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BRIEF ARTICLE

Influence of efflux pump inhibitors on the multidrug resistance of *Helicobacter pylori*

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Abstract

AIM: To evaluate the effect of efflux pump inhibitors (EPIs) on multidrug resistance of *Helicobacter pylori* (*H. pylori*).

METHODS: *H. pylori* strains were isolated and cultured on Brucella agar plates with 10% sheep's blood. The multidrug resistant (MDR) *H. pylori* were obtained with the inducer chloramphenicol by repeated doubling of the concentration until no colony was seen, then the susceptibilities of the MDR strains and their parents to 9 antibiotics were assessed with agar dilution tests. The present study included periods before and after the advent of the EPIs, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), reserpine and pantoprazole), and the minimum inhibitory concentrations (MICs) were determined accordingly. In the same way, the effects of 5 proton pump inhibitors (PPIs), used in treatment of *H. pylori* infection, on MICs of antibiotics were evaluated.

RESULTS: Four strains of MDR *H. pylori* were induced successfully, and the antibiotic susceptibilities of MDR strains were partly restored by CCCP and pantoprazole, but there was little effect of reserpine. Rabeprazole was the most effective of the 5 PPIs which could decrease the MICs of antibiotics for MDR *H. pylori* significantly.

CONCLUSION: *In vitro*, some EPIs can strengthen the activities of different antibiotics which are the putative substrates of the efflux pump system in *H. pylori*.

Key words: Multidrug efflux pump; *Helicobacter pylori*; Multidrug resistance; Proton pump inhibitor; Real-time polymerase chain reaction

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is an important human pathogen that infects 50% of the world's population[1,2]. *H. pylori* infection in humans is associated with the development of numerous gastric pathologies, such as peptic ulcer, chronic gastritis and gastric cancer. Once *H. pylori* is established in the gastric submucosa, infected

individuals usually carry it for life unless treated[1,2]. In recent years, the rate of resistance of *H. pylori* to standard therapies has increased as a result of the widespread use of antibiotics. *H. pylori* resistance to metronidazole and clarithromycin has increased worldwide, and multidrug resistant (MDR) strains that are simultaneously resistant to amoxicillin, metronidazole and clarithromycin have been reported[3,4]: Boyanova *et al*[3] reported that 26.4% of the strains were resistant to metronidazole and clarithromycin among antibiotic-treated patients; Wueppenhorst *et al*[4] found that 15% of the *H. pylori* isolated from patients showed resistance to 2 or 3 types of antibiotic.

Efflux pump systems in bacteria which can eject the drugs and toxic compounds, including antibiotics, have a critical role in the development of multidrug resistance[5]. We have observed previously that the efflux pump gene *hefA* of *H. pylori* has valuable applications in multidrug resistance[6]. Until now, efflux pump inhibitors (EPIs) as promising therapeutic agents, have been widely investigated in other bacteria, where they could decrease the intrinsic bacterial resistance to antibiotics, and reverse the acquired resistance associated with efflux pump overexpression. Different classes of EPIs have been exploited and studied, including analogues of antibiotic substrates and new molecules[7,8]. However, whether the EPIs can also reverse the multidrug resistance of *H. pylori* has not been fully researched. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP), as an energy blocker, can induce a decrease in the transmembrane electrochemical gradient, and inhibit the efflux pumps driven by hydrogen ion gradients. Previous research showed that it can inhibit the multidrug and toxic compound extrusion (MATE) family efflux transporter pumps, such as NorM in *Bacteroides thetaiotaomicron*, and the resistance/nodulation/division superfamily (RND) family multidrug efflux transporter (MexAB-OprM system in *P. aeruginosa*), the small multidrug resistance subfamily of the DMT (drug/metabolite transporters) superfamily, such as QacE in *Escherichia coli*, and thus restore the antibiotic sensitivity of the bacteria[9-11]. Reserpine belongs to the alkaloid family, and could inhibit the major facilitator superfamily (MFS) and the adenosine triphosphate (ATP)-binding cassette (ABC multidrug efflux) superfamilies[12,13]. CCCP and reserpine are employed in the present study as different types of EPI, and a chloramphenicol-induced multidrug resistance model was developed. The aim of this investigation was to elucidate whither EPIs can influence the antibiotic susceptibilities of *H. pylori* MDR strains.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from TaKaRa Biotechnology Co. Ltd (Dalian, China). Chloramphenicol, tetracycline, and CCCP were purchased from Sigma Co. Ltd (Shanghai, China). Quant SYBR Green PCR Kits, polymerases and other molecular biology reagents were purchased from Biosail Biotechnology Co. Ltd (Beijing, China), and used according to their manufacturers' instructions. Clarithromycin, amoxicillin, penicillin G, cefotaxime, polymyxin B, piperacillin ciprofloxacin clindamycin ceftriaxone ampicillin, metronidazole, erythromycin and other drugs were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Bacterial strains and culture conditions

H. pylori isolates were obtained from routine cultures of clinical gastric biopsies from patients with peptic ulcer or chronic active gastritis at the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). *H. pylori* NCTC11637 obtained from Henan Key Laboratory of Molecular Medicine was used as a reference. Isolates were cultured on Brucella agar medium plates containing 7% lysed sheep blood at 37℃ under microaerobic conditions (50 mL/L O_2, 100 mL/L CO_2, 850 mL/L N_2) for 48-72 h. Identification of *H. pylori* isolates was based on the results of Gram staining, cell morphology, and positive reactions for catalase, oxidase and urease activities.

Induction of multiple antibiotic resistances of clinical isolates

Susceptible strains were isolated from gastric biopsy samples, with an established protocol[6], and the MDR strains were developed. Induction of chloramphenicol resistance in susceptible isolates was performed by selecting resistant colonies that arose in the agar plates containing 1/2 × minimum inhibitory concentration (MIC) chloramphenicol. The resistant colonies were incubated for 48-72 h under microaerobic conditions with repeated doubling of the chloramphenicol concentration until no colony was seen. The induced strains were further incubated on fresh plates with no chloramphenicol for 4 generations, to insure that the induced strains were non-adaptive resistance strains, and then transferred onto plates containing 4 × MIC chloramphenicol. Induced colonies were maintained on plates containing 4 × MIC of tetracycline, ampicillin, penicillin G, cefotaxime, piperacillin, ciprofloxacin, clindamycin, ceftriaxone, and erythromycin. The colonies were incubated for 48-72 h under microaerobic conditions.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

RNA was isolated using the total RNA kit (SBS Genetech Co., Ltd Beijing, China) and reverse transcribed into cDNA. *hefA*, *versus gyrB* (a housekeeping gene encoding for gyrase B), was utilized to study the relative expression of the *hefA* gene in 4 MDR strains and their parent strains. cDNA of *hefA* and *gyrB* was amplified using a real-time PCR 5700 sequence detector (Perkin Elmer Company) in the presence of Real Master Mix (SYBR Green). The gene-specific primers used were designed based on the sequence alignments of the genes from *H. pylori* 11637 in GenBank. The sequences of *hefA* (accession No: AF059041) are F: (5'-CTCGCTCGCATGATCGC-3')

Table 1 Induced multidrug resistant MIC profiles of *H. pylori* (μg/mL)

Strains	Exptl condition	ERY	CIP	TET	CTX	CRO	PIP	CLI	PEN	CAM
4151	Before induction	0.25	0.25	0.5	0.25	0.25	0.25	0.5	0.125	0.5
	After induction	2	0.5	4	1	2	0.5	1	0.25	32
8022	Before induction	0.125	1	1	0.5	0.5	0.5	0.5	0.063	0.25
	After induction	2	1	4	4	4	0.5	0.5	0.25	16
11021	Before induction	0.5	1	1	0.5	0.125	0.25	0.25	0.063	0.5
	After induction	2	2	2	4	4	1	1	0.5	32
11637	Before induction	0.063	0.5	0.25	0.25	0.125	0.25	1	0.25	0.5
	After induction	2	1	4	2	2	0.25	2	1	16
Geometric mean	Before induction	0.177	0.595	0.595	0.354	0.210	0.297	0.500	0.105	0.420
	After induction	2.000	1.000	3.364	2.378	2.828	0.500	1.000	0.420	22.627

ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; CTX: Cefotaxime; CRO: Ceftriaxone; PIP: Piperacillin; CLI: Clindamycin; PEN: Penicillin G; CAM: Chloramphenicol; Exptl: Experimental; MIC: Minimum inhibitory concentration.

and R: (5'-CGTATTCGCTCAAATTCCCT-3'). The PCR primers were predicted to amplify a 162-bp amplicon. Expression of the housekeeping gene *gyrB* (accession No: AB084049) was assessed in parallel with the primer pair *gyrB*: F: (5'-TTACTACGACTTATCCTGGGGCT AGCGCTG-3') and R: (5'-CCCCATCAATTTCCACA TTCTCCGC-3'). The PCR primers were predicted to amplify a 267-bp amplicon. According to the methods reported[6,14,15], *hefA* gene expressions in MDR and parent strains were determined.

Assessment of susceptibilities to antimicrobials

H. pylori strains grown for 48 h on sheep blood agar plates were resuspended in phosphate-buffered saline. Suspensions of *H. pylori* were adjusted to an optical density of 0.1 at 625 nm, and 1 μL of these suspensions containing approximately 10^5 colony forming units/mL, was spread on horse blood agar plates containing approximately 5×10^4 bacteria within 5 mm. (1) MICs of tetracycline, ampicillin, penicillin G, cefotaxime, piperacillin ciprofloxacin clindamycin ceftriaxone, erythromycin in MDR strains and their parent isolates were determined with the conventional 2-fold agar dilution tests; (2) After addition of CCCP (100 μmol/L), reserpine (20 μg/mL) and pantoprazole (10 μg/mL) to the agar, MICs of the antibiotics were determined again; and (3) To determine whether proton pump inhibitors (PPIs) can affect the *H. pylori* eradication effect of the antibiotics, 5 PPIs (omeprazole, rabeprazole, pantoprazole, lansoprazole, esomeprazole, 10 μg/mL) usually used in treatment of *H. pylori* infection were added to the incubation media as above, and MICs of metronidazole, amoxicillin, furazolidone and clarithromycin were tested. A 4-fold increase in MIC of antibiotics for the MDR strains was considered significant[16].

RESULTS

Induction of multidrug resistance in H. pylori strains

From 65 *H. pylori* isolates, 9 strains with no antibiotic resistance phenotype were selected. Following consecutive doubling of the concentration of chloramphenicol, MICs of chloramphenicol-induced strains to 9 antibiotics were determined. The MICs in 4 MDR strains (including 3 clinical isolates and *H. pylori* NCTC11637) were significantly increased (≥ 4-fold) compared with their parent strains (Table 1).

Table 2 Relative expression of hefA in PT and MDR strains

Strains	PT (*hefA*/*gyrB*)	MDR (*hefA*/*gyrB*)
4151	1.93	4.67
8022	1.85	5.84
11021	1.35	5.94
11637	2.45	6.88
mean ± SD	1.90 ± 0.45	5.83 ± 0.91

PT: Parent isolates; MDR: Multidrug resistant strains.

Expression of hefA in MDR strains and their parent isolates

The expression of *hefA* in MDR strains and their parent isolates was assessed by real-time RT-PCR. Each relative expression value was the mean of 3 replications. The relative expression of *hefA versus gyrB* in 3 clinical isolates and *H. pylori* 11637 was significantly higher in MDR strains (5.83 ± 0.91) than in parent strains (1.90 ± 0.45). The difference in *hefA* expression was statistically significant ($P < 0.001$) (Table 2).

MICs of the antibiotics in H. pylori strains after treatment with CCCP

When treated with CCCP (100 μmol/L), MICs of the 9 antibiotics were tested. MICs of 5 antibiotics decreased at least 4-fold in MDR strains: 19-fold with chloramphenicol, 10-fold with tetracycline, 7-fold with erythromycin, 4-fold with cefotaxime and ceftriaxone (geometric mean), while there was little difference in the MIC of antibiotics in parent strains (sensitive strains) after challenge with CCCP (Figure 1).

Difference in MICs of the antibiotics in H. pylori strains after treatment with pantoprazole or reserpine

After treatment with pantoprazole (10 μg/mL), MICs of the 9 antibiotics were tested. MICs of 5 antibiotics

Table 3 MICs of the antibiotics after treatment with pantoprazole or reserpine (μg/mL)

Antibiotic	Treatment	PT					MDR				
		4151	8022	11021	11637	Geometric mean	4151	8022	11021	11637	Geometric mean
Erythromycin	+Res	0.250	0.125	0.250	0.063	0.149	2.000	2.000	2.000	2.000	2.000
	+Pan	0.063	0.016	0.125	0.016	0.037	0.250	0.250	0.130	1.250	0.318
Ciprofloxacin	+Res	0.250	1.000	1.000	0.250	0.500	0.250	0.500	1.000	1.000	0.595
	+Pan	0.250	0.500	0.500	0.500	0.420	0.500	1.000	2.000	0.500	0.841
Tetracycline	+Res	0.500	0.500	1.000	0.125	0.420	4.000	4.000	2.000	4.000	3.364
	+Pan	0.125	0.500	0.250	0.063	0.177	0.250	0.250	0.250	0.250	0.250
Cefotaxime	+Res	0.125	0.500	0.250	0.250	0.250	1.000	4.000	1.000	2.000	1.682
	+Pan	0.063	0.250	0.250	0.063	0.125	0.063	0.500	0.500	0.250	0.250
Ceftriaxone	+Res	0.250	0.500	0.063	0.125	0.177	2.000	2.000	4.000	2.000	2.378
	+Pan	0.063	0.125	0.031	0.031	0.053	0.250	0.250	0.500	0.250	0.297
Piperacillin	+Res	0.125	0.500	0.250	0.250	0.250	0.500	0.500	1.000	0.063	0.354
	+Pan	0.125	0.250	0.125	0.125	0.149	0.250	0.250	0.500	0.125	0.250
Clindamycin	+Res	0.500	0.500	0.125	1.000	0.420	0.500	0.500	1.000	2.000	0.841
	+Pan	0.500	0.500	0.125	0.250	0.297	0.250	0.125	0.250	0.500	0.250
Penicillin G	+Res	0.125	0.032	0.063	0.250	0.089	0.250	0.250	0.125	1.000	0.297
	+Pan	0.063	0.032	0.016	0.125	0.044	0.125	0.063	0.125	0.500	0.149
Chlor-amphenicol	+Res	0.500	0.250	0.250	0.500	0.354	16.000	8.000	16.000	8.000	11.314
	+Pan	0.500	0.125	0.125	0.500	0.250	8.000	8.000	8.000	4.000	6.727

PT: Parent strain; MDR: Multidrug resistant strain; Pan: Pantoprazole; Res: Reserpine.

Table 4 Influence of proton pump inhibitors on MICs of the antibiotics in MDR strains (μg/mL)

	Before treatment	Omeprazole	Pantoprazole	Lansoprazole	Rabeprazole	Esomeprazole
Metronidazole	4.757	3.364	1.414	2.378	1.189	2.828
Amoxicillin	3.364	2.000	1.189	1.682	1.000	1.682
Furazolidone	2.378	2.000	2.378	2.000	2.378	2.378
Clarithromycin	5.657	4.757	3.364	5.657	4.000	4.757

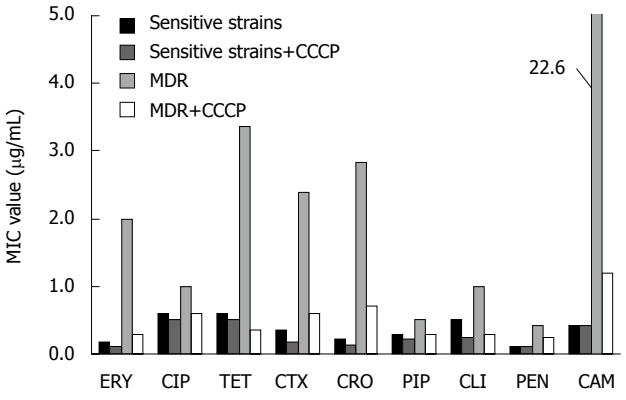

Figure 1 MICs of the antibiotics in *H. pylori* strains after treatment with CCCP (carbonyl cyanide m-chlorophenyl hydrazone). ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; CTX: Cefotaxime; CRO: Ceftriaxone; PIP: Piperacillin; CLI: Clindamycin; PEN: Penicillin; CAM: Chloramphenicol.

decreased at least 4-fold in MDR strains: 6-fold with erythromycin, 13-fold with tetracycline, 9-fold with cefotaxime and ceftriaxone, 4-fold with clindamycin (geometric mean), while MICs with erythromycin and ceftriaxone were also decreased 4-fold in their parent strains. MICs of antibiotics in MDR strains and parent strains had no obvious difference after reserpine (20 μg/mL) was added to the agar (Table 3).

Influence of PPIs on MICs of the antibiotics in MDR strains

To further clarify the effect of PPIs on MICs of antibiotics used in eradicating *H. pylori* in the clinic, and to determine which PPI was the most efficient in *H. pylori* treatment when combined with 2 antibiotics, MICs of metronidazole, amoxicillin, furazolidone and clarithromycin in MDR *H. pylori* strains were measured after treatment with one of the 5 PPIs (10 μg/mL). Our data indicated that MICs of metronidazole and amoxicillin were decreased 4-fold by treatment with rabeprazole, while there was a 3-fold decrease with pantoprazole. There was little effect of omeprazole, lansoprazole and esomeprazole on the MICs of antibiotics (Table 4).

DISCUSSION

With the extended use of antibiotics, the rate of resistance of antibiotics for *H. pylori* has increased worldwide, and appearance of an MDR strain that is simultaneously resistant to amoxicillin, metronidazole and clarithromycin, has been reported. In addition to chromosomally-encoded drug resistance, resistance to toxic compounds through increased export may be of importance in the multidrug resistance of *H. pylori*. Efflux systems have been identified in *H. pylori*[17], but the mechanism of multidrug resistance

in *H. pylori* is far from well understood, and a tetracycline resistance-related gene (*HP*1165) was found in 2006[18]. A non-specific MDR protein has also been reported, though we have little knowledge about the function of multidrug resistance[19,20]. Preliminary studies suggested that overexpression of the efflux pump systems in *H. pylori* were associated with multidrug resistance to antibiotics[6,21,22]. In the current investigation, the findings suggested that efflux pump systems were overexpressed under the pressure of increasing concentrations of antibiotics, which led to multidrug resistance to antibiotics in *H. pylori* strains, indicating that the long-term administration of antibiotics in the clinic may be responsible for *H. pylori* multidrug resistance.

Five families of multidrug efflux transporters have been described, which are primarily active transporters with energy from ATP hydrolysis or driven by ion gradients[23,24]. MDR efflux pumps are recognized as an important component of resistance in *H. pylori*. To combat resistant pathogens, in recent years EPIs, as promising therapeutic agents, have been widely investigated in other bacteria[25,26]. EPIs can be divided into several categories according to their mechanisms[27]; to interfere with the structural synthesis of the efflux pumps, to block the energy supply of the bacterial membrane, or to act as a competitive substrate of efflux pumps. CCCP, as an energy blocker, can reduce the transmembrane electrochemical gradient, and inhibit the efflux pumps driven by hydrogen ion gradients. The results of this study showed that CCCP could decrease MICs of the 5 antibiotics at least 4-fold in MDR *H. pylori* strains, while it had little influence on MICs of antibiotics in parent strains, indicating that overexpression of efflux pump systems in *H. pylori*, as in other bacteria, can cause multidrug resistance. Only MICs of 5 out of 9 antibiotics were reduced after treatment with CCCP, suggesting that these types of antibiotic are the substrates of the *HefA*BC-RND efflux pump in MDR *H. pylori*. Reserpine could inhibit MFS and the ATP-binding cassette (ABC multidrug efflux) superfamilies[12,13]. MICs of antibiotics in MDR strains and parent strains showed little difference when treated with reserpine, confirming that MFS and ABC family efflux pumps may be not responsible for multidrug resistance in *H. pylori*.

PPIs including omeprazole, rabeprazole, pantoprazole, lansoprazole and esomeprazole combined with 2 or 3 kinds of antibiotics have been used widely in the treatment of *H. pylori* infection. As proton motive forced uncoupling agents, however, less is known about whether PPIs could restore the antibiotic sensitivity of the MDR *H. pylori* strains. Other studies reported that PPIs, as one class of EPI, could decrease the MICs of norfloxacin, ciprofloxacin and levofloxacin by 4-8 fold by inhibiting the NorA efflux pump in MDR *Staphylococcus aureus*[28]. In this study, when treated with pantoprazole, MICs of the 5 antibiotics were decreased at least 4-fold in MDR strains, indicating that not only could PPIs act as antacids in the treatment of *H. pylori* infection, but also might inhibit the efflux pumps, and strengthen the effect of antibiotics by increasing their intracellular accumulation. Previous studies have indicated that 5 PPIs used in treatment of *H. pylori* infection had little effect on the eradication rate of *H. pylori*[29,30]. While 5 different PPIs were compared *in vitro* for their effects on the MICs of antibiotics in MDR *H. pylori* strains, our data showed that rabeprazole was the most efficient in reducing the multidrug resistance of these strains, with pantoprazole in second place. The underlying mechanisms require further study, but, based on the results in this study, it is suggested that rabeprazole could be used in the treatment of MDR *H. pylori* strains rather than other PPIs.

The antibiotic resistance rate of *H. pylori* has increased worldwide, and MDR strains have been reported, though limited information is available on the mechanisms of multidrug resistance and the role of EPIs in MDR *H. pylori*. To our knowledge, this study demonstrates for the first time that EPIs could partly reverse some types of antibiotic resistant phenotype in MDR *H. pylori* strains, and some PPIs contribute to enhance the antibacterial effect in *H. pylori* treatment.

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COMMENTS

Background

More than 50% of the world's population have *Helicobacter pylori* (*H. pylori*) infection, which is the major pathogen in human gastritis, peptic ulcers and gastric cancer. Nowadays, because of the extensive application of antibiotics, not only does the *H. pylori* resistance rate continue to rise, but also multidrug resistant (MDR) strains have emerged. The efflux pump systems of bacteria play an important role in the mechanism of multidrug resistance.

Research frontiers

To deal with the problem of multidrug resistance, in recent years, efflux pump inhibitors (EPIs) have been given extensive attention in many other bacteria, such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, *etc*. However, whether EPIs could influence the resistance of *H. pylori* has not yet been reported.

Innovations and breakthroughs

In the present study, some types of EPI were used to explore their effect on multidrug resistance of *H. pylori*, and for the first time it was demonstrated that EPIs could partly reverse an antibiotic-resistant phenotype in MDR *H. pylori* strains, and some proton pump inhibitors (PPIs) contributed to enhance the antibacterial effect in *H. pylori* treatment.

Applications

In vitro, the study found that EPIs could improve antibiotic sensitivity of MDR strains of *H. pylori*, and for clinical application, the EPI most efficient in decreasing the resistance should be investigated.

Peer review

The study by Zhang *et al*. explores the hypothesis that bacterial efflux pumps play an important role in the development of MDR *H. pylori*. Three chloramphenicol-induced MDR strains (out of 9 strains) were isolated from stomachs of patients and were shown to express higher levels of *hefA* (a efflux pump gene) mRNA.

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