A Novel Approach for Screening of New Anti-*Helicobacter pylori* Substances

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Gastric mucosal cell-derived t-lactic acid strongly enhances proliferation of *Helicobacter pylori*, and may contribute to the long-term colonization of *H. pylori* in the stomach. Therefore it is assumed that inhibitory substances active against t-lactic acid-dependent growth of *H. pylori* will be useful candidates as novel therapeutic agents for *H. pylori* infection. In this study, we developed a new assay system for screening anti-*H. pylori* substances, and baicalein and glycyrrhetinic acid were found as potent inhibitory substances against t-lactic acid-dependent *H. pylori* growth but not t-lactic acid-independent growth. The newly developed assay system described in this study also may facilitate the development of novel therapeutic agents for *H. pylori* infection.

Key words  *Helicobacter pylori*; t-lactic acid; glycyrrhetinic acid; baicalein; novel assay system

MATERIALS AND METHODS

Materials  Brain Heart Infusion (BHI) broth was obtained from Difco Laboratories (Detroit, MI, U.S.A.). Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12) and fetal bovine serum (FBS) were obtained from Sigma (St. Louis, MO, U.S.A.). Defibrinated horse blood was obtained from Nippon Biotest Laboratories (Tokyo, Japan). Clarithromycin was kindly provided by Dr. S. Omura (Taisho Pharmaceutical Co., Ltd., Tokyo, Japan). *H. pylori* SS-1 was provided by Dr. A. Kai (Tokyo Metropolitan Research Laboratory of Public Health, Japan). *H. pylori* RC-1 was isolated and established from human gastric biopsy specimen in the Kitasato Institute Hospital (Tokyo, Japan). *H. pylori* RC-1 is a clarithromycin-resistant strain, and both *H. pylori* SS-1 and RC-1 are pathogenic factors such as cytotoxin-associated gene A (*cagA*) and the vacuolating cytotoxin gene (*vacA*-positive strains).

Culture of *H. pylori* for Assay  The bacteria were grown on BHI agar plate supplemented with 10% defibrinated horse blood, 1 mg/ml glucose, and 250 μg/ml bovine serum albumin for 4 d in a microaerobic environment (15% CO₂, 5% O₂, and 80% N₂). The colonies were harvested from the plates, suspended in 20 ml BHI broth containing 10% FBS and 1 mg/ml glucose, and incubated at 37°C overnight with agitation on a rotary shaker at 100 rpm in a microaerobic condition. Two milliliters of the seed culture of *H. pylori* was inoculated into 200 ml BHI broth containing 10% FBS and 1 mg/ml glucose, and incubated at 37°C for 48 h with agitation on a rotary shaker at 100 rpm in a microaerobic condition. The resulting *H. pylori* was used for the assay.

Measurement of Inhibitory Activity against t-Lactic Acid-Dependent *H. pylori* Growth  In brief, *H. pylori* was suspended in DMEM/F-12 containing 5 mm t-lactic acid at a turbidity of 0.0018 (1×10⁵ CFU/ml). One hundred microliters of the suspension was added to each well of the 96 well culture plate then incubated with or without test compound at 37°C for 24 h under microaerobic condition. After incubation, the growth of *H. pylori* was determined by Alamar blue assay as described elsewhere. In brief, 10 μl of...
Alamar blue solution (Alamar Bio-Sciences, Sacramento, CA, U.S.A.) was added to each well and incubated for 1 h, and the fluorescence intensity was measured on a fluorescence reader, Fluoroskan II (Labsystems, Helsinki, Finland) at an excitation wavelength 544 nm and emission wavelength 590 nm.

Ordinary Assay for Anti-H. pylori Activity  

*H. pylori* was suspended in BHI broth containing 10% FBS and 1 mg/ml glucose (1 × 10^5 CFU/ml). One hundred microliters of suspension of *H. pylori* was added to the each well of a 96 well culture plate then incubated with or without test compound at 37 °C for 24 h under microaerobic condition. The growth of *H. pylori* was measured by Alamar blue assay as described above.

**Calculation of H. pylori Growth Inhibitory Activity**  

*H. pylori* growth inhibitory activity of the tested compound was calculated by the following formula:

\[
A: \text{cultured without test sample. } B: \text{cultured with test sample. } C: \text{ medium alone.}
\]

**RESULTS AND DISCUSSION**

In the course of our screening for anti-*H. pylori* substances of plant origin using our newly developed assay system, it was found that baicalein and glycyrrhetinic acid showed potent inhibitory effects against 1-lactic acid-dependent growth of *H. pylori*. The structures of these compounds are shown in Fig. 1. Each compound inhibited 1-lactic acid-dependent growth of both *H. pylori* SS-1 (clarithromycin sensitive) and RC-1 (clarithromycin resistant) strains in a dose dependent manner (Fig. 2). Whereas, no or negligible activity was observed by the ordinary assay for anti-*H. pylori* activity (Fig. 2). The IC_{50} value of the each compound is listed in Table 1. These results suggest that the inhibitory effect of baicalein and glycyrrhetinic acid is specific to 1-lactic acid-dependent growth of *H. pylori*.

Although baicalein and glycyrrhetinic acid showed potent inhibitory effects (Fig. 2), neither baicalin nor glycyrrhizin, which are glycoside forms of baicalein and glycyrrhetinic acid, respectively, showed inhibitory activity (data not shown). Therefore it is assumed that the cell membrane permeability of the compound is an important factor to exert inhibitory activity against 1-lactic acid-dependent growth of *H. pylori*.

The new assay method is based on the observation that 1-lactic acid strongly enhances growth of *H. pylori*. The mechanism of the growth-enhancing activity of 1-lactic acid is now not known. It has been reported that whole genomic analyses of *H. pylori*, strains 26695 and J99, predict the absence of a gene that codes for 1-lactate dehydrogenase (1-LDH).^{8,9} 1-LDH is a key enzyme for utilization of 1-lactic acid by tricarboxylic acid (TCA) cycle.^{10} Therefore it is suggested that *H. pylori* cannot utilize 1-lactic acid as an energy source by the TCA cycle. Because *H. pylori* has been predicted to code two lactic permease genes,^{8,9} *H. pylori* may be able to incorporate 1-lactic acid by the permease, and then it may be utilized through an unknown metabolic pathway for energy-yielding reaction or act as a signal transducer. The compounds that showed potent inhibitory activity against 1-

![Fig. 1. Structures of Inhibitory Substances against 1-Lactic Acid-Dependent Growth of *H. pylori*](image)

**Fig. 1.** Structures of Inhibitory Substances against 1-Lactic Acid-Dependent Growth of *H. pylori*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibitory activity against <em>H. pylori</em> growth (IC\text{50, } \mu M)</th>
<th>Growth of <em>H. pylori</em> in BHI broth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC\text{50} SS-1</td>
<td>IC\text{50} RC-1</td>
</tr>
<tr>
<td>Baicalein</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Glycyrrhetinic acid</td>
<td>0.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.01</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*H. pylori* SS-1 and RC-1 are clarithromycin-sensitive and -resistant strains, respectively. One hundred microliters of *H. pylori* suspension (1 × 10^5 CFU/ml) was added to each well of a 96 well plate then incubated with or without test compound at 37 °C for 24 h under microaerobic condition. After incubation, growth of *H. pylori* was determined by fluorometric method using Alamar blue as mentioned in Materials and Methods. Inhibitory activity against 1-lactic acid-dependent *H. pylori* growth was measured by ordinary assay (open circle). Anti-*H. pylori* activity measured by ordinary assay (closed circle).

![Fig. 2. Inhibitory Effects of Baicalein and Glycyrrhetinic Acid against Growth of *H. pylori*](image)

**Fig. 2.** Inhibitory Effects of Baicalein and Glycyrrhetinic Acid against Growth of *H. pylori*
lactic acid-dependent growth of *H. pylori* may inhibit such metabolic and/or signal transduction pathways. Further investigations are required to clarify the mode of action of the inhibitory compounds as well as how l-lactic acid enhances the growth of *H. pylori*.

In a recent observation, when the growth of *H. pylori* was assessed by both colony forming unit (CFU) method and fluorometric method using Alamar blue, comparable data were obtained.\(^3\) This result suggests that Alamar blue method is considered as an alternative method to CFU. Although CFU method is useful for counting the number of living *H. pylori*, it is hard to analyze multiple samples, and by this technique, a prolonged period (usually 4—5 d) is required for the incubation to obtain visible colonies on agar plate. Because estimation using Alamar blue method can be done within a few hours, this method was used for estimation of living *H. pylori* in this study. In addition to the advantage of shortening the assay time, Alamar blue method has made it possible for the assay to be performed on a 96 well microplate. The assay using 96 well microplate is suitable for the analyses of multiple samples such as column fractions or the screening of anti-*H. pylori* substances.

The newly developed assay system is rapid, simple, sensitive, and a useful method for estimation of living *H. pylori*, and may facilitate the development of novel therapeutic agents for *H. pylori* infection.

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**REFERENCES**