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ORIGINAL ARTICLE

Inhibitory effect of obovatol from *Magnolia obovata* on the *Salmonella* type III secretion system

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In many pathogenic Gram-negative bacteria, such as *Salmonella*, *Escherichia coli*, *Yersinia* and *Chlamydia* spp., which cause diseases in humans, the type III secretion system (TTSS) is an important virulence factor that translocates effector proteins into the cytosol of host cells. Thus, the TTSS is a good target for antibacterial agents. Here we used a hemolysis assay to search for TTSS inhibitors and found that a compound from *Magnolia obovata* called obovatol blocks the TTSS of *Salmonella*. Obovatol showed potent inhibitory activity ($IC_{50} = 19.8\ \mu M$) against the TTSS-related hemolysis of *Salmonella*, which was not due to a reduction of bacterial growth. Instead, the compound inhibited bacterial motility, TTSS-related mRNA expression and effector protein secretion. These data demonstrate the inhibitory effect of obovatol on the *Salmonella* TTSS and suggest that it could be useful for the prevention and supplementary treatment of bacterial infections.
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INTRODUCTION

Type III secretion systems (TTSSs) are essential for the virulence of many Gram-negative pathogens of plants, animals and humans.[1,2] TTSSs contain more than 20 different proteins[3] and the proteins that make up the TTSS apparatus are termed structural proteins. The TTSS apparatus is composed of three definite substructures: a cytoplasmic bulb, a region spanning the inner and outer bacterial membranes and a hollow 'needle' produced from the bacterial surface.[4–7] The TTSS shows structural similarity to the bacterial flagella and some proteins in the TTSS also share amino acid sequence homology to flagellar proteins.[8,9] The TTSS is activated upon contact with the host cell and it aids the translocation of several bacterial proteins into the host cell cytoplasm.[10] Protein translocation is facilitated by several proteins, called translocators, which produce a pore in the host cell membrane. The translocated proteins are termed effectors. In other words, effector proteins are delivered through a hollow tube-like needle and directly into the host cell through the pore formed in the host cell membrane by translocator proteins.[11] The injected proteins alter the signal transduction pathways in the host cell to enhance bacterial attachment, invasion and survival. They can also induce necrosis, apoptosis, actin cytoskeleton disruption and immune response attenuation in the host cells. These effects promote pathological outcomes, such as diarrhea and inflammation.[12,13]

The human and animal Gram-negative pathogens that utilize TTSS for infection include enteropathogenic *E. coli* and *Salmonella*, *Shigella*, *Vibrio*, *Chlamydia*, *Yersinia* and *Pseudomonas* species. *Salmonella* species are major human and animal bacterial pathogens that cause a broad spectrum of diseases ranging from mild diarrhea to systemic infections like typhoid fever.[14] *Salmonella* encodes two TTSSs in discrete pathogenicity islands; *Salmonella* pathogenicity island 1 (SPI-1), which is required for efficient invasion of the intestinal epithelium to cause intestinal diseases, and SPI-2, which is essential for replication and survival within macrophages and systemic infections.[15] Expression of the SPI-1 and SPI-2 TTSS is regulated by many environmental and genetic signals.[16]

The prevalence of antibiotic-resistant bacteria is increasing to dangerously high levels worldwide.[17] Although traditional antibiotics were previously effective against many bacteria, new antibiotic-resistant strains are continuously emerging and spreading globally, threatening our ability to treat common infectious diseases.[18,19] Thus, there is a high possibility of untreatable bacterial diseases appearing in the near future. The rising incidence of antibiotic-resistant pathogenic bacteria warrants the development of novel strategies to treat bacterial infections. $TTSS_S$ are attractive targets for novel antibiotics, as with such compounds there should be less selective pressure for the development of resistance, because they can inhibit infection without killing the bacteria.[20–22]

To enable the identification of novel antibacterial agents, we utilized a TTSS-induced hemolysis assay. This assay can be used to identify compounds that directly or indirectly affect the TTSS of *Salmonella*.

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Table 1 Sequences of the RT-PCR primers used in this study

Gene	*Forward primers*	*Reverse primers*
sipA	5′-TGT TCG GCT ATT ATC AAT CGT CT-3′	5′-CGC AGC AAT CTT ACG CAC CT-3′
sipB	5′-CTG ACT GGG CTG CGG TAT TCG TG-3′	5′-CTG CGG TGG GAC TTG CGG TAA-3′
sipC	5′-GCC TTC AGC ACC GAG TTT G-3′	5′-TG TCA CGA CTA AAG CGA ATG AG-3′
sopA	5′-ATT CAG ACA CGG CGA TGA TG-3′	5′-TGG CGT CCG TCA GGT GAT AAG CA-3′
sopB	5′-TGA GTA ACC CGA CGG ATA CCA GT-3′	5′-AGC ATC AGA AGG CGT CTA ACC AC-3′
sopD	5′-TTA CTA TCA AGA TGG ACG CTT CT-3′	5′-GTG CAT TTC CCG TCA CTT-3′
sop E2	5′-CGG CGT AAC CTC TTT CAT AAC GA-3′	5′-AGG GTA GGG CGG TAT TAA CCA GT-3′
mdh	5′-ATG AAA GTC GCA GTC CTC GGC GCT GCT GGC GG-3′	5′-ATA TCT TTT TTC AGC GTA TCC AGC AT-3′

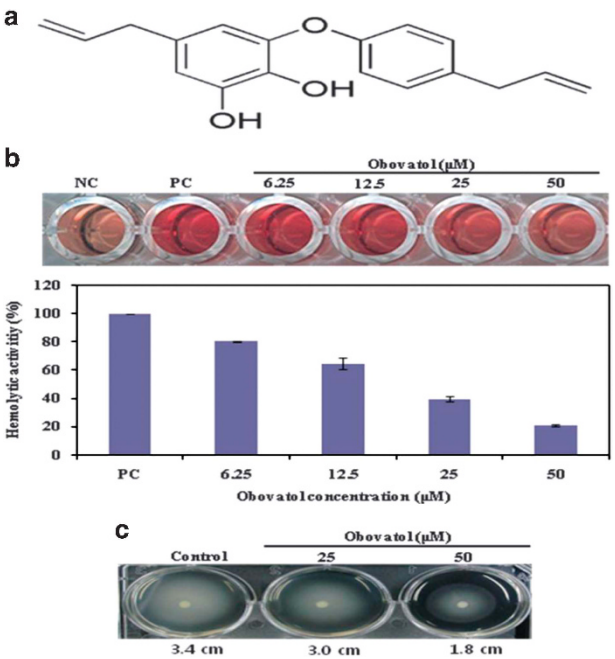

Figure 1 Identification of the *Salmonella* TTSS inhibitor obovatol and its effects on *Salmonella* motility. (**a**) Structure of the TTSS inhibitor obovatol. (**b**) Obovatol suppresses TTSS-mediated contact-dependent hemolysis. Obovatol had observable effect on the TTSS-mediated contact-dependent hemolysis of sheep red blood cells. *S. typhimurium* SL1344 was incubated with sheep blood cells for 2 h in the absence or presence of various concentrations of obovatol and hemolytic activity was measured as the absorbance at 530 nm (A_{530}) with a spectrophotometer. NC, without *Salmonella*; PC, *Salmonella* without obovatol; Obovatol, *Salmonella* treated with various concentrations of obovatol (in μM). (**c**) Effect of obovatol on *Salmonella* motility. A motility assay was performed by spot inoculation (1 μl) on 0.3% agar plates containing 0, 25 or 50 μM obovatol. The plates were incubated at 37 °C for 20 h and the migration distance was measured. The mean zone diameters in the presence of 0, 25 and 50 μM obovatol were 3.4 ± 0.10, 3.0 ± 0.20 1.8 ± 0.06 cm, respectively. The data represent the averages of three independent experiments with s.d.

We screened a compound library of 5000 small molecules for compounds that inhibit the TTSS at concentrations that do not reduce bacterial growth. We then investigated the effects of an identified small molecule inhibitor, obovatol, on the *Salmonella* TTSS.

MATERIALS AND METHODS

Bacteria strain and growth conditions

The bacterial strain used in this study, *Salmonella typhimurium* SL1344, has been described previously.[23] The strain was inoculated into 10 ml of brain heart infusion (BHI) broth (Difco, Franclin Lakes, NJ, USA) for growing at 37 °C for 14 h and was used for hemolysis and motility assays.

Hemolysis assay

A TTSS-mediated hemolytic assay was performed as described by Galyov *et al.*[24] Briefly, *S. typhimurium* SL1344 was cultured overnight in BHI broth at 25 °C with shaking. Overnight cultures were diluted 10-fold in fresh medium and incubated at 37 °C with shaking for 1 h. Sheep blood was mixed with cold phosphate-buffered saline and centrifuged three times at 10 000 *g* for 5 min to obtain red blood cells. After incubation, the bacterial cells were recovered by centrifugation, washed in phosphate-buffered saline, resuspended in fresh BHI medium. The bacteria were then mixed with the prepared red blood cells in a microcentrifuge tube and centrifuged for 10 min at 6000 *g*. Then, the mixture was incubated for 4 h at 37 °C. After incubation, the cell pellets were resuspended in phosphate-buffered saline and then centrifuged for 5 min at 6000 *g*. An aliquot (100 μl) of the supernatant was carefully transferred to a 96-well plate and the absorbance at 530 nm (A_{530}) was measured.

Motility assay

S. typhimurium SL1344 was grown overnight at 37 °C. Motility plates were prepared for the assay by pouring 4 ml of motility agar (0.3%) into the wells of a six-well plate. Motility agar was supplemented with the test compounds (at final concentrations of 25 and 50 μM), which was added before the agar (50 °C) was poured into the plates. Then, 1 μl of *S. typhimurium* SL1344 was inoculated onto the surface of the agar. The plates were incubated at 37 °C for 20 h and motility was measured as the diameter of the halo zone.

Bacterial growth

S. typhimurium SL1344 was inoculated into BHI broth and cultivated for 14 h at 25 °C. Then, these overnight cultures were diluted 10-fold into fresh medium and the test compounds were added. The cultures were incubated at 37 °C under aerobic conditions for 4 h. An aliquot (100 μl) of each culture was spread onto an agar plate and incubated for 24–48 h at 37 °C. After incubation, the colonies on the agar plates were manually counted using a click counter.

Determination of MICs

The MICs of the test compounds were determined against various human pathogens, including *S. typhimurium* ATCC14028, *S. typhimurium* SL1344, *E. coli* ATCC25922, *Pseudomonas aeruginosa* ACTT278553, and *Staphylococcus aureus* ACTT6538p, using the serial dilution method.[25,26] *Salmonella* species were cultivated in BHI broth for 14 h at 37 °C. These overnight cultures were diluted into fresh medium, and adjusted to $\sim 10^5$ CFU ml^{-1}. Then, an aliquot (200 μl) of each culture was added to the first well of a 96-well plate followed by the addition of 5 μl of a test compound (20.48 mg ml^{-1}). Then, 2-fold serial dilutions of each compound were added to subsequent wells. After 24–48 h of incubation at 37 °C, growth was measured to assess antibacterial activity. The MIC was defined as the concentration that completely inhibited the growth of an organism when compared with a control well containing no test compound.

Assessment of test compound effects on protein secretion

Protein secretion by the *Salmonella* TTSS can be induced *in vitro* by changing the cultivation temperature from 25 to 37 °C.[27] Thus, to test the functionality of

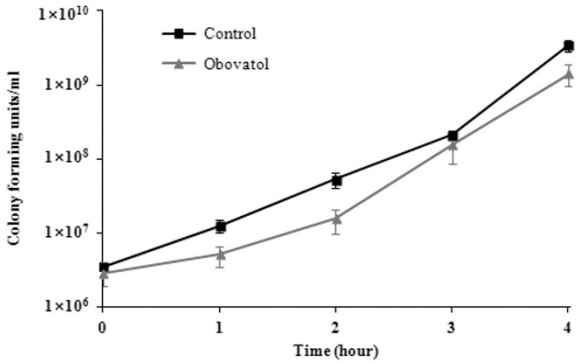

Figure 2 Effect of obovatol on the growth of *S. typhimurium* SL1344. Obovatol inhibits the *Salmonella* TTSS, but it does not inhibit bacterial growth. An overnight culture of *S. typhimurium* SL1344 was diluted 10-fold into fresh medium and grown in the absence or presence of 400 μM obovatol for 4 h. Aliquots were obtained at 1 h intervals and spread plated to determine the number of viable bacteria. Each point represents the mean of three independent experiments. A full colour version of this figure is available at the *Journal of Antibiotics* journal online.

the TTSS after exposure to the test compounds, overnight cultures of *S. typhimurium* SL1344 (BHI broth, 14 h, at 25 °C) were inoculated into fresh medium containing test compounds and incubated for 4 h at 37 °C. The proteins secreted by *S. typhimurium* SL1344 into the culture supernatant were precipitated with trichloroacetic acid (final concentration, 10% (vol/vol)) and collected by centrifugation (12 000 r.p.m., 20 min, 4 °C). The protein pellets were washed with cold acetone, resuspended in SDS-PAGE loading buffer, separated by SDS-PAGE and stained with Coomassie blue.

RNA isolation and RT-PCR

Overnight cultures of *S. typhimurium* SL1344 (BHI broth, 25 °C) were diluted 10-fold into fresh BHI. Then, test compounds were added and the mixtures were incubated for 4 h at 37 °C. The cells were collected by centrifugation (30 min, 7000 r.p.m.) and total RNA was isolated from the bacteria using TRIzol reagent (MRC TRI-Reagent; Molecular Research Center, Cincinnati, OH, USA). Next, cDNA was generated from the RNA using the SuperScript III Fist-Strand Synthesis system (Invitrogen, catalog number 18080-051, Carlsbad, CA, USA) according to the manufacturer's directions. Then, RT-PCR was performed to assess the expression of TTSS-related genes. Each RT-PCR contained 50 ng of cDNA, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 1.5 mM $MgCl_2$, 10 pM primer, 250 μM dNTP and 1 unit of Taq polymerase (AccuPower PCR PreMix, BIONEER, Daejeon, Korea) in a total volume of 20 μl. The PCR primers used to amplify the specific cDNAs are shown in Table 1. PCR was performed under the following cycling conditions: denaturation at 94 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C of 30 s, with a final extension at 72 °C for 3 min. PCR products were separated by electrophoresis through 1% agarose gels and stained with ethidium bromide. DNA bands were visualized with a BioDoc-it Imaging System (UVP, Upland, CA, USA).

RESULTS

Identification of TTSS inhibitors by their reduction of TTSS-mediated hemolytic activity

A hemolysis assay was used to screen a compound collection for inhibitors of the *Salmonella* TTSS. One of the compounds identified was obovatol (Figure 1a). As shown in Figure 1b, obovatol showed dose-dependent inhibition of TTSS-mediated hemolysis. The concentration of obovatol that caused 50% inhibition of hemolysis (IC_{50}) was 19.8 μM.

The bacterial flagellum is a large complex structure that is used for motility by many bacteria. The flagella is evolutionarily and structurally related to the TTSS.[28] As the type III secretion apparatus and the flagella share many similarities, the effect of obovatol on bacterial motility was also examined.[29] The results of the motility assay showed that *S. typhimurium* SL1344 motility was remarkably reduced in the presence of 25 μM or 50 μM obovatol. The mean diameter in the presence of 50 μM obovatol (Figure 1c), 1.8 cm, was obviously smaller than that of the control (3.4 cm). Therefore, the bacteria were much less motile in the presence of obovatol than in its absence (untreated) and dose-dependent inhibition of motility was observed. These results indicated that obovatol inhibited both the TTSS and motility of *S. typhimurium* SL1344.

The TTSS inhibitor obovatol does not affect bacterial growth

To test whether obovatol inhibited bacterial growth, we performed assays to determine the viable bacterial counts in the presence of

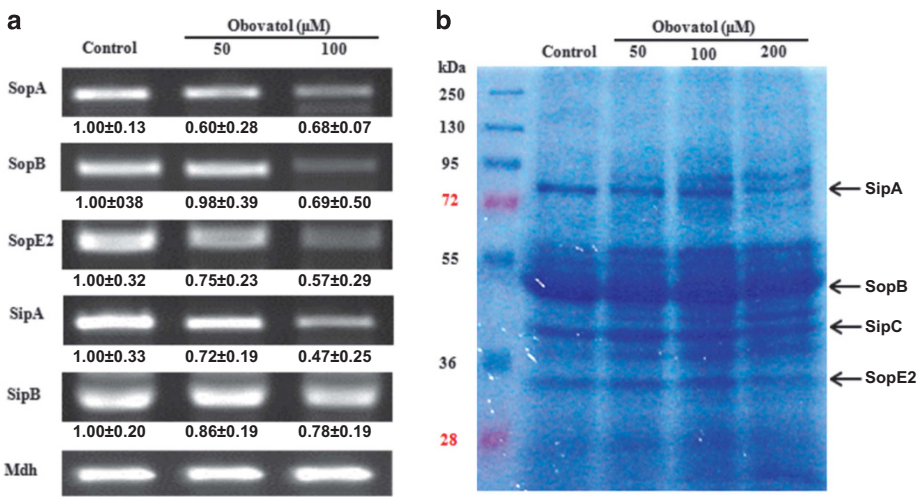

Figure 3 Suppression of TTSS-related mRNA expression and protein secretion by obovatol. (**a**) Effector gene expression in *S. typhimurium* SL1344 as determined by RT-PCR. Representative RT-PCR results for effector mRNA expression. *Mdh* was used as an internal control. Control, untreated *Salmonella*; Obovatol, *Salmonella* treated with obovatol. (**b**) Secretory protein profile of untreated control and obovatol-treated *Salmonella*. Obovatol decreased the production of type III system-associated proteins. The expression of secretory proteins was assessed by SDS-PAGE and Coomassie blue staining. Arrows on the figure indicate the suppressed proteins. The molecular sizes of the marker proteins are shown on the left.

Table 2 ***In vitro*** **antibacterial activities of obovatol against various pathogenic bacteria**

Pathogenic bacteria	*MIC in μg ml⁻¹ (MIC in μM)*	
	Obovatol	*Nalidixic acid*[a]
E. coli ATCC 25922	>512 (>1813.4)	4 (17.2)
P. aeruginosa ATCC 278553	>512 (>1813.4)	128 (551.2)
S. typhimurium ATCC 14028	>512 (>1813.4)	8 (34.4)
Salmonella typhimurium SL1344	>512 (>1813.4)	8 (34.4)
S. aureus ATCC 6538p	32 (113.3)	16 (68.9)

[a]Positive control.

obovatol and the MIC. The growth of *S. typhimurium* SL1344 was assessed by cultivating the bacteria in BHI medium with obovatol and plotting the CFU ml^{-1} against exposure time to obovatol (400 μM) and comparing it to bacteria grown in BHI without obovatol (control). Bacteria were counted at 1 h intervals for 4 h. The growth curve showed that 400 μM obovatol in BHI broth did not inhibit the growth of *S. typhimurium* SL1344 (Figure 2), as the growth curve in the presence of obovatol was similar to that of the control. The MIC was determined by growing bacteria at 37 °C in a 96-well plate containing BHI medium with different concentrations of obovatol. The MICs of obovatol for *Salmonella* species and *S. aureus* ATCC 6538p were >512 and 32 μg ml^{-1}, respectively (Table 2). In contrast, the MIC of nalidixic acid for *E. coli* ATCC25922, *P. aeruginosa* ATCC278553, *Salmonella* species and *S. aureus* ATCC 6538p were in range of 4–128 μM (Table 2). These results suggest that the inhibition of TTSS did not reduce bacterial growth, as the viability of the bacterial cells did not differ on plates with or without obovatol.

The TTSS inhibitor obovatol regulates the expression of TTSS effector genes and secreted proteins

To determine whether obovatol affected the expression of TTSS effectors, we investigated the mRNA expression of *Salmonella* effector proteins (virulence factors) in *S. typhimurium* SL1344 in the presence and absence of obovatol. In this experiment, 50–100 μM obovatol was used. As shown in Figure 3a, the mRNA expression of *sipA*, *sipB*, *sopA*, *sopB* and *sopE2* was downregulated in the presence of obovatol. Although mRNA expression was not significantly inhibited in the presence of 50 μM obovatol, expression was more affected in the presence of 100 μM obovatol. *Mdh* is used as bacterial reference gene for normalize RT-PCR between samples.

The protein expression level may differ from the mRNA expression level. Therefore, protein secretion by the *Salmonella* TTSS was monitored by using SDS-PAGE and Coomassie blue staining. As shown in Figure 3b, effector protein secretion by S. *typhimurium* SL1344 was suppressed in the presence of obovatol. Obovatol (at 50–200 μM) exhibited dose-dependent inhibitory effects on TTSS-mediated protein secretion. The RT-PCR and SDS-PAGE data suggest that the mRNA expression and TTSS-mediated secretion of certain effectors were inhibited by obovatol.

DISCUSSION

TTSS are used by various bacteria to infect host cells. The TTSS has a remarkable ability to deliver effector proteins directly into the host cell cytoplasm. There, these translocated effectors can disrupt various cellular processes by altering signaling pathways, modulating transcription, hijacking intracellular transport, modifying the cytoskeleton and suppressing the host immune response. The TTSS is a common virulence-associated system present in many pathogenic Gram-negative bacteria. Hence, this system is a good target for the development of novel antibacterial agents.

Type III secretion has been correlated with contact-dependent hemolysis of red blood cells *in vitro* and lysis of erythrocytes by TTSS has been observed in a number of bacteria such as *Shigella*, enteropathogenic *E. coli*, *Yersinia*, *Bordetella* and *Salmonella*. In *Salmonella*, the TTSS translocator proteins SipB, SipC and SipD mediate translocation into the host cell, which causes contact-dependent hemolysis of red blood cells.[30,31] Contact-dependent hemolysis of sheep red blood cells by *Salmonella* can be observed by measuring the A_{530} of the culture supernatant to detect the presence of hemoglobin leakage.[32] Thus, a TTSS-mediated hemolytic assay was used to screen for inhibitors of the *S. typhimurium* SL1344 TTSS. We found that obovatol blocks the TTSS of *S. typhimurium* SL1344. This small-molecule compound is present in the bark of the *M. obovata* tree and it is known to have various biological activities, including anti-proliferative, neurotrophic, antioxidant, anti-platelet, anti-fungal and anti-inflammatory activities.[33–35] In this study, obovatol showed *Salmonella*-TTSS inhibition, with an IC_{50} of 19.8 μM, but had no effects on bacterial growth.

The TTSS of *Salmonella* is related to the flagellum assembly. A great number of TTSS proteins show amino acid sequence and/or structural similarity to those in the basal body of the bacterial flagellum. Therefore, we also tested whether obovatol could inhibit the motility of *S. typhimurium* SL1344. We demonstrated that the motility of this strain was significantly decreased in the presence of obovatol.

Pathogenicity islands are present in the genomes of many Gram-negative and Gram-positive bacteria. These pathogenicity islands have essential roles in the virulence of several bacterial pathogens of humans, animals and plants. The *Salmonella* TTSS is encoded by SPI-1 and SPI-2, which encode effector proteins such as PrgI, SifA, SifB, SipA, SipB, SpaO, SptP, SopA, SopB, SopD, SopE2 and so on. We performed RT-PCR and SDS-PAGE to test whether SPI gene expression and effector protein secretion, respectively, were affected by obovatol. First, we assessed the mRNA expression levels of *S. typhimurium* SL1344 effectors. The expression of some effectors, including *sipA*, *sopA* and *sopE2*, were strongly downregulated in the presence of obovatol, and *sipB* and *sopB* were slightly downregulated in the presence of obovatol. Finally, we investigated protein secretion by *S. typhimurium* SL1344 with and without obovatol. In the presence of 50 μM obovatol, secretory protein levels were slightly reduced, and in the presence of 200 μM obovatol, protein secretion was completely inhibited.

In conclusion, our results show that obovatol inhibited the TTSS of *Salmonella* and we expect that this knowledge will be useful for the development of novel antibacterial agents. In future studies, we will focus on determining the toxicity of obovatol and clarifying the mechanisms underlying TTSS inhibition.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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