Aspirin increases susceptibility of *Helicobacter pylori* to metronidazole by augmenting endocellular concentrations of antimicrobials

Xiao-Ping Zhang, Wei-Hong Wang, Yu Tian, Wen Gao, Jiang Li

**Abstract**

**AIM:** To investigate the mechanisms of aspirin increasing the susceptibility of *Helicobacter pylori* (*H. pylori*) to metronidazole.

**METHODS:** *H. pylori* reference strain 26695 and two metronidazole-resistant isolates of *H. pylori* were included in this study. Strains were incubated in Brucella broth with or without aspirin (1 mmol/L). The *rdxA* gene of *H. pylori* was amplified by PCR and sequenced. The permeability of *H. pylori* to antimicrobials was determined by analyzing the endocellular radioactivity of the cells after incubated with [7-3H]-tetracycline. The outer membrane proteins (OMPs) of *H. pylori* 26695 were depurated and analyzed by SDS-PAGE. The expression of 5 porins (hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC of *H. pylori*) were analyzed using real-time quantitative PCR.

**RESULTS:** The mutations in *rdxA* gene did not change in metronidazole resistant isolates treated with aspirin. The radioactivity of *H. pylori* increased when treated with aspirin, indicating that aspirin improved the permeability of the outer membrane of *H. pylori*. However, the expression of two OMP bands between 55 kDa and 72 kDa altered in the presence of aspirin.

**CONCLUSION:** Although aspirin increases the susceptibility of *H. pylori* to metronidazole, it has no effect on the mutations of *rdxA* gene of *H. pylori*. Aspirin increases endocellular concentrations of antimicrobials probably by altering the OMP expression.

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Key words: *Helicobacter pylori*; Aspirin; Metronidazole; Resistance; Minimum inhibitory concentrations

**INTRODUCTION**

Aspirin, referred to as non-steroidal anti-inflammatory drugs (NSAIDs), is one of the most widely used drugs worldwide. It inhibits cyclooxygenases (COX), thereby irreversibly blocking the conversion of arachidonic acid to prostanooids. In addition, aspirin is also considered to offer some protection against coronary heart disease, due in part to inhibition of the thromboxane A2, a potent platelet aggregator. It has been reported that aspirin demonstrates chemopreventative activity against cancers in the esophagus, stomach and colon by inducing apoptosis in epithelial cells and regulating angiogenesis. Aspirin also has numerous effects in different bacterial species. Previous studies reported that aspirin could inhibit the growth of some bacteria, affect the production of virulence factors of some bacteria.
and alter the susceptibility of bacteria to some antibiotics by influencing the gene expression and inducing a number of morphological and physiological alterations in bacteria. We previously reported that NSAIDs, including sodium salicylate, aspirin, indomethacin and celecoxib, inhibited the growth of _H. pylori_ in a dose-dependent manner when incubated in brucella broth _in vitro_. These drugs also significantly affected the activity of virulence factors of _H. pylori_, for example, urease and vaculating cytotoxin. In addition, the minimum inhibitory concentrations (MICs) of clarithromycin, metronidazole and amoxicillin to _H. pylori_ decreased when treated with a low concentration of aspirin, indicating that aspirin increased the susceptibility of _H. pylori_ to these antimicrobials.

The aim of the present study was to investigate the mechanisms of aspirin increasing the susceptibility of _H. pylori_ to metronidazole. The _rdxA_ gene of _H. pylori_ treated with and without aspirin was analyzed by PCR amplification and sequencing. The effect of aspirin on the permeability of the outer membrane of _H. pylori_ was determined using [7-3H]-tetracycline. The effects of aspirin on the expression of outer membrane proteins (OMPs) of _H. pylori_ were also determined.

MATERIALS AND METHODS

**Chemicals**

Aspirin (Sigma Chemical Co, St Louis, MO, USA) and proton conductor carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma Chemical Co.) were dissolved in DMSO (Sigma Chemical Co.) in advance. [7-3H]-tetracycline (0.6 Ci/mmol; 22.2 GBq/mmol; Dupont/NEN Research Products, Boston, Mass.) was freshly dissolved in thin hydrochloric acid.

**Strains and culture conditions**

_H. pylori_ reference strain 26695 (susceptible to metronidazole) and two clinical isolates of _H. pylori_ (metronidazole resistant, R1 and R2) were included in this study. Strains were cultured on Columbia agar plates containing 8% sheep blood in a microaerobic atmosphere (< 1%, vehicle control) were added for 6 h at 37 °C.

**Determination of MICs**

Bacteria were prepared in Brain Heart Infusion broth to yield a viable count of 3 × 10^9 CFU/mL (equivalent to 1 McFarland turbidity standard unit) and used as the inocula for susceptibility testing. Bacterial suspension (100 μL) was spread, in duplicate, on Columbia agar plates with or without aspirin (1 mmol/L). A single E-test strip of metronidazole (OXOID Ltd, England) was applied to each plate. The MIC of metronidazole and the possible effect of aspirin on the MIC of metronidazole were determined after 72 h of incubation at 37 °C under microaerobic conditions.

**Extraction of genomic DNA**

_H. pylori_ genomic DNA was extracted using silicon dioxide method. Cells were harvested and washed twice in phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.2). The cell precipitation was suspended in 100 μL TE. Then 5 μL SiO2. Liq. and 400 μL binding buffer (containing 4 mol/L guanidinium isothiocyanate, 50 mmol/L Tris-HCl, 20 mmol/L EDTA) was added and incubated at 55 °C for 5 min with shaking once every minute. The suspension was centrifuged at 8000 r/min for 30 s at room temperature and the precipitate was washed thrice in cleaning buffer (containing 20 mmol/L Tris-HCl, 1 mmol/L EDTA, 100 mmol/L NaCl and dehydrated alcohol). The resulting suspension was dried at 55 °C and stored at -20 °C.

**Amplification of _H. pylori rdxA_ gene and sequencing**

The fragments (886 bp) containing the complete _rdxA_ gene was amplified by PCR. Forward primer: 5'-AGGGATTTATTGATGCTACAAG-3'; Reverse primer: 5'-AGGAGCATCAGATAGTTCTGA-3'. The PCR amplification was carried out in 25 μL reaction solution containing 2 μL of _H. pylori_ genomic DNA, 4.5 μL of 10 × PCR buffer (with 15 μmol/L MgCl2), 2 μL of dNTPs (each 2.5 mmol/L), 2 μL of forward and reverse primers (each 5 μmol/L), 0.5 μL of TaqDNA polymerase (1 U/μL) and 9 μL of ddH2O. The reaction was denatured initially at 94 °C for 5 min, followed by 30 cycles, with each cycle composed of 30 s at 94 °C (denaturation), 1 min at 52 °C (annealing), and 1 min at 72 °C (extension). After a final extension of 10 min at 72 °C, the amplicons were electrophoresed in a 1.5% agarose gel and purified using the silicon dioxide method as described above. The resulting _rdxA_ gene was sequenced by the dideoxy chain termination procedure at Beijing Li-Jia-Tai-Cheng Technology Company. The _rdxA_ genes of _H. pylori_ treated with and without aspirin were analyzed on line (http://align.genome.jp).

**Uptake studies using [7-3H] tetracycline**

Strain 26695 was grown to mid-logarithmic phase (approximately from 3 × 10^8 to 5 × 10^9 CFU/mL) in Brucella broth and then 1 mmol/L of aspirin or DMSO (< 1%, vehicle control) were added for 6 h at 37 °C on a shaker at 60 r/min under microaerobic conditions. Cell suspension was centrifuged at 8000 r/min for 10 min at room temperature and the precipitate washed and suspended in HEPES buffer (pH 7.2, containing 100 μmol/L MgCl2). At room temperature, 5 μCi [7-3H] tetracycline (0.6 Ci/mmol; 22.2 GBq/mmol; Dupont/NEN Research Products, Boston, USA) was added to 10 mL cell suspension. After 20 min, each cell suspension was divided into two halves, and 100 μmol/L CCCP was added to one half. One milliliter aliquots were
taken at 10 min intervals and washed three times in PBS. The resulting pellets were then diluted scintillation fluid and analyzed for radioactivity in an scintillation counter (TRI-CARB 2100TR).

**Purification of OMPs**

*H pylori* 26695 was incubated in Brucella broth for 48 h. The suspension was centrifuged at 8000 r/min for 10 min, washed, and suspended in ice-cold Tris-Mg buffer (10 mmol/L Tris-HCl containing 5 mmol/L MgCl₂, pH 7.3) and sonicated (once 30 s at 3-5 s interval for 5 min) until most of the cells were disrupted as visualized microscopically. Unbroken cells were removed by centrifugation at 8000 r/min for 20 min at 4°C. The inner and outer membranes were concentrated by centrifugation at 50000 r/min for 60 min at 4°C. The precipitate was suspended in 2% Triton Tris-Mg buffer (pH 7.5) and incubated for 30 min at room temperature, and then centrifuged and incubated again under the same condition. The resulting pellets, OMPs, were washed twice in 10 mmol/L Tris-HCl and resolved in ddH₂O. The final concentration of OMPs was determined by Coomassie brilliant blue R250 method.

**SDS-PAGE gel electrophoresis**

Ten microgram OMPs were used for SDS-PAGE gel electrophoresis at permanent voltage (5% stacking gel at 60 V, 10% separating gel at 100 V). After incubation for 30 min in fixing liquid, the gel was dyed with Coomassie brilliant blue G250 for 30 min.

**Isolation of total RNA and reverse transcription**

Total RNA was obtained by the TRIzol method as described by manufacturer (Invitrogen, Burlington, Ontario, Canada), and the contaminating DNA was removed by DNase I treatment according to the manufacturer (Sigma). For cDNA synthesis, 4 μg RNA diluted with DEPC H₂O was heated to 70°C for 5 min and chilled quickly on ice for 15 min. The samples were then added to a 20 μL reaction mixture containing 2 μL random hexamer primers (1 μg/μL), 0.4 μL of RNasin, 1 μL of M-MLV, 4 μL of dNTPs (each 2.5 mmol/L) and 4 μL of 5 × RT buffer. The cDNA synthesis reaction was performed for 60 min at 37°C. The enzyme was subsequently inactivated at 95°C for 5 min. Aliquots of cDNA were stored at -70°C.

**Real-time quantitative PCR**

The mRNA levels of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC were determined by real-time PCR using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA.). Specific primers and TaqMan probes were designed with the aid of the Primer Express program 3.0 (Perkin-Elmer Applied Biosystems) (Table 1). A standard curve was constructed using 10-fold serial dilutions of each cDNA. Reaction mixtures for PCR (50 μL) were prepared by mixing 5 μL synthesized cDNA solution with 5 μL of 10 × PCR buffer (containing 15 μmol/L MgCl₂), 3 μL forward and reverse primers (each 5 μmol/L), 4 μL of dNTPs (each 2.5 mmol/L), 2 μL TaqMan probe, 1 μL of ROX, 0.5 μL TaqDNA polymerase (1 U/μL). PCR was carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s according to the manufacturers’ instructions. The levels of the protein mRNA were expressed as the ratio of the protein mRNA to 16S rRNA mRNA ([protein mRNA (U/mL)/16S rRNA mRNA (U/mL)] × 100 000). The PCR was carried out in quintuple using samples prepared at the same time.

**Statistical analysis**

Statistical analysis was performed using SPSS, 13.0. Representative data of endocellular radioactivity and quantitative PCR were presented as mean ± SD. The Student’s *t* test was used to compare data. *P* < 0.05 was considered statistically significant.

### RESULTS

**Effects of aspirin on MICs of metronidazole**

For strain R1, MIC of metronidazole decreased from 256 μg/mL to 0.25 μg/mL in the presence of aspirin (1 mmol/L), and for strain R2, MIC of metronidazole reduced from 64 μg/mL to below the readable value (0.016 μg/mL), indicating that aspirin increased the susceptibility of *H pylori* to metronidazole and converted these two resistant strains to susceptible strains.

**Effects of aspirin on mutations of rdxA gene**

The 886 bp DNA fragments containing the complete *rdxA* gene were amplified by PCR for *H pylori* reference...
strain 26 695 and the two metronidazole resistant strains (R1, R2) treated with or without aspirin. Sequence analysis revealed that there were point mutations, insertions and deletions in \( \text{rdxA} \) gene of strain R2 when compared with the reference strain 26 695, and the nucleotide homology between the two strains (26 695 and R2) was 94.4%. However, the nucleotide homology of \( \text{rdxA} \) gene between strain R2 and its corresponding isolate R2(A) (strain R2 treated with aspirin) was over 99.5% (Figures 1 and 2).

**Effects of aspirin on permeability of outer membrane of \( \text{H pylori} \)**

Irrespective of the presence of CCCP, the radioactivity of \( \text{H pylori} \) treated with aspirin was higher when compared to that of the vehicle control (DMSO), indicating that aspirin enhanced the permeability of the outer membrane of \( \text{H pylori} \) (Figure 3).

Irrespective of the presence of aspirin, the radioactivity of \( \text{H pylori} \) cells decreased when the efflux pump inhibitor CCCP was added, indicating that aspirin did not interfere with the collapse effect of CCCP on the proton gradient across the cytoplasmic membrane of \( \text{H pylori} \) (Figure 4).

**Effects of aspirin on OMP profiles**

The OMP profiles of \( \text{H pylori} \) treated with aspirin were similar to that in control. However, the expression of two OMPs (Band 1 and 2) between 55 kDa and 72 kDa altered in the presence of aspirin (Figure 5).

**DISCUSSION**

\( \text{H pylori} \) is a spiral-shaped gram-negative bacterium and an important human pathogen that colonizes in the stomach of 50% of the world human population[10,11].

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**Table 2** cDNAs levels of porins and efflux system of \( \text{H pylori} \) tested by real-time quantitative PCR

| Gene   | Aspirin group | DMSO control | Blank control |
|--------|---------------|--------------|--------------|
| hopA   | 965.60 ± 23.33 | 1087.75 ± 26.42 | 856.27 ± 25.43 |
| hopB   | 84.00 ± 15.97  | 91.45 ± 14.83  | 100.15 ± 16.28 |
| hopC   | 52.98 ± 11.67  | 51.77 ± 12.01  | 52.79 ± 10.27  |
| hopD   | 83.86 ± 14.09  | 92.46 ± 17.78  | 89.92 ± 13.72  |
| hopE   | 54.07 ± 10.58  | 52.77 ± 11.93  | 52.12 ± 9.92   |
| hefA   | 12.31 ± 2.12   | 12.51 ± 1.99   | 12.97 ± 2.76   |
| hefB   | 25.47 ± 4.73   | 28.48 ± 5.07   | 25.60 ± 4.99   |
| hefC   | 55.62 ± 8.89   | 50.08 ± 9.97   | 53.37 ± 10.04  |

P > 0.05 (Aspirin group compared with DMSO control or blank control).

**Effects of aspirin on expression of mRNA of porins and efflux system**

Real-time quantitative PCR indicated that there was no significant difference in the levels of the cDNA of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC for strains treated with aspirin when compared with that in control. This indicated that, irrespective of the presence of aspirin, the expression of the levels of mRNA of hopA, hopB, hopC, hopD, hopE and hefA, hefB, hefC did not change (Table 2).
Infection results in chronic inflammation of the gastric mucosa and peptic ulcer, and it has been proven that 
*H pylori* infection is strongly associated with adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. International Agency for Research on Cancer affiliated to World Health Organization (WHO) defined 
*H pylori* as one of the first class human carcinogens\(^\text{[12]}\). Recent Studies revealed that 
*H pylori* infection plays important roles in the invasion of heart and brain vascular disorders, autoimmune diseases, nutritional and metabolic diseases, hematopathy and dermatologic diseases. Eradication of 
*H pylori* infection is very important to prevent and cure these diseases. The most successful treatment regimens use combinations of two or more antibiotics, such as amoxicillin, clarithromycin, metronidazole, or tetracycline, along with a proton pump inhibitor or bismuth. However, with the wide use of antimicrobials in clinical practice, antibiotic resistance is more and more apparent and is considered one of the major causes of treatment failure\(^\text{[13]}\).

Early studies suggested that salicylate inhibited the growth of some bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis*, and affected the activity of fimbriae, flagellum and the production of biofilm, slime, and thus might alter the pathogenicity of bacteria\(^\text{[14-21]}\). It has been reported that *in vitro*, salicylate could alter the susceptibilities of bacteria to some antimicrobials. Salicylate induced the intrinsic multiple antimicrobial resistance phenotype in many bacteria, such as *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*, and increased the susceptibilities of some bacteria to aminoglycosides\(^\text{[22-29]}\). Our previous studies also found that *in vitro* aspirin not only inhibited the growth of 
*H pylori*\(^\text{[6-9]}\), but also decreased the MICs of metronidazole, clarithromycin and amoxicillin to 
*H pylori*, and even converted some resistant strains to susceptible ones\(^\text{[6-8]}\). Therefore, the present study investigated the mechanisms of aspirin increasing the susceptibility of

Figure 3 Radioactivity of 
*H pylori* cells treated with aspirin (1 mmol/L) or vehicle control (DMSO). A: CCCP; B: No CCCP.

Figure 5 OMP profiles of 
*H pylori* 26695. M: Size marker; C: Control; D: DMSO; A: Aspirin. Band 1: OMP increased in the presence of aspirin; Band 2: OMP decreased in the presence of aspirin.

Figure 4 Radioactivity of 
*H pylori* cells treated with CCCP (100 µmol/L) or without CCCP. A: Aspirin; B: Vehicle control.
Metronidazole is a prodrug activated by nitroreductases in bacteria cells. Resistance of metronidazole is caused by either the absence or the inactivation of the nitroreductase\(^{[10]}\). It has been reported that the resistance of \( H\). \( pylori\) to metronidazole was mainly due to null mutations in the \( rdxA\) gene, which encoded an oxygen-insensitive NADPH nitroreductase\(^{[12]}\). However, studies also reported involvement of other reductases in the development of the resistant phenotype. In addition to oxygen-insensitive NADPH nitroreductases, several other nitroreductases in \( H. pylori\), such as NADPH flavin oxidoreductase, ferredoxin-like protein, flavodoxin, \( \alpha\)-ketoglutarate oxidoreductase and pyruvate: flavodoxin oxidoreductase, have been found to reduce metronidazole and to generate active compounds\(^{[35-38]}\). In our study, mutations in \( rdxA\) gene might be involved for the resistance of the isolated strain (R2). However, in the presence of aspirin, the strain converted from metronidazole resistant to susceptible, while the mutations in \( rdxA\) gene did not change. By using isotope scintillation technique with \([7-^{3}H]\)-tetracycline, our study revealed that aspirin increased the endocellular concentration of antimicrobials in \( H. pylori\) cells, indicating that aspirin increased the outer membrane permeability of \( H. pylori\) to antimicrobials. With the higher endocellular concentration in the presence of aspirin, metronidazole might be reduced and activated by other nitroreductases in \( H. pylori\). Therefore, the MIC of \( H. pylori\) to metronidazole decreased, and in some circumstances, resistant strains even converted to susceptible ones.

Two pathways may be involved in the mechanisms for the increasing concentration of antimicrobials in bacteria cells. One is the augmentation of anti-microbials entering into the bacteria cells passively; the other is the impairment of antimicrobials pumping out of the bacteria actively. Previous studies on \( Escherichia coli\) revealed that salicylate increased resistance to multiple antibiotics, including quinolones, cephalosporins, ampicillin, nalidixic acid, tetracycline and chloramphenicol\(^{[22]}\). Aspirin could induce multiple antibiotic resistance (\( mar\) gene), alter the expression of OMPs, and decrease the outer membrane permeability to antimicrobials or increase the efflux of antimicrobials\(^{[35-38]}\). Therefore, the MIC of \( H. pylori\) to metronidazole decreased, and in some circumstances, resistant strains even converted to susceptible ones.

The alteration of the permeability of outer membrane of \( H. pylori\) should be accompanied by the modification of some related OMPs. In the present study, the expression of two OMPs of \( H. pylori\) between 55 kDa and 72 kDa altered in the presence of aspirin. However, the functions and identifications of these OMPs need to be determined by two-dimensional electrophoresis and protein mass-spectrum analysis. If these OMPs were associated with the increase of the permeability of outer membrane of \( H. pylori\), further studies should be performed to determine whether the functional and phenotypic alterations of these OMPs in the presence of aspirin occurred at the levels of protein translation or modification, or some other porins or efflux systems were involved.

Park et al\(^{[43]}\) conducted a pilot study aimed at comparing the efficacy of the standard omeprazole-amoxicillin-clarithromycin (OAC) regimen with a combined OAC regimen and aspirin (OACA). Follow-up endoscopic findings showed that the previous ulcers were completely healed in all subjects. Although the eradication rate for the OACA group (86.7%) was higher than that of the OAC group (80.3%), there was no statistically significant difference between the two groups. The overall adverse events were similar in the two groups. The OACA regimen was well tolerated in the group of patients with peptic ulcer disease. The potential of aspirin and other NSAIDs for clinical use to augment the efficacy of \( H. pylori\) eradication may warrant further investigations.

With the increasing attention paid to the detriment of \( H. pylori\) and the resistance of antimicrobials to this microorganism, it is urgent to investigate new effective therapeutic regimens. Investigating the molecule...
mechanisms of aspirin increasing the susceptibility of \( H \) pylori to antimicrobials will help discover a more effective eradication regimen in clinical practice.

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**COMMENTS**

**Background**

It was reported that aspirin inhibited the growth of Helicobacter pylori \((H \) pylori) and the minimal inhibitory concentration (MICs) of clarithromycin, metronidazole, and amoxicillin to \( H \) pylori decreased when treated with aspirin. This indicated that aspirin increased the susceptibility of \( H \) pylori to these antimicrobials, and even converted some resistant strains to susceptible ones.

**Research frontiers**

\( H \) pylori infection results in chronic inflammation of gastric mucosa, peptic ulcer and is strongly associated with adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. Recent research revealed that \( H \) pylori infection played important roles in the invasion of heart and brain vascular disorders, autoimmune diseases, nutritional and metabolic diseases, hemopathies and dermatologic diseases. Eradication of \( H \) pylori infection is, therefore, very important to prevent and cure these diseases. However, with the wide use of antimicrobials in clinical practice, antibiotic resistance has become apparent and is considered one of the major causes of treatment failure.

**Innovations and breakthroughs**

In vitro, aspirin decreased the MICs of metronidazole, clarithromycin and amoxicillin to \( H \) pylori, and even converted some resistant strains to susceptible ones. This study investigated the mechanisms of aspirin increasing the susceptibility of \( H \) pylori to metronidazole.

**Applications**

Investigating the molecule mechanisms of aspirin increasing the susceptibility of \( H \) pylori to antimicrobials will help understand the mechanisms of the resistance of \( H \) pylori to antibiotics more intensively and discover a more effective eradication regimen in clinical practice.

**Terminology**

Carbonyl cyanide m-chlorophenylhydrazone (CCCP), a kind of efflux pump inhibitor that is effective at a micromolar concentration, can alter the pH gradient across the cytoplasmic membrane, therefore, deprives the energy provision of the transport protein.

**Peer review**

The authors intensively reported that non-steroidal anti-inflammatory drugs (NSAIDs), including sodium salicylate, aspirin, indomethacin and celecoxib, inhibited the growth of \( H \) pylori in a dose-dependent manner and changed the susceptibility of \( H \) pylori to antibiotics. In this study, the authors demonstrated that although aspirin increased the susceptibility of \( H \) pylori to metronidazole, it had no effect on the mutations of \( \text{rdxA} \) gene of \( H \) pylori and that aspirin increased endocellular concentrations of antimicrobials probably by altering the outer membrane proteins (OMPs) expression of \( H \) pylori. This theme is interesting, and will give new insights of \( H \) pylori eradication for physicians.

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