Plumbagin Inhibits Expression of Virulence Factors and Growth of *Helicobacter pylori*

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**Introduction**

*Helicobacter pylori* primarily colonizes the human stomach. Infection by this bacterium is associated with various gastric diseases, including inflammation, peptic ulcer, and gastric cancer. Although there are antibiotic regimens for the eradication of *H. pylori*, the resistance of this species against antibiotics has been continuously increasing. The natural compound plumbagin has been reported as an antimicrobial and anticancer molecule. In this study, we analyzed the inhibitory effect of plumbagin on *H. pylori* strain ATCC 49503 as well as the expression of various molecules associated with *H. pylori* growth or virulence by immunoblotting and reverse transcription polymerase chain reaction (RT-PCR) analyses. We demonstrated the minimal inhibitory concentration of plumbagin on *H. pylori* through the agar dilution and broth dilution methods. Furthermore, we investigated the effect of plumbagin treatment on the expression of the RNA polymerase subunits and various virulence factors of *H. pylori*. Plumbagin treatment decreased the expression of RNA polymerase subunit alpha (rpoA), which is closely associated with bacterial survival. Moreover, the mRNA and protein levels of the major CagA and VacA toxins were decreased in plumbagin-treated *H. pylori* cells. Likewise, the expression levels of urease subunit alpha (ureA) and an adhesin (alpA) were decreased by plumbagin treatment. Collectively, these results suggest that plumbagin may inhibit the growth, colonization, and pathogenesis of *H. pylori* by the mechanism demonstrated in this study.

**Keywords:** *Helicobacter pylori*, plumbagin, anti-microbial, virulence factor

Plumbagin is a naturally occurring compound originated from Plumbaginaceae, Droseraceae and Ebenceae families [13]. Crude extracts of plumbagin have been used to cure rheumatoid arthritis, dysmenorrhea, and toothache in the folk remedy [24]. Many reports have been suggested anti-bacterial, anti-fungal, anti-diabetic and anti-cancer effect of plumbagin [6, 7, 11, 19, 30]. Park *et al.* previously reported inhibitory function of plumbagin on Western type *H. pylori* strain (ATCC 43504), although the mechanism is yet to be elucidated [25].

*Helicobacter pylori* is a Gram-negative bacterium possessing its characteristic helical appearance. *H. pylori* primarily colonizes on human stomach and it has been reported to infect approximately half of the world population [33]. Infection of *H. pylori* on gastric mucosa is associated with various gastric disease including inflammation, chronic gastritis, peptic ulcer and gastric adenocarcinoma [4]. Moreover, because of its involvement in the gastric cancer development, *H. pylori* was classified as a group I carcinogen by WHO [10].
RNA polymerase is an indispensible protein responsible for transcription of DNA into RNA. Bacterial RNA polymerase consists of α, β and σ subunits (α2β'σ') [1]. β and β' are the largest subunits of bacterial RNA polymerase possessing catalytic site, and β and β' subunits in *H. pylori* exist as a single fused protein [1, 5]. α subunit binds to β and β' subunits and stabilizes the complex, and σ subunit has both structural and functional role [1, 20]. In addition, there are σ factors which binds to core complex and facilitate promoter specific initiation of transcription [1, 28].

Various bacterial proteins are associated with virulence and pathogenesis of *H. pylori* such as toxins, ureases, adhesion proteins and flagella proteins. The most studied virulence factors of *H. pylori* are cytotoxin-associated protein A (CagA) and vacuolating cytotoxin A (VacA). CagA protein is translocated to the host cells by Cag pathogenicity island (cagPAI) type IV secretion system [23]. Once injected, CagA proteins are phosphorylated by host cell Src kinases at its EPIYA motif and subsequently deregulate intracellular signaling transcription pathways, disrupt epithelial cell junctions, and induce inflammation [4, 9, 26, 29]. VacA has been known to induce cytoplasmic vacuole formation [3]. Moreover, VacA increases β-catenin level in the host cells by inhibiting glycogen synthase kinase 3-β and leads to uncontrolled cell growth [21].

Urease is one of the major virulence factors, which enables *H. pylori* to successfully colonize on gastric mucosa. The bacterium typically colonizes the strongly acidic mucosal lining of the stomach [16]. Therefore, increase in pH of the environment by secretion of urease allows the bacterium to persist in the hostile conditions [16]. The *H. pylori* urease comprises α and β that were known to form a dodecameric complex ((αβ)5)4 [16].

The adherence of *H. pylori* on the gastric mucosa is the first step of the bacterial colonization. Therefore, adhesins are also an important virulence factor that helps the bacterium to overcome the mucus and exfoliation of the epithelium [12, 27]. Adhesins of *H. pylori* belong to the largest outer membrane protein (OMP) family, namely, the Hop family which involves BabA, SahA, AlpA, AlpB, HopZ, and OipA [12].

In this study, we demonstrated minimal inhibitory concentration (MIC) of plumbagin on East Asian type reference strain of *H. pylori* (ATCC 49503) by agar dilution method and investigated expression of RNA polymerase subunits as well as various virulence factors after plumbagin treatment.

### Materials and Methods

#### Materials

*H. pylori* reference strain was purchased from ATCC (ATCC49503, VA, USA). Mueller-Hinton broth, Mueller-Hinton agar and Brucella agar were purchased from Becton-Dickinson (MA, USA). Bovine serum was purchased from Gibco (NY, USA). Trizol reagent, random hexamer, and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) were purchased from Invitrogen (CA, USA). Plumbagin and protease inhibitor cocktail were obtained from Sigma-Aldrich (MO, USA). Antibodies to detect CagA and VacA were purchased from Santa Cruz Biotechnology (TX, USA) and polyclonal antibody against whole *H. pylori* (ATCC 49503) was produced as previously described [14]. ECL kit was purchased from Thermo Scientific (MA, USA).

#### Bacterial culture

*H. pylori* were grown on the Brucella agar plate supplemented with 10% bovine serum at 37°C for 72 h in a humidified atmosphere with 5% CO₂. For cultivation in broth, bacterial colonies were collected and suspended in Mueller-Hinton broth supplemented with 10% bovine serum. The number of bacterial particles in the *H. pylori* suspension was set to MacFarland 0.33 and incubated at 37°C for 72 h in a humidified atmosphere with 5% CO₂.

#### Agar dilution method to determine MIC

*H. pylori* grown on the Brucella agar plate were collected and suspended in saline. The number of bacterial particles in the *H. pylori* suspension was set to MacFarland 4.0. Thirty µl of the bacterial suspension was placed on the Mueller-Hinton agar supplemented with 10% bovine serum including indicated concentrations of plumbagin. The bacteria were incubated for 72 h and MIC was determined based on the lowest concentration at which inhibition of growth was observed.

#### Broth dilution method to determine MIC

*H. pylori* grown on the Brucella agar plate were collected and suspended in Mueller-Hinton broth supple-
mented with 10% bovine serum. The number of bacterial particles in the *H. pylori* suspension was set to MacFarland scale 0.5. Various concentrations of plumbagin (125 nM–4 μM) were treated and the bacteria were incubated for 72 h and final optical density (600 nm wavelength) of the bacterial suspension was measured by spectrophotometry.

**RT-PCR (reverse transcription-polymerase chain reaction)**

*H. pylori* was grown in the Mueller-Hinton broth for 72 h. Cultured *H. pylori* were washed twice with PBS and total RNA was extracted using Trizol reagent as described in the manufacturer’s instructions. cDNA was synthesized by reverse transcription with 2 μg total RNA, 0.25 μg of random hexamer and 200 U of MMLV-RT for 50 min at 37°C and 15 min at 70°C. Subsequent PCR amplification using 0.2 U of Taq polymerase was performed in a thermocycler using specific primers. The PCR primer sequences used in this study are listed in Table 1.

**Westernblotting**

Cultured *H. pylori* were washed twice with PBS and then lysed with RIPA buffer containing protease inhibitor cocktail. The cell lysates were sonicated for 1 min and incubated on ice for 10 min. The cell lysates were then centrifuged and the supernatants were collected. The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with optimal concentrations of primary antibody at 4°C overnight and then incubated with the appropriate secondary antibody (anti-mouse or anti-rabbit) for 1 h at room temperature. The immunolabeled proteins were visualized using ECL. Anti-*H. pylori* antibody was used as an internal control.

**Statistical analysis**

Data in the bar graphs are presented as mean ± standard error of mean (SEM). All the statistical analyses were performed using GraphPad Prism 5.02 software (GraphPad Software, CA, USA). All the data were analyzed by unpaired Student’s t-test and *p* < 0.05 was considered to be statistically significant (*p* < 0.05, **p** < 0.01 and ***p*** < 0.001).

**Results**

**Inhibitory effect and MIC of plumbagin against *H. pylori***

Park *et al.* previously reported the inhibitory effect of plumbagin on *H. pylori* growth. However, the inhibitory mechanism and effect of plumbagin on virulence factors of *H. pylori* have not been studied yet. In this study,

<table>
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<th>Product length (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Cycles</th>
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Plumbagin Inhibits *H. pylori* Growth and Virulence Factors

Therefore, we investigated whether plumbagin treatment has an influence on the expression of virulence factors in *H. pylori*. First, we investigated inhibitory effect and MIC of plumbagin on *H. pylori* ATCC 49503 strain. To define MIC, agar dilution method was performed. Various concentrations of plumbagin (250 nM–64 μM) was diluted in Mueller-Hinton agar and 30 μl of bacterial suspension set to McFarland scale 4.0 was placed on the agar, then MIC was defined after 72 h of incubation. In the agar dilution test result, MIC of plumbagin on *H. pylori* ATCC 49503 strain was 8 μM which was lower than 4 μg/ml (approximately 21.3 μM) as previously reported by Park et al. (Fig. 1). The discordance of MIC was presumably came from the difference of bacterial strain. Furthermore, we also determined MIC of plumbagin by broth dilution method and found that MIC in the broth dilution test was 1 μM which was lower than the result from agar dilution test. In the agar dilution method, drug should diffuse to the bacteria growing on the surface of the medium whereas drug in the broth can directly react with bacteria. This is presumably the reason why MIC in the broth dilution method showed higher than that of agar dilution method. Based on this result, we reconfirmed the inhibitory effect of plumbagin on *H. pylori* and especially we defined MIC of plumbagin on East Asian type strain of *H. pylori* (ATCC 49503).

**Effect of plumbagin on RNA polymerase of *H. pylori***

RNA polymerase is indispensible enzyme for living organisms necessary for transcription and subsequent synthesis of proteins. RNA polymerase in *H. pylori* consists of several subunits including α subunit, β subunit, σ-70 factor, and σ-64 factor that are respectively encoded by *rpoA*, *rpoB*, *rpoD*, and *rpoN*. Therefore, we investigated expression of the RNA polymerase subunits in *H. pylori* after plumbagin treatment. Protein and RNA were hardly extractable after 3 days of plumbagin treatment because plumbagin inhibits bacterial growth in 3 days, thus we conducted experiments in a time dependent manner in a shorter time period. *H. pylori* was inoculated in Mueller-Hinton broth supplemented with 10% bovine serum and treated with plumbagin for various time periods (0, 1, 3, 6, and 12 h) then RNA was extracted and subjected to RT-PCR. The final concentration of plumbagin was set to 2 μM, because 2 μM was enough to completely inhibit the bacterial growth in the broth. In the results, we found that expression of *rpoA* was decreased by plumbagin treatment in *H. pylori* (Fig. 2). The expression of *rpoA* began to decrease in 12 h of plumbagin treatment (Fig. 2). In *E. coli*, the four RNA polymerase subunits assemble in a sequential manner (α – α2 – α2β – α2ββ) [15]. RNA polymerase α subunit takes part in the initiation of the enzyme assembly, because dimerization of α subunits is the first step in assembly of the RNA polymerase suggesting the key role of α subunit in assembly of the enzyme [15]. Therefore, decreased expression of RNA polymerase α subunit seems to be sufficient to inhibit the function of RNA...
polymerase and subsequent growth of \( H. pylori \), although expressions of other RNA polymerase subunits were not changed by plumbagin treatment. This result indicates that inhibitory mechanism of plumbagin on \( H. pylori \) growth is at least in part associated with reduced expression of RNA polymerase \( \alpha \) subunit.

**Effect of plumbagin on virulence factors of \( H. pylori \)**

There are various virulence factors necessary for \( H. pylori \) to colonize on gastric mucosa, to survive in the hostile environment, or to induce pathological changes in the host. The best investigated virulence factors in \( H. pylori \) are CagA and VacA toxins, and both toxins are tightly associated with bacterial pathogenesis. Therefore, we investigated whether plumbagin affects expression of the toxins in \( H. pylori \). The expression levels of \( cagA \) and \( vacA \) genes were evaluated by RT-PCR after treatment of plumbagin in \( H. pylori \). Moreover, expression of \( secA \), which is responsible for secretion of VacA, was also investigated after plumbagin treatment. In the results, we found that mRNA expression of both \( cagA \) and \( vacA \) genes were decreased by plumbagin in \( H. pylori \), but \( secA \) expression was not changed (Fig. 3A). In the Westernblot, the protein levels of CagA and VacA toxins were also decreased in 18 h following decrease of their mRNA expression (Fig. 3B). These results suggest that plumbagin inhibits synthesis of CagA and VacA toxins in \( H. pylori \), although it does not affect secretion of VacA mediated by SecA.

\( H. pylori \) possess virulence factors for successful colonization and survival in the host gastric mucosa. Ureases are one of the essential virulence factors for \( H. pylori \) to infect in the host gastric mu cosa. This is because \( H. pylori \) utilize ureases as a strategy to survive from the acidic condition in the stomach. Therefore, inhibition of urease synthesis will seriously threat the survival of \( H. pylori \) from the hostile environment. Our investigations on expression of the urease subunits showed that \( ureA \) mRNA level is decreased by plumbagin, but \( ureB \) expression remained constant (Fig. 3C). Furthermore, we investigated expression of adhesins, which are also closely associated with colonization of the bacteria, in \( H. pylori \) after plumbagin treatment. Among the adhesins examined in this study, \( alpA \) mRNA level was reduced by plumbagin treatment (Fig. 3D). Collectively, our data regarding expression of various virulence factors of \( H. pylori \) suggest that plumbagin treatment on \( H. pylori \) inhibits expression of \( cagA, vacA, ureA \) and \( alpA \) all of which are necessary for bacteria to successfully infect with and to subsequently induce disease in the host.

**Discussion**

In this study, we confirmed inhibitory effect of plumbagin on East Asian type \( H. pylori \) strain (ATCC49503) and defined MIC by agar dilution method. Next, we investigated expression of RNA polymerase subunits in \( H. pylori \) and found that plumbagin inhibits expression...
Fig. 3. Effect of plumbagin on the expression of *H. pylori* virulence factors. *H. pylori* suspension set to McFarland scale 0.33 (1 × 10⁸/ml) was treated with 2 µM of plumbagin for indicated time periods. (A) mRNA expression level of cagA and vacA toxins and secA after plumbagin treatment, (B) Protein level of CagA and VacA toxins after plumbagin treatment. Polyclonal anti-*H. pylori* antibody was used as an internal control (Control), (C) mRNA expression level of ureases after plumbagin treatment, (D) mRNA expression level of adhesins after plumbagin treatment. Density of the bands were illustrated as a graph, and the results from triplicate experiments were analyzed by unpaired Student’s t-test (*p < 0.05, **p < 0.01 and ***p < 0.001).
of RNA polymerase α subunit. Furthermore, we also investigated expression of various virulence factors in *H. pylori* and found that expressions of *cagA, vacA, ureA* and *alpA* were decreased by plumbagin treatment in *H. pylori*.

Although anti-microbial activity of plumbagin has been studied in a few types of *H. pylori* strains (ATCC 43504, BCRC 17021, BCRC 17023, BCRC 17026, BCRC 17027 and BCRC 15415), the mechanism in which plumbagin inhibits growth of *H. pylori* has not been reported yet [25, 32]. To elucidate the inhibitory mechanism, we investigated expression of the molecules associated with DNA replication or transcription in *H. pylori* after plumbagin treatment. DNA replication is a crucial step for survival and propagation of living organisms. At first, therefore, we investigated whether plumbagin has an influence on the expression of the molecules involved in the *H. pylori* replication. In bacteria, DNA replication comprises three steps: initiation, elongation and termination [31]. Various molecules take part in the replication process that include a chromosomal replication initiator protein (DnaA), DNA helicase (DnaB), DNA polymerase III core polymerases (DnaE, DnaQ and HolE), sliding clamp (DnaN) and multiprotein clamploaders (DnaX, HolA, HolB, HolC and HolD) all of which are necessary to appropriately function as single multicomponent machinery [22]. We treated plumbagin on *H. pylori* and investigated expression of *dnaA, dnaB, dnaE, dnaN, dnaQ, and holB* by RT-PCR, but no change was observed among the molecules we investigated (Supplementary Fig. 1). Transcription is also tightly associated with growth of living organisms because it is indispensible for synthesis of protein in the end. In the investigation on the RNA polymerase subunits, which are key molecules for transcription, we found that mRNA level of α subunit (*rpoA*) in *H. pylori* was reduced by plumbagin treatment (Fig. 2). α subunit, which is one of the core subunits of RNA polymerase, binds to β subunit to initiate assembly of functional RNA polymerase and stabilizes the structure of the RNA polymerase [15]. Based on our data, therefore, decrease of *rpoA* expression is at least in part involved in the inhibition of *H. pylori* growth by plumbagin.

As many virulence factors in *H. pylori* are associated with successful infection of the bacteria or bacteria-induced pathogenesis, down-regulation of virulence factors may decrease chance for *H. pylori* to colonize on the host gastric epithelium as well as reducing pathogenesis by the bacteria. Among the various virulence factors, we found plumbagin treatment inhibited expression of *cagA, vacA, ureA* and *alpA* in *H. pylori*. *CagA* and *VacA* toxins are closely associated with tumorigenesis by *H. pylori*. They disrupt intracellular signaling in host cells that lead to uncontrolled growth of the cells and inflammatory responses [9, 21]. These reports imply that decreased expression of *CagA* and *VacA* toxins by plumbagin treatment will alleviate the pathogenesis in gastric mucosa by *H. pylori*. Urease is an essential virulence factor of *H. pylori* because it is closely associated with survival of the bacteria in the acidic environment of gastric mucosa [16]. In this study we investigated expression of two urease subunits, *ureA* and *ureB*, and found that plumbagin treatment reduced *ureA* expression. The two urease subunits form dodecameric complex ((αβ)₄₃)₄ to function as an enzyme [16]. Therefore, reduced expression of one of them may decrease the enzyme activity which in turn impedes survival of *H. pylori* in the hostile condition of host stomach. There are adherence associated proteins enabling *H. pylori* to adhere to mucosal epithelial cells during the first step of bacterial colonization. AlpA is an adhesin involved in adhering to gastric tissue, and we found plumbagin treatment down-regulated *alpA* expression in *H. pylori* [12]. Therefore, decreased expression of AlpA by plumbagin treatment may reduce the colonization of *H. pylori*. We further investigated expression of flagella molecules but they were not affected by plumbagin treatment (Supplementary Fig. 1). Collectively, plumbagin treatment reduced expression of various virulence factors in *H. pylori* and these results imply that plumbagin may inhibit the colonization and pathogenesis by *H. pylori*.

The first-line regimen currently recommended for eradication of *H. pylori* is triple therapy including clarithromycin, amoxicillin and proton pump inhibitor [18]. However, numerous reports are alarming the limitation of current empirical regimen for eradication of *H. pylori*, because of the prevalence of clarithromycin resistance worldwide and decreased eradication rate of *H. pylori* by first-line therapy [8, 34, 35]. In particular, clarithromycin resistance in Asia has been surprisingly increased from 15.28% in 2009 to 32.46% in 2014 according to the recent review by Ghotaslou *et al.* [8]. In addition, resis-
tance rate against levofloxacin has been increased and metronidazole resistance rate is also high in *H. pylori* [8]. Continuous increase of the antimicrobial resistance of *H. pylori* corresponding to the use of the antibiotics is a significant limitation for effective eradication of *H. pylori* in the future [17]. These reports collectively show the importance of surveillance on antibiotic resistance and selection of appropriate antibiotic regimen as well as development of a new therapeutic agent for eradication of *H. pylori*. Therefore, natural compounds such as plumbagin can be potentially suggested as an alternative choice for eradication of *H. pylori* in the future, although further studies seem to be necessary to completely understand the inhibitory mechanism of plumbagin on *H. pylori* and to confirm the effectiveness in *vivo*.

**Acknowledgments**

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**References**


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**Plumbagin에 의한 헬리코박터 파이로리균의 성장 및 병원성 인자 발현 억제효과**

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4 임상병리학과

혈리코박터 파이로리균은 인간의 위에 감염하여 위염, 위궤양, 심지어 위암을 포함한 다양한 위장질환의 발생시키는 원인으로 알려져 있다. 이러한 혈리코박터균의 세균은 위장내의 병원성과 관련된 다양한 독소들의 발현에 영향을 미치는 액체배지혈액법을 이용하여 조사하였다. 본 연구에서는 혈리코박터 표준균주(ATCC 49503)에 plumbagin을 처리한 후 항균효과를 확인하였으며, 세균의 성장 및 병원성과 관련된 다양한 독소들의 발현에 미치는 영향을 imunoblotting 및 RT-PCR 방법을 이용하여 조사하였다. plumbagin의 혈리코박터균 역제효과를 확인하기 위해 한천의 실험법 및 액체배지혈액법을 이용해 최소역제농도를 도출하였다. 위와 같은 Plumbagin에 의한 혈리코박터균의 역제기전을 이해하기 위하여 혈리코박터균에 plumbagin을 처리한 후 세균의 증식과 관련된 독소들을 대상으로 RT-PCR을 수행한 결과 RNA polymerase subunit α (rpoA)의 mRNA 발현이 감소한 것을 확인하였다. 또한, 혈리코박터균에 plumbagin을 처리한 후 주요 병원성인자의 발현은 조사한 결과 CagA와 VacA 독소들의 mRNA 및 단백질량이 감소한 것을 확인하였으며, 유제체(ureA)와 부착단백(alpa)의 발현도 plumbagin 처리에 의해 감소한 것을 확인하였다. 위와 같은 결과들을 토대로, plumbagin은 본 연구에서 발현 기전들을 통해 혈리코박터균의 성장, 감염 및 발병을 억제하는 것으로 사료된다.