Effect of plaunotol in combination with clarithromycin against clarithromycin-resistant *Helicobacter pylori* in vitro and in vivo

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**Objectives:** Recently, there has been a decrease in the eradication rate of *Helicobacter pylori* due to the increase in antibiotic resistance of this bacterium. Plaunotol, a cytoprotective anti-ulcer agent, exhibits antibacterial activity against *H. pylori*. The purpose of the present study was to investigate the effect of plaunotol in combination with clarithromycin against clarithromycin-resistant *H. pylori* clinical isolates.

**Methods and results:** In the chequerboard titration method, the combination of plaunotol and clarithromycin showed a synergistic effect against 67% (10/15) clarithromycin-resistant strains and an additive effect against the other strains. No indifferent and antagonistic effects were observed against any of the strains tested. In a gastritis model of Mongolian gerbils infected with clarithromycin-resistant *H. pylori*, the plaunotol (40 mg/kg) and clarithromycin (66.6 mg/kg) combination exhibited synergistic effects; however, neither plaunotol nor clarithromycin alone showed bactericidal effects.

**Conclusions:** These results suggest that plaunotol may play a useful role in combination with anti-*H. pylori* drugs in the treatment of diseases associated with clarithromycin-resistant *H. pylori*.

Keywords: synergistic effect, cytoprotective anti-ulcer agent, eradication

**Introduction**

*Helicobacter pylori* is a Gram-negative bacterium associated with peptic ulcer disease, gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer. The discovery of *H. pylori* has already changed the prognosis of peptic ulcer disease, with most patients being cured at their first presentation.1,2 Eradication of *H. pylori* is very important to prevent relapse and accelerate the healing of peptic ulcer disease,3 and only triple-drug therapy [proton pump inhibitor (PPI) + amoxicillin + clarithromycin] is currently permitted in Japan. Triple-drug therapy has a strong antibacterial effect against *H. pylori* and has a high eradication rate.4 However, the eradication rate of *H. pylori* using triple-drug therapy has decreased5,6 due to the increase in clarithromycin-resistant strains of *H. pylori*.7,8 Plaunotol, a cytoprotective anti-ulcer agent, enhances the antibacterial activity of clarithromycin.9,10 However, it is unclear whether plaunotol enhances the antibacterial activity of clarithromycin against clarithromycin-resistant *H. pylori*. Plaunotol shows antibacterial activity against *H. pylori* and plaunotol in combination with clarithromycin exhibits synergistic or additive activity against clarithromycin-susceptible *H. pylori in vitro* and *in vivo*. Triple-drug therapy is instituted before the detection of clarithromycin-resistant *H. pylori*. Therefore, it is useful to evaluate the combined effect of plaunotol with clarithromycin against clarithromycin-resistant *H. pylori*. In this study, we evaluated the antibacterial activity of plaunotol in combination with clarithromycin against clarithromycin-resistant *H. pylori* isolates by using the agar dilution chequerboard method. The *in vivo* activity of the plaunotol/clarithromycin combination was also

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examined in a gastritis model in Mongolian gerbils infected with a clinical isolate of clarithromycin-resistant *H. pylori*.

**Materials and methods**

**Antibacterial agents**

Plaunotol (Daiichi Sankyo Co., Ltd, Tokyo, Japan) and clarithromycin (Taisho Pharmaceutical Co., Ltd, Tokyo, Japan) were used.

**Microorganisms**

Twenty-one *H. pylori* clinical isolates used in this study were isolated from gastric biopsy samples obtained during a routine endoscopy. The gastric biopsy material was processed according to the standard methodology, and *H. pylori* was identified based on colony morphology, Gram staining and catalase-, oxidase- and urease-positive reactions. Twenty-one clarithromycin-resistant strains (MIC ≥ 1 mg/L) were maintained at −80°C in Brucella broth with 25% glycerol until usage. Stock culture was grown in Brucella broth (Nippon Becton Dickinson Co. Ltd) containing 3% heat-inactivated fetal bovine serum (FBS) and antibiotics (2.5 mg/L amphotericin B, 5 mg/L vancomycin, 2.5 mg/L trimethoprim and 125 IU/L polymyxin B) under microaerobic conditions with gas generator envelopes (AnaeroPack, Mitsubishi Gas Chemical Co., Inc.) in gas jars at 37°C for 4 days.

**Animals**

A total of 36 specific-pathogen-free, 5-week-old male Mongolian gerbils (MGS/Sea, Kyudo Co., Ltd) were housed in plastic cages on hardwood chip bedding in an air-conditioned biohazard room with a 12:12 h light:dark cycle. The experimental design was approved by the Animal Care Committee of the Nagoya City University Animal Research Institute and the animals were cared for in accordance with institutional guidelines, which comply with the instructions of the Health, Labour and Welfare Ministry concerning animal experiments. Stock clarithromycin-resistant *H. pylori* culture was grown in Brucella broth containing 3% FBS under microaerobic conditions. After 1 day of fasting, 6-week-old Mongolian gerbils were orally inoculated with 2 mL of bacterial suspension having a density of 1 × 10^8 cfu/mL. Uninfected gerbils were mock-inoculated with sterile Brucella broth containing 3% FBS.

**Determination of MIC**

Stock cultures of bacteria were grown on brain heart infusion agar (BHIA; Difco Laboratories) supplemented with 5% defibrinated horse blood (Nippon Bio-Supply) at 37°C for 3 days in gas jars with CampyPack. The MICs were measured on BHIA supplemented with 5% defibrinated horse blood by using the 2-fold dilution method. Plaunotol and clarithromycin were dissolved in DMSO and methanol, respectively. Inocula were prepared by suspending the bacterial growth on BHIA supplemented with 5% defibrinated horse blood in physiological saline to achieve a density equivalent to that of a 2.0 McFarland standard. A final inoculum of 10^3–10^5 cfu/spot was employed using an Auto-Inoculator (Dai Nippon Seiki Co., Ltd, Kyoto, Japan). All the plates were incubated for 3 days at 35°C under microaerobic conditions. The MIC was defined as the lowest concentration of the drug that prevented visible growth after 3 days of incubation.

**Determination of in vitro interaction**

Antimicrobial interactions between plaunotol and clarithromycin against 15 clinical isolates were evaluated by the chequerboard titration method. The inoculum, media and culture conditions for this evaluation were the same as those described for the MIC determination.

The fractional inhibitory concentrations (FICs) were calculated as follows:

\[
\text{FIC} = \left( \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} \right) + \left( \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}} \right)
\]

The FIC indices were interpreted as follows: ≤0.5, synergy; >0.5–1, additive; >1–4.0, indifference; >4, antagonism.

**Determination of in vivo interaction**

The experimental design is illustrated in Figure 1. Thirty-six gerbils were grouped into five groups. *H. pylori* was inoculated into four groups (Groups A, B, C and D) of five groups at experimental week 1. The remaining group (Group E) received Brucella broth containing 3% FBS. Plaunotol and clarithromycin were dissolved in 0.5% sodium carboxymethyl cellulose (CMC solution, Kanto Chemical Co., Inc.) as described in the Materials and methods section. The gerbils were sacrificed humanely at 12 weeks.

![Figure 1](image-url)
Groups D and E received oral CMC. The gerbils were sacrificed humanely at 12 weeks. All gerbils were deeply anaesthetized with ether, laparotomized and exsanguinated from the inferior vena cava, followed by excision of their stomachs. Each glandular stomach was fixed in 10% formalin neutral buffer solution, deodorized (Wako Pure Chemical Industries, Ltd) and routinely processed for histopathological examination.

**Histopathological analyses**

Tissue sections were immunohistochemically stained for examination of *H. pylori* (anti-*H. pylori* serum, Dako, Copenhagen). The degree of *H. pylori* density was graded according to the criteria modified from the Updated Sydney System by scoring the following parameters: mononuclear cell infiltration (0–3: 0, normal; 1, mild infiltration into lamina propria; 2, moderate infiltration into lamina propria; 3, marked infiltration into lamina propria and multiple lymphoid follicle formation); neutrophil infiltration (0–3: 0, none; 1, number of neutrophils in the pyloric mucosa in a line from the forestomach to the duodenum <50/mm²; 2, 50–100/mm²; 3, >100/mm²); and *H. pylori* density (0–3: 0, none; 1, mild *H. pylori* density; 2, moderate; 3, marked). Two independent individuals (M. S. and T. Mizoshita) were blind with regard to information concerning *H. pylori* infection and treatment, and judged the histology.

**Statistical analysis**

The Mann–Whitney *U*-test was used to establish the significance of differences in microscopic *H. pylori* density. *P* values of <0.05 were considered to be statistically significant.

**Results and discussion**

Regarding the genetic diversity of clarithromycin-resistant *H. pylori*, Masaoka *et al.* have demonstrated that dilution agar methods should be combined with PCR-restriction fragment length polymorphism (PCR-RFLP) analysis before second-line eradication to increase the accuracy of clarithromycin susceptibility testing and to improve eradication efficacy. In this study, we demonstrated the effect of plaunotol in combination with clarithromycin against clarithromycin-resistant *H. pylori*.

Table 1 shows the MIC<sub>50</sub>, MIC<sub>80</sub> and MIC<sub>90</sub> values and range of MICs of both clarithromycin and plaunotol for the 21 clarithromycin-resistant *H. pylori* strains tested. The MIC<sub>50</sub>/MIC<sub>80</sub>/MIC<sub>90</sub> values (mg/L) of clarithromycin and plaunotol for the 21 clarithromycin-resistant *H. pylori* strains were 4/16/16 and 2/4/4, respectively. By using the checkerboard titration method, the antibacterial effect of plaunotol in combination with clarithromycin was examined against 15 of the 21 clarithromycin-resistant *H. pylori* strains. The clarithromycin/ plaunotol combination demonstrated a synergistic effect against 67% of the strains and an additive effect against 33% of the strains. No indifferent or antagonistic effects were observed against any *H. pylori* strain (Table 2). Thus, by using the checkerboard titration method, we demonstrated that plaunotol synergistically enhanced the antibacterial activity of clarithromycin against clarithromycin-resistant *H. pylori* in vitro. This tendency is in accordance with a previous report of clarithromycin-susceptible *H. pylori* from Koga *et al.* As plaunotol is suggested to cause a change in fluidity with the associated increased membrane permeability, plaunotol might potentiate the anti-*H. pylori* activity of clarithromycin by increasing the permeability of the membranes of even clarithromycin-resistant *H. pylori*.

Table 3 shows the therapeutic effects of plaunotol in combination with clarithromycin. The *H. pylori* infection scores of Groups B and C were lower than that of Group D; however, the difference was not significant. In contrast, the *H. pylori* infection score of Group A receiving the combination therapy was significantly lower than that of Group D; this indicated the synergistic/additive effect of the plaunotol/clarithromycin combination. However, acute inflammation scores and chronic inflammation scores based upon the Updated Sydney System were not significantly different. Fukuda *et al.* have reported that the combination of plaunotol, clarithromycin and a PPI showed a 69% eradication rate as compared with a 38% eradication rate achieved with a combination of clarithromycin and a PPI. Thus far, there has been no study on the synergistic effect of plaunotol in combination with clarithromycin against clarithromycin-resistant *H. pylori* strains. Our study is the first to demonstrate

**Table 1. MIC<sub>50</sub>, MIC<sub>80</sub> and MIC<sub>90</sub> values and MIC range of clarithromycin and plaunotol for the 21 clarithromycin-resistant *H. pylori* strains**

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of strains</th>
<th>MIC (mg/L)</th>
<th>range</th>
<th>50%</th>
<th>80%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>21</td>
<td>1–32</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Plaunotol</td>
<td>21</td>
<td>0.5–4</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Number and percentage of strains in which additive or synergistic effects were observed and the FIC index range for each group**

<table>
<thead>
<tr>
<th>FIC index</th>
<th>No. of strains (%)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synergic</td>
<td>10 (67)</td>
<td>0.0938–0.5</td>
</tr>
<tr>
<td>Additive</td>
<td>5 (33)</td>
<td>0.504–0.75</td>
</tr>
<tr>
<td>Indifferent</td>
<td>0 (0)</td>
<td>—</td>
</tr>
<tr>
<td>Anagonistic</td>
<td>0 (0)</td>
<td>—</td>
</tr>
</tbody>
</table>

with clarithromycin and plaunotol combination. The clarithromycin/plaunotol combination demonstrated a synergistic effect against 67% of the strains and an additive effect against 33% of the strains. No indifferent or antagonistic effects were observed against any *H. pylori* strain (Table 2). Thus, by using the checkerboard titration method, we demonstrated that plaunotol synergistically enhanced the antibacterial activity of clarithromycin against clarithromycin-resistant *H. pylori* in vitro. This tendency is in accordance with a previous report of clarithromycin-susceptible *H. pylori* from Koga *et al.* As plaunotol is suggested to cause a change in fluidity with the associated increased membrane permeability, plaunotol might potentiate the anti-*H. pylori* activity of clarithromycin by increasing the permeability of the membranes of even clarithromycin-resistant *H. pylori*.

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**Table 3. Therapeutic effects of plaunotol in combination with clarithromycin**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th><em>H. pylori</em> infection score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>n = 8</td>
<td>1.58 ± 0.16 *</td>
</tr>
<tr>
<td>Group B</td>
<td>n = 8</td>
<td>2.04 ± 0.16</td>
</tr>
<tr>
<td>Group C</td>
<td>n = 6</td>
<td>2.05 ± 0.29</td>
</tr>
<tr>
<td>Group D</td>
<td>n = 6</td>
<td>2.44 ± 0.22</td>
</tr>
<tr>
<td>Group E</td>
<td>n = 7</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Score, mean plus or minus the standard error.

*P* < 0.05 versus Group D.
that plaunotol can synergistically enhance the antibacterial activity of clarithromycin even against *Helicobacter pylori* strains that exhibit resistance to clarithromycin. The increased occurrence of not only clarithromycin-resistant strains but also metronidazole- and other antibiotic-resistant strains of *H. pylori* has resulted in a decrease in the eradication rate. Regarding the unsuccessful *H. pylori* eradication, a high resistance rate (47.9%) to gatifloxacin (8-methoxy fluoroquinolone) in *H. pylori* strains is also observed.17 Therefore, plaunotol might prevent the decrease in the eradication rate that occurred due to clarithromycin-resistant *H. pylori*.

**Conclusions**

We demonstrated that plaunotol, a cytoprotective anti-ulcer agent, in combination with clarithromycin exhibits a synergistic effect on clarithromycin-resistant *H. pylori* strains in vitro and in vivo. These results suggest that plaunotol could play a useful role in combination with clarithromycin in the treatment of diseases associated with clarithromycin-resistant *H. pylori*.

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**Transparency declarations**

None to declare.

**References**