Original Article
Kinetin inhibits growth of *Helicobacter pylori* by down-regulation of replication genes

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Abstract: *H. pylori* is a Gram-negative curved bacterium which primarily colonizes the epithelial layer of human stomach. Infection with this bacterium may lead to various gastric diseases from asymptomatic gastritis to gastric cancer. Because of its clinical importance, WHO designated *H. pylori* as a class I carcinogen. Kinetin is a type of cytokinins major role of which is to facilitate growth of the plants but it also plays a role to protect plants from infections. However, anti-bacterial effect of kinetin on *H. pylori* has not been elucidated. In this study, we performed agar dilution test and broth dilution test to determine MIC of kinetin against an *H. pylori* reference strain. In particular, we isolated *H. pylori* from gastric biopsies and confirmed inhibitory effect of kinetin against 60 clinical isolates of *H. pylori*. To elucidate the inhibitory mechanism of kinetin on *H. pylori* growth, we performed RT-PCR and found that kinetin down-regulated *dnaA* and *holB* expression both of which are necessary for bacterial replication. We also confirmed kinetin did not show cytotoxicity to gastric epithelial cells (AGS) with the dose completely inhibited *H. pylori* growth and kinetin did not inhibit the eukaryotic replication machinery. Moreover, kinetin reduced *H. pylori*-induced death of gastric cell line in vitro. Many recent reports are alarming the increased antibiotic resistance of *H. pylori* and limitation of current regimen for *H. pylori* eradication indicating necessity of new therapeutic agents. Although further studies seem to be necessary, our study collectively shows that kinetin inhibits growth of *H. pylori* by inhibiting replication of the bacteria and suggests kinetin as one of the potential candidates for *H. pylori* eradication.

Keywords: Kinetin, *H. pylori*, *dnaA*, *holB*

Introduction

*Helicobacter pylori* is a Gram-negative and spiral shaped bacterium. *H. pylori* colonizes the epithelial layer of human stomach for many years and it has been reported to infect more than half of the mankind [1]. Infection with this bacterium may lead to various gastric diseases from asymptomatic gastritis to gastric cancer [2]. Particularly, it has been proposed to act as a carcinogen causing gastric cancer [3]. Because of its clinical importance, WHO designated *H. pylori* as a class I carcinogen [3].

Kinetin is a type of cytokinins, a plant growth substance that plays a major role in cell division and cell differentiation [4]. Kinetin was isolated and identified by Miller and Skoog et al. as a compound from autoclaved herring sperm DNA that had cell division-promoting activity [5]. Although the major role of cytokinins is to facilitate growth of the plants, cytokinins such as kinetin also play a role to protect plants from infections. It was reported that kinetin has anti-fungal function by suppressing fungus spore germination in *Harpophoramaydis* [6]. In addition, cytokinins also reported to inhibit bacterial growth and virulence gene expression of *Agrobacterium tumefaciens* [7].

DNA replication is a biological step and occurs in all living organisms. In prokaryotes, DNA replication can be divided into stages: initiation, elongation, and termination [8]. At the initial phase, DnaA which activates initiation of DNA replication forms complex with oriC site, which has specific AT rich DNA unwinding element (DUE) regions [8]. Once bound to ssDUE, DnaA
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provides docking platform for DnaB helicases to load on to the unwound region [8]. DnaB migrates along the single-stranded DNA in the 5’ to 3’ direction, unwinding the DNA as it travels [9]. The elongation phase begins with loading of DNA polymerase III at the 3’ termini of the primer synthesized by DnaG [9]. DNA polymerase III consists of multicomponents including core polymerases (DnaE, DnaQ and HolE), sliding clamp (DnaN) and multiprotein clamp-loader (DnaX, HolA, HolB, HolC and HolD) all of which are necessary to appropriately function as a single machinery [9]. After polymerization of DNA, termination of the replication process involves the interaction with Ter protein which binds to a termination site sequence in the DNA to physically stop DNA replication, followed by chromosome segregation [9].

Only a few studies have been reported protective effect of cytokinins from infection, and especially anti-bacterial effect of cytokinins on clinically important bacteria is yet to be studied. In this study, therefore, we demonstrated anti-bacterial effect of kinetin on H. pylori and investigated its involved mechanism.

Materials and methods

Materials

H. pylori reference strain was purchased from ATCC (ATCC49503, Manassas, VA, USA). Mueller-Hinton broth, Mueller-Hinton agar and Brucella agar were purchased from Becton-Dickinson (Braintree, MA, USA). Bovine serum was purchased from Gibco (Long Island, NY, USA). Trizol reagent, random hexamer, and Moloneymurine leukemia virus reverse transcriptase (MMLV-RT) were purchased from Invitrogen (Carlsbad, CA, USA). Kinetin was obtained from Sigma-Aldrich (Saint Louis, MO, USA). EZ-Cytox cell viability assay kit was purchased from Daeil Lab Service (Seoul, Korea).

Bacterial culture

Brucella agar plate supplemented with 10% bovine serum was used to grow H. pylori. The bacteria were incubated at 37°C for 72 h in a humidified atmosphere with 10% CO₂. To cultivate bacteria in broth, bacterial colonies were collected and grown in Mueller-Hinton broth supplemented with 10% bovine serum. Turbidity of the bacterial suspension was set to MacFarland 0.33 (1 × 10⁸ /mL) and incubated at 37°C for 72 h in a humidified atmosphere with 10% CO₂.

Agar dilution method to determine MIC

H. pylori were grown on the Brucella agar plate then the bacterial colonies were collected and suspended in saline. Turbidity of the bacterial suspension was set to MacFarland scale 0.5. Kinetin was 2-fold serially diluted in Mueller-Hinton agar supplemented with 10% bovine serum to prepare the media including kinetin in a range of concentrations (0, 31.2, 62.5, 125, 250, and 500 μM). After preparation of the media, the bacterial suspension was plated on the media. The bacteria were incubated for 72 h and growth of the bacterial colonies was observed. The lowest concentration that completely inhibited growth of the bacterial colony was determined as MIC (minimal inhibitory concentration).

Broth dilution method to determine MIC

H. pylori suspension turbidity of which was set to MacFarland scale 0.5 was prepared in Mueller-Hinton broth supplemented with 10% bovine serum. Various concentrations of kinetin were added and the bacteria were incubated at 37°C for 72 h in a humidified atmosphere with 10% CO₂. After 72 h, the final optical density (600 nm wavelength) of the bacterial suspension was measured by spectrophotometry.

Collection of H. pylori clinical isolates

Specimen collection was conducted at Yong-In Severance Hospital in Korea. H. pylori strains, isolated from 60 patients conducted gastroscopic examination to confirm the infection of H. pylori, were evaluated. Strains were identified as H. pylori by colony morphology analysis and urease test. The MICs were determined using a slightly modified agar dilution method (Mueller-Hinton agar base containing 10% bovine serum).

RT-PCR (reverse transcription-polymerase chain reaction)

Cultured H. pylori were collected and washed twice with PBS. After washing, total RNA was extracted by using Trizol reagent as described in the manufacturer’s instructions. cDNA was synthesized by reverse transcription and subjected to PCR amplification as described in a
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Table 1. List of primer sequences and conditions used for RT-PCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product length (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>dnaA</td>
<td>GGGCATGACTTTAGCGGTTA</td>
<td>TTAACGAATTCAGCCGCAAC</td>
<td>128</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>dnaB</td>
<td>AATGGGCGGTATTCGTGCTC</td>
<td>CAATCCCGCTGGCAACTACG</td>
<td>231</td>
<td>55</td>
<td>24</td>
</tr>
<tr>
<td>dnaE</td>
<td>AATCCACCGTCCCAATAC</td>
<td>GCCAAACAAGTGGGAGTA</td>
<td>184</td>
<td>55</td>
<td>22</td>
</tr>
<tr>
<td>dnaN</td>
<td>GTTAGCGCTTTGGAAGCG</td>
<td>CGGTTTCCGCTAGCTGCA</td>
<td>253</td>
<td>55</td>
<td>22</td>
</tr>
<tr>
<td>dnaQ</td>
<td>GCGATGACCTTGGACAAAG</td>
<td>GCATAGGCTCTATGCTGAC</td>
<td>244</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>holB</td>
<td>TGCAAGCGTGGTAAACG</td>
<td>CGCCTTTGAGGCTTACTAC</td>
<td>196</td>
<td>55</td>
<td>24</td>
</tr>
<tr>
<td>ORC1</td>
<td>CTTCCTTTCGCGAGCTGAGT</td>
<td>GTGATGACATTTGAGATCC</td>
<td>189</td>
<td>66</td>
<td>29</td>
</tr>
<tr>
<td>PCNA</td>
<td>TCTCGCTGCAAGTTCTCAG</td>
<td>GTTTGCAACCGTGGAGCTA</td>
<td>376</td>
<td>52</td>
<td>31</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CGGGAAGTTTGTCAATGG</td>
<td>GGCAGTGATGGGACTG</td>
<td>349</td>
<td>55</td>
<td>21</td>
</tr>
</tbody>
</table>

Figure 1. Determination of the MIC of kinetin on a H. pylori ATCC49503 strain by agar dilution and broth dilution method. A. H. pylori was grown on the Mueller-Hinton agar including indicated concentrations of kinetin. MIC of kinetin against H. pylori was determined after 72 h of incubation. B. H. pylori was grown on the Mueller-Hinton broth including indicated concentrations of kinetin. After 72 h of incubation, final optical density of the bacterial suspension was measured at 600 nm wavelength by spectrophotometry and MIC was determined. Data were from triplicate experiments and analyzed by Student’s t-test (**P < 0.05, ***P < 0.01 and ****P < 0.001).

Table 2. MIC of kinetin against the H. pylori clinical isolates

<table>
<thead>
<tr>
<th>MIC (μM)</th>
<th>Number of bacteria (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 15.6</td>
<td>1 (1.7%)</td>
</tr>
<tr>
<td>31.3</td>
<td>1 (1.7%)</td>
</tr>
<tr>
<td>62.5</td>
<td>15 (25.0%)</td>
</tr>
<tr>
<td>125</td>
<td>26 (43.3%)</td>
</tr>
<tr>
<td>250</td>
<td>16 (26.7%)</td>
</tr>
<tr>
<td>500</td>
<td>1 (1.7%)</td>
</tr>
</tbody>
</table>

previous report [10]. The PCR primer sequences and PCR conditions used in this study are described in Table 1.

Western blotting

Cells were washed with PBS and then lysed at 4°C with lysis buffer containing 1% TritonX-100, protease inhibitor cocktail, and PBS. Lysates were centrifuged and the supernatants were subjected to Western blot as described previously [11].

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, San Diego, 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).

Cell viability assay

AGS cells (1.5 × 10⁴ per well) were plated in 96-well plates and incubated for 24 h before they were treated with various concentrations of kinetin. The cells were then further incubated for 24 h and subjected to WST (water soluble tetrazolium salts) assay by using EZ-Cytox cell viability assay kit according to manufacturer’s instruction. Briefly, 10 μl of WST solution was added to the cultured media and incubated in the CO₂ incubator for 1 h. Absorbance of the supernatant at 450 nm wavelength was measured by spectrophotometer.

Results

Inhibitory effect of kinetin on the growth of H. pylori

To determine the MIC of kinetin on H. pylori, we performed agar dilution test. Mueller-Hinton
agel including various concentrations of kinetin was prepared. An *H. pylori* reference strain (ATCC49503) was suspended in PBS and 30 μl of the 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 kinetin against *H. pylori* was 250 μM (Figure 1A). To confirm whether kinetin can inhibit *H. pylori* growth not only in the reference strains but also in the clinical isolates, we collected clinical isolates of *H. pylori* from gastric biopsies and determined MIC of kinetin. Among the 60 clinical isolates, MIC of 26.7% (16/60) was 250 μM, 43.3% (26/60) was 125 μM and 25.0% (15/60) was 62.5 μM (Table 2). In addition, we also determined MIC of kinetin by broth dilution method because bacteria were grown in the broth condition in the next step of our experiment. We found that MIC in the broth dilution test was 250 μM (Figure 1B). Based on these results, we demonstrated kinetin has anti-bacterial effect on *H. pylori* and also determined MIC. In particular, we showed anti-bacterial effect of kinetin was equally applied on the clinical isolates of *H. pylori* as well as the reference strain.

**Kinetin down-regulates dnaA and holB expression in *H. pylori***

To elucidate the mechanism by which kinetin inhibits growth of *H. pylori*, we investigated whether kinetin has an influence on the replication machinery of the bacteria. *H. pylori* was treated with kinetin and incubated in brucella broth supplemented with 10% bovine serum for 72 h. We collected RNA and performed RT-PCR to investigate the expression of the genes involved in the replication of *H. pylori*. In the result, we found that dnaA and holB mRNA expression levels decreased in a kinetin dose dependent manner, though expression of the other genes remained constant (Figure 2A). Kinetin induced reduction of dnaA and holB expression was statistically significant (*P* = 0.0173 and *P* = 0.0006) in the dose with 62 μM or above (Figure 2B). Both dnaA and holB, which are replication initiation protein and clamp-loader protein, are important proteins for bacterial replication, thus our result infers that kinetin inhibits *H. pylori* growth by down-regulating dnaA and holB expression.

**Kinetin inhibits *H. pylori*-induced cell death of gastric cell line in an in vitro cell culture model**

To further investigate whether kinetin can inhibit *H. pylori* growth and infection to gastric epithelial cells, we adopted in vitro model of *H. pylori* infection. We used AGS (gastric adenocarcinoma cell line) in this experiment because *H. pylori* colonizes on the human gastric epithelium. AGS cells were infected with *H. pylori* and treated with kinetin at the same time for 24 h. After incubation we observed cell viability and found that *H. pylori* infection (100 MOI) reduced cell viability of AGS cells (53.87%) but kinetin treatment (250 μM) partially recovered cell viability (76.12%) (Figure 3A). In the Western blotting result, PARP was cleaved by *H. pylori* infection but it was inhibited by kinetin treatment (Figure 3B). Based on our results, kinetin inhibited growth of *H. pylori* and the corresponding dose of kinetin rescued AGS cells from *H. pylori* induced cell death, thus our results collectively indicate that kinetin might inhibit infection of *H. pylori* by bacterial growth thereby reduced *H. pylori*-induced death of gas-

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**Figure 2.** Effect of kinetin on the expression of replication machineries in *H. pylori*. **A.** *H. pylori* was treated with indicated concentrations of kinetin for 72 h and RNA was harvested. Collected samples were subjected to RT-PCR to detect mRNA expression level of replication machineries (dnaA, holB, dnaB, dnaE, dnaN, dnaQ and polA). **B.** Density of dnaA and holB bands were measured and illustrated as graphs. Data were from triplicate experiments and analyzed by Student’s t-test (*P* < 0.05, **P** < 0.01 and ***P*** < 0.001).
Inhibitory effect of kinetin on *H. pylori* growth

**Figure 3.** Inhibitory effect of kinetin on the *H. pylori* induced cell death in a gastric cell line. (A) AGS cells were infected with *H. pylori* (100 MOI) and treated with indicated concentrations of kinetin for 24 h. After incubation, cell viability was measured by WST assay. (B) AGS cells were treated as in (A) and the cell lysates were collected to conduct Western blotting. Full-length PARP (116 KDa) and cleaved PARP (89 KDa) bands were detected by using specific antibody, β-actin was used as an internal control. Data were from triplicate experiments and analyzed by Student’s t-test (*P* < 0.05, **P** < 0.01 and ***P*** < 0.001).

**Figure 4.** Evaluation of the cytotoxicity of kinetin on AGS cells. A. AGS cells were treated with indicated concentrations of kinetin and cell viability was measured by WST assay. B. AGS cells were treated with indicated concentrations of kinetin and RNA was harvested. Collected RNA samples were subjected to RT-PCR to detect mRNA expression level of eukaryotic replication machineries (ORC1 and PCNA).

**Discussion**

*H. pylori* is one of the clinically important bacteria because it causes various gastric disorders. We have demonstrated inhibitory effect of kinetin on *H. pylori* for the first time in this study. Here we have determined MIC of kinetin against an *H. pylori* reference strain by agar dilution test and broth dilution test. Furthermore, we isolated *H. pylori* from gastric biopsies and confirmed inhibitory effect of kinetin against 60 clinical isolates of *H. pylori*. Our study also has shown the putative inhibitory mechanism of kinetin on *H. pylori* growth. In our results, kinetin down-regulated *dnaA* and *holB* expression both of which are necessary for bacterial replication. In the *in vitro* *H. pylori* infection model, kinetin reduced gastric epithelial cell (AGS) death which was induced by *H. pylori* infection. In addition, kinetin did not inhibit the eukaryotic replication machinery corresponding to prokaryotic *dnaA* and *holB* and we also confirmed kinetin did not show cytotoxicity to gastric epithelial cells in the dose completely inhibited *H. pylori* growth.

As many virulence factors in *H. pylori* are associated with successful infection of the bacteria...
H. pylori is a gram-negative curved bacterium associated with the carcinogenesis on the stomach, thus World Health Organization classified H. pylori as a class I carcinogen [12]. Chronic infection of H. pylori on the stomach has been reported to induce various gastric diseases including gastritis, gastric ulcer and gastric cancer in severe case [12]. According to the global cancer statistics, 14.1 million new cancer cases have been occurred in 2012 and 8.2 million were died with cancer worldwide [13]. Among the cancer cases, gastric cancer was the fourth prevalent (8.5%) type worldwide and 35.4% of gastric cancer has occurred in Eastern Asia [13, 14]. Various reasons have been suggested to be responsible for gastric cancer rate such as dietary patterns, food storage, availability of fresh produces and especially prevalence of H. pylori infection [13, 14]. It is estimated that more than half of the adult population is infected with H. pylori worldwide and infection of which is responsible for 75% of all the gastric cancer cases [15]. Therefore, a lot of efforts for eradication of H. pylori infection still seem to be necessary for health promotion worldwide.

Triple therapy (clarithromycin, amoxicillin and proton pump inhibitor) is currently recommended for eradication of H. pylori as a first-line regimen [16]. However, numerous reports have informed the prevalence of clarithromycin resistance worldwide and decreased eradication rate of H. pylori by first-line therapy alarming the limitation of current empirical regimen being used for eradication of H. pylori [17-19]. Especially, clarithromycin resistance in Asia has been significantly increased to 32.46% in 2014 [19]. Moreover, levofloxacin resistance rate has been increased and resistance to metronidazole also showed to be high in H. pylori [19]. Collectively all these reports illuminate the seriousness of the antibiotic resistance and importance of appropriate selection for antibiotic regimen as well as discovery of a new therapeutic agent for eradication of H. pylori. Therefore, we believe that discovery of natural compounds inhibiting H. pylori growth such as kinetin is important and continuous researches are necessary for the development of supportive agents to increase eradication of H. pylori. Further studies seem to be necessary to completely understand the inhibitory mechanism of kinetin on H. pylori, and use of animal models also needs to evaluate the safety and effectiveness of kinetin in vivo.

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Disclosure of conflict of interest

None.

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