocytes (PMN) infiltration in the lamina propria in wild type strain infection group, mice in cinnamaldehyde-treated group only displayed slightly intestinal damage and inflammation (Fig. 4**C-D**). Similarly, the amounts of pro-inflammatory cytokines IL-6, IL-1β and TNF-α in the cecum were significantly higher in wild type strain-treated group than in cinnamaldehyde-treated group (Fig. 4E).

Bacteria in various organs such as spleen, liver and cecum were quantified. SL1344 infected mice had higher bacterial loads in these organs than mice in cinnamaldehyde treatment group, indicating reduced bacterial dissemination in cinnamaldehyde treated mice (Fig. 4F). Taken together, these results indicated that cinnamaldehyde effectively protected mice from *Salmonella* Typhimurium infection.

2.4. Cinnamaldehyde inhibited the transcription of several SPI-1 associated effector proteins and regulator proteins in S. Typhimurium

The observation that cinnamaldehyde reduced the production of SipA, SipB and HilA (Bajaj et al., 1995) suggested that the stability of these proteins was influenced by cinnamaldehyde treatment. To determine whether cinnamaldehyde inhibited the translation of these SPI-1 effector proteins, *sipA*, *sipB* and *sipC* were cloned to the pZLQ plasmid (Luo and Farrand, 1999; Luo and Farrand, 2001) and the level of flag-tagged proteins were examined. The result showed that the level of Flag-SipA, Flag-SipB and Flag-SipC were not influence by cinnamaldehyde treatment (Fig. 5**A-C**), indicating that the translation of these proteins was not inhibited.

To further elucidate the mechanism of how cinnamaldehyde regulates the expression of these effector proteins, we determined the relative mRNA levels of some SPI-1 genes using RT-qPCR. The mRNA levels of *sipA*, *sipB*, *sipC* and *sipD* under 0.1 mM cinnamaldehyde were reduced about 32 folds compared with that of the DMSO group

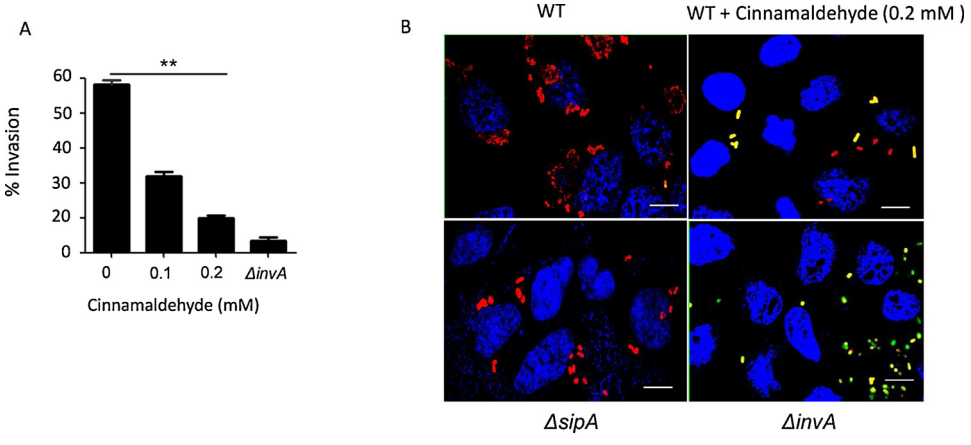

Fig. 3. Protective effect of cinnamaldehyde on *S*. Typhimurium induced cell invasion.
A. Cinnamaldehyde treatment abolished invasion of *S*. Typhimurium into HeLa cells. The indicated bacterial strains were used to infect HeLa cells. Internalized bacteria were counted after gentamicin treatment. The values have been normalized to the internalization level of wild type bacteria, which was considered as 100 %. Bar, S.E.M. (n = 3); **, p < 0.01. Results shown were from three independent experiments.
B. Representative images of immunofluorescence analysis of *S*. Typhimurium strains invasion of HeLa cells. 0.2 mM of cinnamaldehyde was added to bacterial cultures 4 h before infection. Red cells represent intracellular bacteria. Scale bar = 10 μm. Note that cinnamaldehyde reduced the number of red cells.

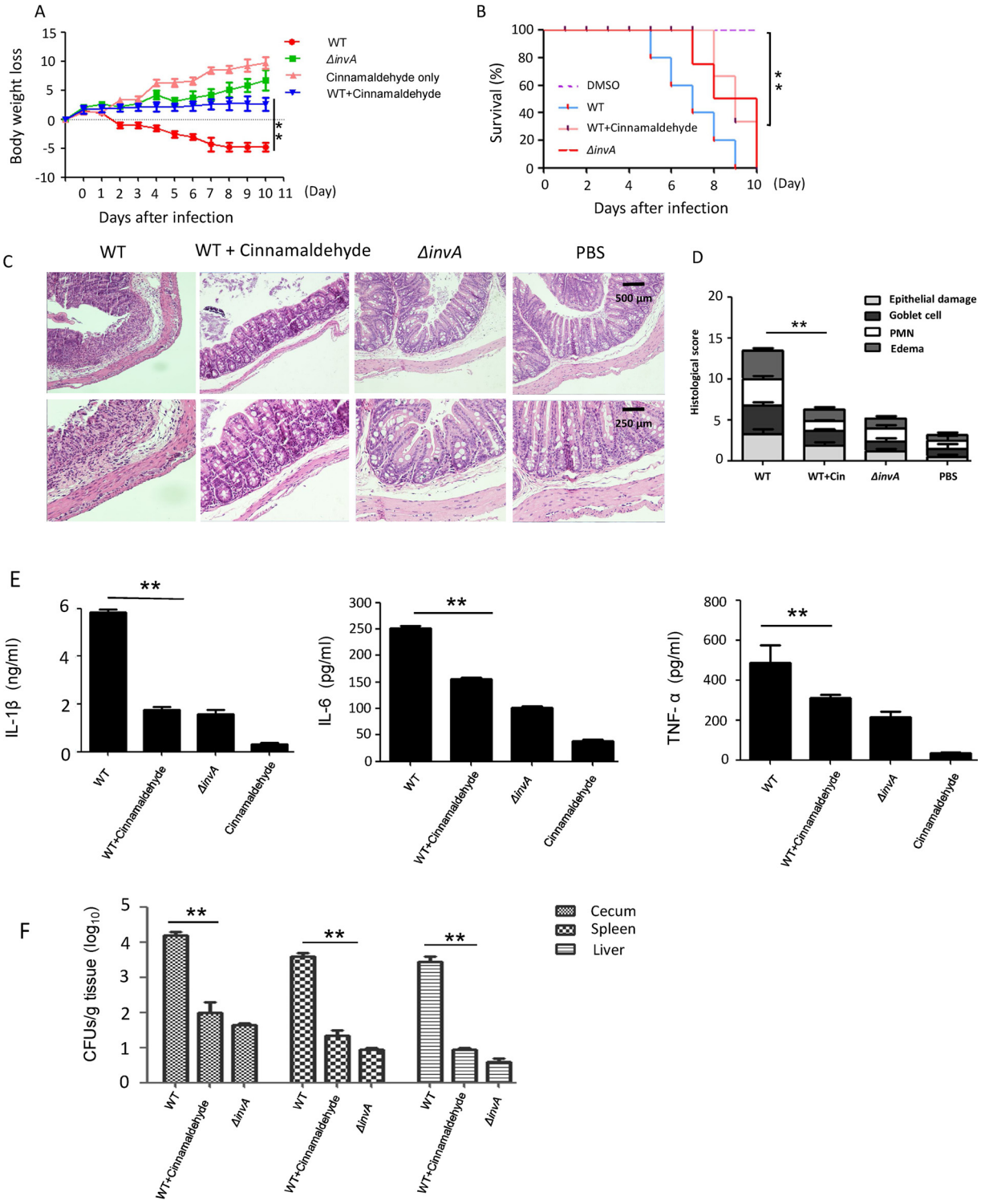

Fig. 4. Protective effect of cinnamaldehyde on *S*. Typhimurium induced damages in mice.
Mice were pre-dosed with streptomycin and then challenged with either wild type or the *invA* mutant *S*. Typhimurium. **A**. Body weight loss in infected animals was examined by low doses (5×10^6/mouse) bacterial infection. Compared with animals challenged with wild type bacteria, animals pretreated with cinnamaldehyde gained weight through the experimental duration. **B**. Mice mortality was measured after high doses bacteria (1×10^8) Challenge. 70 % of mice in the cinnamaldehyde treatment group survived at the 8th day and 30 % of them survived to the 10th day post infection. Similar results were obtained in three independent experiments, and data shown are from one representative experiment done in triplicate. **p < 0.01; *p < 0.05. Statistical analyses were performed by Log-Rank test. **C**. Histopathology analysis of mice cecum. Sections of cecum from different groups of mice were stained with hematoxylin and eosin (H&E). note that cinnamaldehyde treatment significantly alleviated *S*. Typhimurium infection caused epithelial cell damages, goblet cell loss and PMN infiltration. **D**. The histopathological damage was scored. Bar, s.e.m. (n = 3) **p < 0.01. **E** Cecum of infected mice differently treated with cinnamaldehyde were measured for the indicated cytokines. All experiments were performed with tissues from at least three mice and similar results were obtained from at least three independent experiments. **F.** Thymol reduces the bacterial load in several organs of the infected mice. The bacterial load in cecum, liver, and spleen were determined. Results shown were from one representative experiment done in triplicate. Similar results were obtained in three independent experiments. *, $p < 0.05$, **, $p < 0.01$.

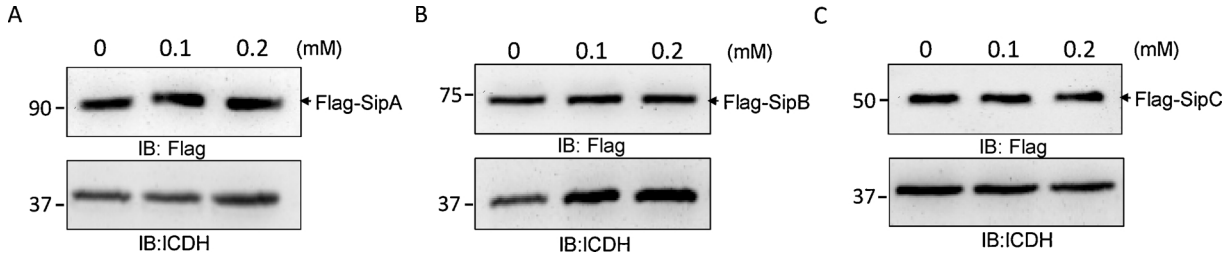

Fig. 5. Cinnamaldehyde has no influence on the translation of SipA, SipB and SipC.
sipA, *sipB* and *sipC* were inserted into pZLQ plasmid and the level of Flag-SipA, Flag-SipB and Flag-SipC were determined under indicated cinnamaldehyde treatment. **A-C**. Cinnamaldehyde did not influence the stability of SipA, SipB and SipC.

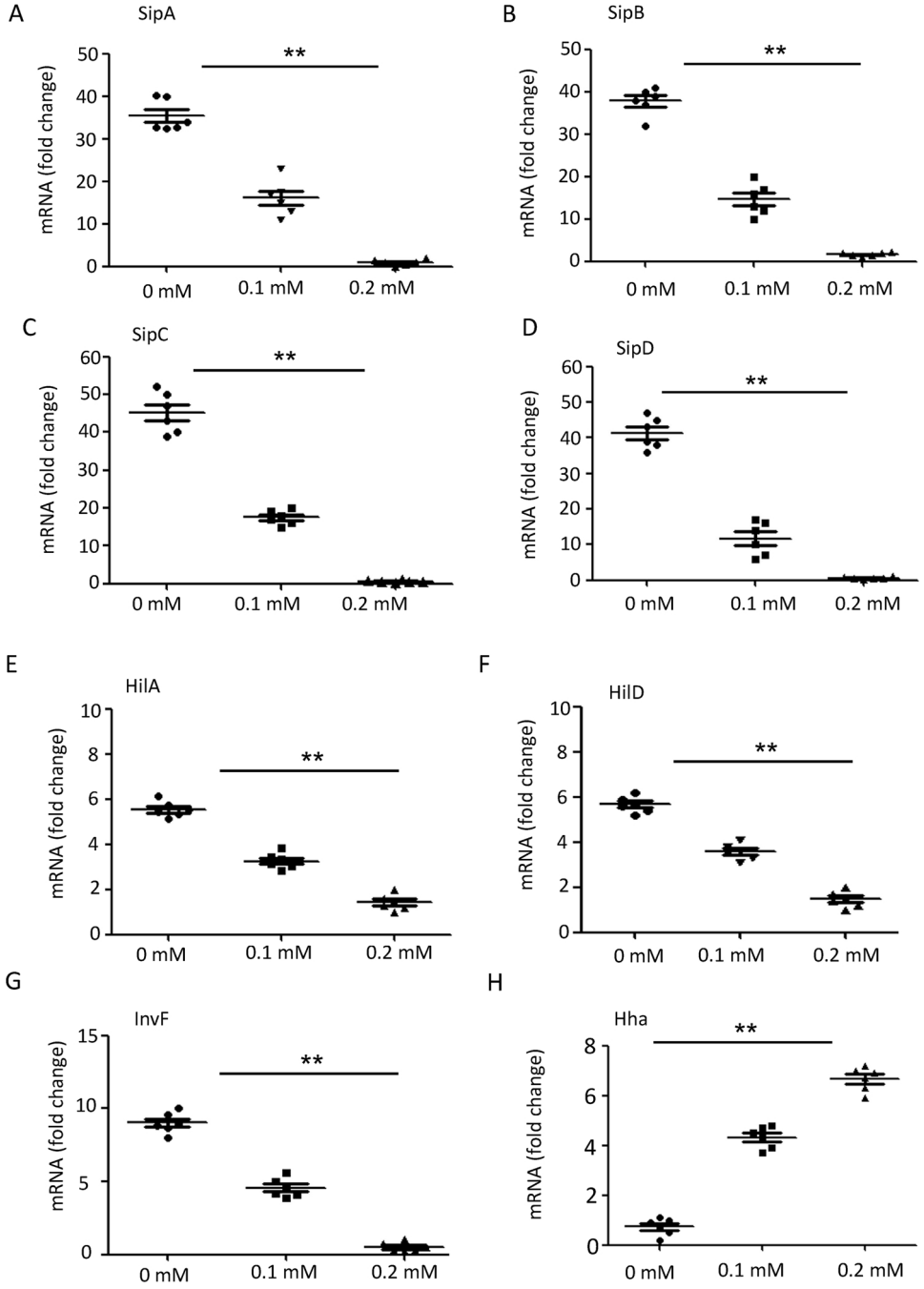

Fig. 6. The mRNA levels of some T3SS-1 proteins were affected upon cinnamaldehyde treatment.
Total bacterial RNA was extracted from *S*. Typhimurium preincubated with cinnamaldehyde at indicated concentrations. cDNAs were analyzed using RT-qPCR. Bar, S.E.M. (n = 3); **, $p < 0.01$. Results shown were from three independent experiments. **A–D** The transcription of genes encoding main SPI-1 effector proteins were inhibited upon cinnamaldehyde treatment. **E–H** transcription of *hilD*, *hilA* and *invF* were decreased while the transcription of *hha* was increased.

(Fig. 6**A–D**). Effector proteins such as SipA, SipB, SipC, SipD and SicA are encoded by the *sic/sip* operon within SPI-1 (Ellermeier and Slauch, 2007) and this operon is induced *via* the activation of InvF (Darwin and Miller, 2001). Moreover, the combined action of three AraC-like transcriptional activators, HilC, HilD and RtsA, with the HilD at the top of the hierarchy, amplify the activation signal and act as a switch for HilA transcription (Ellermeier and Slauch, 2007). Thus, we tested the mRNA levels of four positive regulator genes, *hilD*, *hilC*, *hilA* and *invF,* as well as *hha,* which represses the transcription of SPI-1 genes (Fahlen et al., 2001). Our results showed that cinnamaldehyde exhibited a remarkable inhibitory effect on the transcription of *hilD*, *hilA* and *invF* while promoted the transcription of *hha* (Fig. 6**E–H**). When treated with 0.1 mM cinnamaldehyde, transcription of *hilD*, *hilC*, *hilA* and *invF* were decreased about 4.7, 6.2, 4.5 and 8.2-fold, respectively, while the mRNA level of *hha* was increased about 3.5 and 4-fold, respectively (Fig. 6**E–H**). These results demonstrated that cinnamaldehyde reduced the production of SPI-1 effector proteins by inhibiting the transcription of the positive regulator genes and increasing the relative mRNA level of the negative regulator *hha*.

2.5. Overexpression of HilC, HilD or InvF in S. Typhimurium rescued the level of SipA under cinnamaldehyde treatment

To verify the idea that cinnamaldehyde inhibited the expression of SPI-1 effector proteins *via* regulating the transcription of SPI-1 regulator proteins, HilC, HilD or InvF were overexpressed in wild type *S.* Typhimurium. Wild-type strains carrying [*hilC*]-pZLQ, [*hilD*]-pZLQ, or [*invF*]-pZLQ, respectively, were treated with cinnamaldehyde at indicated concentrations. Expression of the pZLQ constructs were induce by IPTG. No difference was observed in endogenous SipA expression level even under 0.2 mM cinnamaldehyde (Fig. 7**A–C**), which further demonstrated that cinnamaldehyde inhibited the level of SPI-1 effector proteins *via* effecting the transcription of SPI-1 regulator proteins.

3. Discussion

Antibiotic resistance in bacteria has emerged as a medical catastrophe (Cohen, 1992). The excessive use of antibiotics in human beings and animals provides a live or death pressure for multidrug-resistant organisms to become predominant (Levy, 1998). It is essential to develop novel anti-bacterial agents (Boucher et al., 2017). In recent years, anti-virulence approach has emerged as a new strategy to combat multidrug-resistant bacteria due to its non-bactericidal feature (Rasko and Sperandio, 2010). Bacterial virulence mechanisms, especially specialized protein secretion systems harbored by many pathogens, appear to be prime targets for anti-virulence therapeutics (Baron and Coombes, 2007). Among these secretion systems, the highly conserved type III secretion system central to the virulence of many human gram-negative pathogens has been extensively exploited (Charro and Mota, 2015).

Since the introduction of the first anti-T3SS compound, several drugs have been reported, among which several classes of small molecules possessing inhibitory activity towards T3SS in several gram-negative pathogens, such as *Shigella*, *Chlamydia*, *E. coli* and *Salmonella,* were studied extensively (Tsou et al., 2013). For example, a group of salicylidene acylhydrazides was identified as inhibitors of T3SS in *Shigella flexneri*, where they interrupted the assembly of the needle complex and consequently affected the secretion of T3SS proteins (Veenendaal et al., 2009). The natural compound library serves as another source of T3SS inhibitors. Cytosporone B, an octaketide, has been identified as a strong inhibitor of the T3SS of *Salmonella* Typhimurium (Li et al., 2013). Baicalin, a flavonoid, was reported as a T3SS inhibitor which affects microbial virulence mechanisms of *Salmonella* Typhimurium and subsequently detriments invasion of the bacteria into host cells (Tsou et al., 2016). Recently, a whole-genome microarray analysis of *Salmonella* Enteritidis revealed that two plant-derived compound, trans-cinnamaldehyde (TC) and eugenol (EG), reduced the translation of multiple virulence genes of the bacteria (Kollanoor Johny et al., 2017). However, the effect of cinnamaldehyde on *Salmonella* Typhimurium remains elusive. In this study, we added another natural agent to the list of compounds that inhibit the activity of the T3SS in *Salmonella* Typhimurium.

Instead of targeting bacterial central metabolic pathways, our results indicated that cinnamaldehyde decreased the production of key SPI-1 effector proteins SipA, SipB and SipC. We also identified the inhibition effect of cinnamaldehyde upon the invasion of *Salmonella* Typhimurium into HeLa cells. Treatment with cinnamaldehyde attenuated the mortality and pathology changes caused by *Salmonella* Typhimurium infection in mice. Further study revealed that cinnamaldehyde functions by inhibiting the transcription of several SPI-1 key regulator proteins, HilA, HilC, HilD and InvF, and up regulating the transcription of *hha,* which represses the transcription of SPI-1 genes. Furthermore, the effect of cinnamaldehyde on SPI-1 transcription regulators was evaluated in *Salmonella* strains recombinantly overexpressing HilC, HilD or InvF, respectively. Our results showed that levels of SPI-1 effector proteins showed no difference after treatment of cinnamaldehyde.

In summary, our study discovered that cinnamaldehyde effectively inhibits the production of several SPI-1 proteins and thus attenuates the virulence of S. Typhimurium both *in vitro* and *in vivo*. These findings indicate that anti-infective therapy by targeting T3SS-1 may represent a useful strategy for preventing and treating *Salmonella* Typhimurium infection.

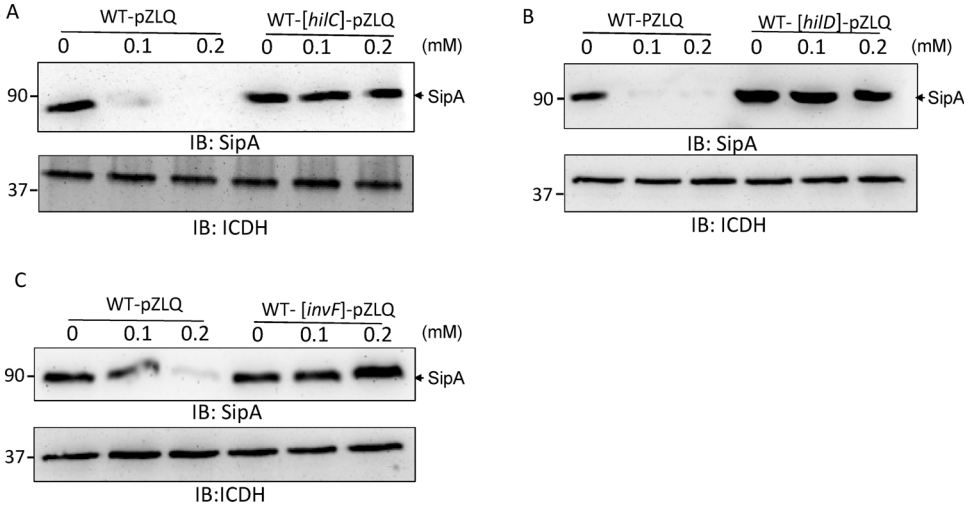

Fig. 7. Overexpression of HilC, HilD or InvF rescue the decreased level of SipA caused by cinnamaldehyde.
A. Cinnamaldehyde did not influence the level of SipA in HilC overexpression strain at concentration of 0.1 and 0.2 mM.
B. Overexpression of HilD also rescued the decreased level of SipA caused by cinnamaldehyde.
C. Cinnamaldehyde did not influence the level of SipA in *S.* Typhimurium strain carrying [*invF*]-PZLQ plasmid.

4. Experimental procedures

4.1. Bacterial strains and reagents

The wild type *Salmonella* Typhimurium strain SL1344 used in this study was generously provided by Dr. Xiaoyun Liu at Peking University, China. *S*. Typhimurium strains harboring 3xFLAG fused to *sipA* and *sipB* were generated by inserting 3xFlag into the chromosome (Zhang et al., 2018). Briefly, the opening reading frame of *sipA* or *sipB* was inserted into pKS3xFlag which provides the 3xFlag prior to the insertion of fragments of 807 and 820 bp upstream and downstream of the fusion, respectively. The *pir* protein dependent R6K vector pSR47S (Luo and Isberg, 2004) carrying the cassette was introduced into SL1344 using triparental mating (Datsenko and Wanner, 2000). Successful transconjugants were identified by PCR with appropriate primers after 15 % sucrose LB plates selection. SL1344 Δ*sipA* was constructed using the λ Red and FLP-mediated site-specific recombination as describe before (Datsenko and Wanner, 2000). The Δ*invA* mutant was a gift from Dr. Daoguo Zhou at Purdue University. Bacteria were grown in LB medium containing 0.3 M NaCl. When required, medium was supplemented with kanamycin 50 μg/ml, chloramphenicol 30 μg/ml or ampicillin 100 μg/ml. Cinnamaldehyde was purchased from Sigma. The anti-HilA antibody was generously provided by Dr. Yufeng Yao at Shanghai Jiaotong University. The anti-ICDH antibody was given by Dr. Zhaoqing Luo at Purdue University. The SipA-specific antibody and SipC-specific antibody was produced in our animal facility.

4.2. Measurement of bacterial growth

Salmonella Typhimurium SL1344 culture grown overnight was diluted at 1:100 in new LB broth and incubated with cinnamaldehyde at indicated concentrations. At the time points indicated, the OD_{600} of the culture was measured using an UV spectrophotometer. Three independent experiments were performed, and three replicates were analyzed in each experiment.

4.3. Bacterial invasion and immunostaining

HeLa cells were suspended in DMEM supplemented with 10 % fetal bovine serum (Biological Industry, USA) containing 100 U/mL penicillin and 100 μg/ml streptomycin (Invitrogen, USA). For invasion related assay, penicillin and streptomycin in the medium were removed and HeLa cells were seeded in 24-cell plates at a density of 1.2×10^5 per well and incubated for 12 h at 37 °C and 5 % CO_2. Overnight cultured wild type strain SL1344 and SL1344 Δ*sipA* were diluted 20-fold in LB broth and treated with cinnamaldehyde at indicated concentrations. After incubation in a shaker for 4 h at 37 °C, bacteria were added to HeLa cells at a MOI of 50:1 followed by centrifugation for 10 min at 1000 rpm. 50 min after incubation in 37 °C, cells were incubated with DMEM containing 100 μg/ml gentamicin at 37 °C for 1 h to kill extracellular bacteria. After washed three times with PBS, 1 mL 0.2 % saponin (Sigma Aldrich, USA) was added to lyse the cells. The CFU of bacteria were counted by plating 1:10 dilution in LB.

For the immunofluorescence assay, cell monolayers seeded on glass coverslips were infected as descried above in invasion assay and were washed with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for three times. 20 min after 4 % paraformaldehyde fixation, coverslips were washed with PBS for three times and incubated with PBS containing 50 mM NH_4Cl for 5 min. After washed another three times, cells were incubated with anti-*S.* Typhimurium antibody (Abcam, USA) (1:1000) diluted in PBS for 1 h. After 3x washings, goat anti-mouse secondary antibody conjugated to Alex Fluor 488 (Abcam, USA) was added and incubated for 30 min. Cells were then permeabilized by 0.3 % triton X-100 for 15 min and then washed three times. The primary antibody was used as described before and donkey anti-mouse antibody conjugated to Alex Fluor 594 (Abcam, USA) was used for 30 min. Finally, cells were washed and mounted on glass slides using DAPI (Life Technologies, USA). All images were obtained using a confocal laser-microscope (Olympus, Japan).

4.4. Gene cloning and overexpression of HilC, HilD and InvF in Salmonella typhimurium

To construct strains overexpressing HilC, HilD or InvF, gene sequences were amplified from the genomic DNA of the wild type strain SL1344 using primers described as follows: *hilC* forward, 5'-CGCGGATCCGTATTGCCTTCAATGAATAAATCAG-3' (BamHI); *hilC* reverse, 5'-CCGGAATTCTCAATGGTTCATTGTACGCATAAAG-3' (EcoRI). *hilD* forward, 5'-CGCGGATCCGAAAATGTAACCTTTGTAAGTAATAGTC-3' (BamHI); *hilD* reverse, 5'-CCGGAATTCTTAATGGTTCGCCATTTTTATGAAT-3' (EcoRI). *invF* forward, 5'-CGCGGAATGTCATTTTCTGAAAGCCGACA-3' (BamHI); *invF* reverse, 5'- CCGGAATCATTTGTCTGCCAATTGAAT. The DNA fragments and the plasmid pZLQ were digested by BamHIand EcoRIand then ligated by a T4 DNA ligase to construct *hilC*-pZLQ, *hilD*-pZLQ and *invF*-pZLQ. The constructed plasmids were then introduced into electrocompetent *Salmonella* Typhimurium.

4.5. Bacterial cell fractionation and Western blot assay

To study the influence of cinnamaldehyde on the level of SPI-1 associated effector proteins, *S*. Typhimurium wild type strain SL1344, strains expressing 3xFLAG fused to *sipA* and *sipB*, respectively, strains overexpressing HilC, HilD or InvF were grown overnight and diluted 100 times in fresh LB. Cells treated by cinnamaldehyde at indicated concentrations were cultured for 4 h at 37 °C. Bacteria were collected based on OD_{600} values of the cultures to confirm the equivalent numbers of bacteria in each sample. Cells were centrifuged for 2 min at 10,000 × g and the pellets were re-suspended with 100 μl 1 × SDS loading buffer. Samples were immediately heated for 5 min at 95 °C to denature the proteins.

Proteins were separated by 12 % SDS-PAGE and then transferred to a PVDF membrane using a semi-dry transfer apparatus (Bio-Rad, USA). The blotted membrane was incubated in blocking solution (5 % milk powder) for 2 h at room temperature. The membrane was incubated in 5 % milk powder supplemented with anti-SipA monoclonal antibody, anti-Flag monoclonal antibody, anti-SipC monoclonal antibody or anti-HilA monoclonal antibody for 2 h at room temperature or overnight at 4 °C and then washed three times with TBST for 10 min. Followed by incubated with anti-mouse IgG conjugated with horseradish peroxidase (HRP) for 1 h at room temperature and three times washing, the membrane was detected by the enhanced chemiluminescence (ECL) method.

4.6. RNA isolation and real-time RT-PCR

RT-qPCR was used to determine the effect of cinnamaldehyde on the transcription of SPI-1 related genes in *Salmonella* Typhimurium. Overnight culture of SL1344 at 37 °C was diluted 100-fold in 0.3 M NaCl LB and grown with cinnamaldehyde at indicated concentrations for 4 h. *S*. Typhimurium cells were collected, and total bacterial RNA was isolated using TRIzol (Simms et al., 1993) as described before. Briefly, cell pellets were resuspended by 1 mL TRIzol, and 200 μL chloroform was added to stand for 5 min. The mixture was vortexed for 30 s and centrifuged at 10,000 *g* for 10 min at 4 °C. The upper clear phase was transferred to a fresh tube and equal volume of isopropanol was added and the mixture was shaken vigorously. The precipitated RNA was centrifuged for 10 min at 4 °C and precipitated by ethanol again. RNA was resuspended in DEPC H_2O and stored in −80 °C. RNA concentrations were read by a micro volume spectrophotometer (Quawell Q6000).

Equal amounts of RNA were reverse transcribed into cDNAs using the TIANScript cDNA kit (Tian gen, China) according to the

manufacturer's instructions. The cDNAs were amplified using the FastStart Universal SYBR Green Master kit (Roche, Germany) and 10-pmol primers for target genes on a Bio-Rad iCycler Thermal Cycler. Followed by 40 PCR cycles, the melting curve was generated to ascertain template-independent amplification. All samples were analyzed in triplicate and the change in gene transcription was calculated using the method described before.

4.7. Animal experiment

Female BALB/c mice were obtained from the Experimental Animal Center of Jilin University. Mouse model for *Salmonella* infection was induced as described before (Suar et al., 2006). Briefly, mice were provided *ad libitum* with water containing streptomycin (5 g/L) for two days before treated with 50 mg cinnamaldehyde /kg of body weight by oral gavage at 8 h intervals for one day. Drinking water as libitum was offered 6-h before *Salmonella* infection, followed by 5×10^7 CFU of *Salmonella* Typhimurium infection or sterile PBS (control) treatment. Thereafter, mice were treated with cinnamaldehyde as described above for another five days. At day 6 post-infection, mice were sacrificed by cervical dislocation. To evaluate bacteria loads in spleen, liver and cecum, tissue samples were homogenized in cold PBS and serial dilutions of the homogenates were plated on LB plates under streptomycin selection, followed by overnight incubation in 37 °C. For histopathological analysis, segments of cecum, liver and spleen were fixed and embedded in paraffin according to standard procedures. Cryosections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Pathological evaluation was performed by two pathologists in a blinded manner. Cytokine levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacture's protocol (BioLegend, USA).

Acknowledgments

We thank Dr. Yufeng Yao (Shanghai Jiaotong University) for the HilA-specific antibody; Dr. Bradley Jones (University of Iowa), Dr. Daoguo Zhou (Purdue University), Dr. James Slauch (University of Illinois) for bacterial strains and Dr. Zhaoqing Luo (Purdue University) for the pZLQ plasmid. This work was supported by grant from the National Natural Science Foundation of China (31620103918).

References

Aiello, D., Williams, J.D., Majgier-Baranowska, H., Patel, I., Peet, N.P., Huang, J., et al., 2010. Discovery and characterization of inhibitors of Pseudomonas aeruginosa type III secretion. Antimicrob. Agents Chemother. 54 (5), 1988–1999.
Bajaj, V., Hwang, C., Lee, C.A., 1995. HilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes. Mol. Microbiol. 18 (4), 715–727.
Baron, C., Coombes, B., 2007. Targeting bacterial secretion systems: benefits of disarmament in the microcosm. Infect. Disord. Drug Targets 7 (1), 19–27.
Boucher, H.W., Ambrose, P.G., Chambers, H.F., Ebright, R.H., Jezek, A., Murray, B.E., et al., 2017. White paper: developing antimicrobial drugs for resistant pathogens, narrow-spectrum indications, and unmet needs. J. Infect. Dis. 216 (2), 228–236.
Charro, N., Mota, L.J., 2015. Approaches targeting the type III secretion system to treat or prevent bacterial infections. Expert Opin. Drug Discov. 10 (4), 373–387.
Coburn, B., Grassl, G.A., Finlay, B.B., 2007. Salmonella, the host and disease: a brief review. Immunol. Cell Biol. 85 (2), 112–118.
Cohen, M.L., 1992. Epidemiology of drug resistance: implications for a post—-antimicrobial era. Science 257 (5073), 1050–1055.
Darwin, K.H., Miller, V.L., 2001. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in Salmonella typhimurium. EMBO J. 20 (8), 1850–1862.
Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. 97 (12), 6640–6645.
Du, J., Reeves, A.Z., Klein, J.A., Twedt, D.J., Knodler, L.A., Lesser, C.F., 2016. The type III secretion system apparatus determines the intracellular niche of bacterial pathogens. Proc. Natl. Acad. Sci. 113 (17), 4794–4799.
Ellermeier, J.R., Slauch, J.M., 2007. Adaptation to the host environment: regulation of the SPI-1 type III secretion system in Salmonella enterica serovar Typhimurium. Curr. Opin. Microbiol. 10 (1), 24–29.
Fahlen, T.F., Wilson, R.L., Boddicker, J.D., Jones, B.D., 2001. Hha is a negative modulator of transcription of hilA, the Salmonella enterica serovar Typhimurium invasion gene transcriptional activator. J. Bacteriol. 183 (22), 6620–6629.
Forshell, L.P., Wierup, M., 2006. Salmonella contamination: a significant challenge to the global marketing of animal food products. Rev. Sci. Tech. Off. Int. Epiz 25 (2), 541–554.
Galán, J.E., 2009. Common themes in the design and function of bacterial effectors. Cell Host Microbe 5 (6), 571–579.
Galán, J.E., Bliska, J.B., 1996. Cross-talk between bacterial pathogens and their host cells. Annu. Rev. Cell Dev. Biol. 12 (1), 221–255.
Galán, J.E., Wolf-Watz, H., 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature 444 (7119), 567.
Gauthier, A., Robertson, M.L., Lowden, M., Ibarra, J.A., Puente, J.L., Finlay, B.B., 2005. Transcriptional inhibitor of virulence factors in enteropathogenic Escherichia coli. Antimicrob. Agents Chemother. 49 (10), 4101–4109.
Ginocchio, C.C., Galan, J.E., 1995. Functional conservation among members of the Salmonella typhimurium InvA family of proteins. Infect. Immun. 63 (2), 729–732.
Hayward, R.D., Koronakis, V., 1999. Direct nucleation and bundling of actin by the SipC protein of invasive Salmonella. EMBO J. 18 (18), 4926–4934.
Hayward, R.D., McGhie, E.J., Koronakis, V., 2000. Membrane fusion activity of purified SipB, a Salmonella surface protein essential for mammalian cell invasion. Mol. Microbiol. 37 (4), 727–739.
Iwatsuki, M., Uchida, R., Yoshijima, H., Ui, H., Shiomi, K., Matsumoto, A., et al., 2008. Guadinomines, type III secretion system inhibitors, produced by Streptomyces sp. K01-0509. J. Antibiot. 61 (4), 222.
Kauppi, A.M., Andersson, C.D., Norberg, H.A., Sundin, C., Linusson, A., Elofsson, M., 2007. Inhibitors of type III secretion in Yersinia: design, synthesis and multivariate QSAR of 2-arylsulfonylamino-benzanilides. Bioorg. Med. Chem. 15 (22), 6994–7011.
Kline, T., Felise, H.B., Barry, K.C., Jackson, S.R., Nguyen, H.V., Miller, S.I., 2008. Substituted 2-imino-5-arylidenethiazolidin-4-one inhibitors of bacterial type III secretion. J. Med. Chem. 51 (22), 7065–7074.
Kollanoor Johny, A., Frye, J.G., Donoghue, A., Donoghue, D.J., Porwollik, S., McClelland, M., Venkitanarayanan, K., 2017. Gene expression response of Salmonella enterica serotype Enteritidis phage type 8 to subinhibitory concentrations of the plant-derived compounds trans-cinnamaldehyde and eugenol. Front. Microbiol. 8, 1828.
Koraimann, G., 2003. Lytic transglycosylases in macromolecular transport systems of gram-negative bacteria. Cell. Mol. Life Sci. CMLS 60 (11), 2371–2388.
Levy, S.B., 1998. The challenge of antibiotic resistance. Sci. Am. 278 (3), 46–53.
Li, J., Lv, C., Sun, W., Li, Z., Han, X., Li, Y., Shen, Y., 2013. Cytosporone B, an inhibitor of the type III secretion system of Salmonella enterica serovar Typhimurium. Antimicrob. Agents Chemother. 57 (5), 2191–2198.
Linington, R.G., Robertson, M., Gauthier, A., Finlay, B.B., van Soest, R., Andersen, R.J., 2002. Caminoside A, an antimicrobial glycolipid isolated from the marine sponge Caminus sphaeroconia. Org. Lett. 4 (23), 4089–4092.
Luo, Z.Q., Farrand, S.K., 1999. Signal-dependent DNA binding and functional domains of the quorum-sensing activator TraR as identified by repressor activity. Proc. Natl. Acad. Sci. 96 (16), 9009–9014.
Luo, Z.Q., Farrand, S.K., 2001. The Agrobacterium tumefaciens rndHomolog is required for TraR-Mediated quorum-dependent activation of Ti plasmid tra gene expression. J. Bacteriol. 183 (13), 3919–3930.
Luo, Z.Q., Isberg, R.R., 2004. Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer. Proc. Natl. Acad. Sci. 101 (3), 841–846.
Piddock, L.J., White, D.G., Gensberg, K., Pumbwe, L., Griggs, D.J., 2000. Evidence for an efflux pump mediating multiple antibiotic resistance in Salmonella enterica serovar Typhimurium. Antimicrob. Agents Chemother. 44 (11), 3118–3121.
Rasko, D.A., Sperandio, V., 2010. Anti-virulence strategies to combat bacteria-mediated disease. Nat. Rev. Drug Discov. 9 (2), 117.
Simms, D., Cizdziel, P.E., Chomczynski, P., 1993. TRIzol: a new reagent for optimal single-step isolation of RNA. Focus 15 (4), 532–535.
Sockett, P.N., 1995. The epidemiology and costs of diseases of public health significance, in relation to meat and meat products. J. Food Saf. 15 (2), 91–112.
Suar, M., Jantsch, J., Hapfelmeier, S., Kremer, M., Stallmach, T., Barrow, P.A., Hardt, W.D., 2006. Virulence of broad-and narrow-host-range Salmonella enterica serovars in the streptomycin-pretreated mouse model. Infect. Immun. 74 (1), 632–644.
Thanassi, D.G., Hultgren, S.J., 2000. Multiple pathways allow protein secretion across the bacterial outer membrane. Curr. Opin. Cell Biol. 12 (4), 420–430.
Threlfall, E.J., 2002. Antimicrobial drug resistance in Salmonella: problems and perspectives in food-and water-borne infections. FEMS Microbiol. Rev. 26 (2), 141–148.
Tsou, L.K., Lara-Tejero, M., RoseFigura, J., Zhang, Z.J., Wang, Y.C., Yount, J.S., et al., 2016. Antibacterial flavonoids from medicinal plants covalently inactivate type III protein secretion substrates. J. Am. Chem. Soc. 138 (7), 2209–2218.
Tsou, L.K., Dossa, P.D., Hang, H.C., 2013. Small molecules aimed at type III secretion systems to inhibit bacterial virulence. Medchemcomm 4 (1), 68–79.
Veenendaal, A.K., Sundin, C., Blocker, A.J., 2009. Small-molecule type III secretion system inhibitors block assembly of the Shigella type III secreton. J. Bacteriol. 191 (2), 563–570.
Waterman, S.R., Holden, D.W., 2003. Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system. Cell. Microbiol. 5 (8), 501–511.
Yuan, R., Lin, Y., 2000. Traditional Chinese medicine: an approach to scientific proof and clinical validation. Pharmacol. Ther. 86 (2), 191–198.
Zhang, Y., Liu, Y., Wang, T., Deng, X., Chu, X., 2018. Natural compound sanguinarine chloride targets the type III secretion system of Salmonella enterica Serovar Typhimurium. Biochem. Biophys. Rep. 14, 149–154.
Zhou, D., Galán, J., 2001. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. 3 (14-15), 1293–1298.
Zhou, D., Mooseker, M.S., Galán, J.E., 1999. Role of the S. typhimurium actin-binding protein SipA in bacterial internalization. Science 283 (5410), 2092–2095.