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In Vitro Activity of Diphenyleneiodonium toward Multidrug-Resistant *Helicobacter pylori* Strains

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Background/Aims: The increased resistance of *Helicobacter pylori* to antibiotics has increased the need to develop new treatments for this bacterium. The aim of our study was to identify new drugs with anti-*H. pylori* activity. **Methods:** We screened a small molecule library—the library of pharmacologically active compounds (LOPAC), which includes 1,280 pharmacologically active compounds—to identify inhibitors of *H. pylori* growth. The minimal inhibitory concentrations (MICs) of antibiotics against multidrug-resistant *H. pylori* strains were determined using the agar dilution method. **Results:** We identified diphenyleneiodonium (DPI) as a novel anti-*H. pylori* agent. The MIC values for DPI were <0.03 μg/mL against all tested *H. pylori* strains. DPI also exhibited strong antibacterial activity against common gram-negative and gram-positive pathogenic bacteria. **Conclusions:** DPI may be a candidate anti-*H. pylori* drug for future development. **(Gut Liver 2017;11:648-654)**

Key Words: *Helicobacter pylori*; Diphenyleneiodonium; Drug resistance, multiple; Anti-bacterial agents; Minimal inhibitory concentration

INTRODUCTION

Helicobacter pylori infection is the main cause of peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric cancer. The eradication of *H. pylori* has been shown to dramatically decrease the recurrence of peptic ulcer disease, including gastric and duodenal ulcers.[1] Currently, it is estimated that around 50% of the world's population has this bacterium.[2] Hence, eradicating this organism is of significant clinical importance. According to various guidelines published since 1993, the first-line choice of treatment for *H. pylori* eradication consists of conventional triple therapy, which includes a proton pump inhibitor (PPI), clarithromycin, and amoxicillin for 7 to 14 days. Over the past few years, however, the efficacy of conventional triple therapy has decreased and now demonstrates eradication rates of less than 80%.[3,4] This decrease is mainly due to the emergence of clarithromycin-resistant *H. pylori* strains.

To improve first-line treatments for *H. pylori*, a four-drug treatment (including metronidazole), with sequential and concomitant bismuth-based quadruple therapy for 7 to 14 days, was introduced. In many previous studies, this regimen has demonstrated a better eradication rate than that of conventional triple treatment as the first-line treatment.[5-7] However, all of these treatments include metronidazole, which has been conventionally used as a second-line treatment. Hence, there is concern that these four-drug regimens could worsen antibiotic resistance and decrease the second-line eradication rate. Another drawback to these complex regimens is that they increase both the cost of therapy and patient noncompliance. In addition, quinolone is popularly used as a second-line treatment after a four-drug treatment regimen.[8] The quinolone resistance rate also has dramatically increased in recent years.[9]

Various methods have recently been introduced to overcome the drawbacks of four-drug treatment and fluoroquinolone-containing therapy. Rifabutin-containing therapy, probiotics, and tailored therapy are alternative methods.[10,11] However, these

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methods involve some challenges. Rifabutin causes serious complications, such as myelosuppression, and is difficult to use in countries with a high incidence of *Mycobacterium tuberculosis*.[12,13] Probiotics are relatively invulnerable agents, but the cost of eradication has increased and they have not been clinically effective in some randomized control trials.[14,15] Most importantly, the ultimate disadvantage of the various methods presented so far is that they could increase antibiotic resistance. Antibiotic resistance has been increasing gradually since *H. pylori* eradication began, and *H. pylori* will eventually become resistant to several antibiotics.[10,16] This will increase the incidence of multidrug-resistant *H. pylori*, and effective antibiotic regimens will gradually disappear. Therefore, resistance to diverse antibiotics indicates the need to develop new drugs that are effective against resistant strains.

The library of pharmacologically active compounds (LOPAC) is a collection of high-quality innovative molecules, such as antibiotics, enzyme inhibitors, cell-cycle regulators, and various other substances. It is designed for the identification of novel drug discovery assay and commonly used for screening of novel agents in drug discovery fields. LOPAC includes a large number of small molecules, and small molecules have certain benefits, such as high chemical stability and simple synthesis. Additionally, small molecules registered with LOPAC are commercially available and their effects on human cells are well known. Therefore, it is easier to apply these substances in clinical practice. Many studies have demonstrated that LOPAC is useful and effective for detecting new drugs against various pathogens, such as fungi, tuberculosis, malaria, and viruses.[17-20] Therefore, we utilized LOPAC as a way to identify anti-*H. pylori* drugs. The primary purposes of our present study were to (1) identify new chemical agents with potential anti-*H. pylori* activity among the 1,200 compounds included in the LOPAC Chemical Library; and (2) measure the minimal inhibitory concentrations (MICs) of these candidates against reference and resistant strains of *H. pylori*.

MATERIALS AND METHODS

1. Bacterial strains and culture conditions

The well-characterized ATCC 43504 *H. pylori* strain (ATCC, Manassas, VA, USA) was used as the reference strain for the chemical library screening assay and the initial antimicrobial susceptibility test. Twenty clinical isolates (known to be resistant to antibiotics currently used to treat *H. pylori*) and three susceptible strains were used to test the anti-*H. pylori* activity of diphenyleneiodonium (DPI; Sigma D2926; Sigma-Aldrich Co., St Louis, MO, USA). Resistant strains included single drug- and multidrug-resistant strains. Single drug-resistant strains were resistant to clarithromycin (Sigma C9742), metronidazole (Sigma M1547), levofloxacin (Sigma 28266), or amoxicillin (Sigma A8523), all of which are frequently used to eradicate *H. pylori*. Multidrug-resistant strains were resistant to at least two of these four drugs. Antibiotic resistance was assessed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.[21] *H. pylori* strains were cultured using a selective medium that contained Brucella agar (BD 211088) and 7% defibrinated sheep blood. Incubation of the cultured isolates was performed at 37°C under microaerobic conditions (10% CO_2) for 72 hours.

2. Screening for inhibitors of *H. pylori* growth

The LOPAC Chemical Library was purchased from Sigma-Aldrich Co. and is composed of 1,280 small pharmacologically active molecules. These chemical compounds (2 mM) were serially diluted in 96-well source plates to select the growth inhibition concentration in a 50-μL volume. Each well of the 96-well microplate contained 180 μL of culture medium to which 10 μL of the chemical compound and 10 μL of a stock solution of 10^6 *H. pylori* ATCC 43504 bacteria/mL was added. The plates were then incubated under microaerobic conditions at 37°C for 72 hours to allow for bacterial growth. After 3 days, the chemical compounds that prevented the growth of *H. pylori* were regarded as candidates for anti-*H. pylori* activity and further analyzed in antimicrobial susceptibility testing.

3. Determination of MIC

The susceptibilities of the *H. pylori* isolates to antibiotics were examined using the serial 2-fold agar dilution method, as previously described.[21] Briefly, the bacteria were subcultured on Mueller–Hinton agar supplemented with 5% defibrinated sheep blood for 48 hours. The bacterial suspension was adjusted to 10^7 colony-forming units and directly inoculated onto each antibiotic-containing agar dilution plate. After incubation for 72 hours, the MIC of each antibiotic was determined. The MIC range for amoxicillin, clarithromycin, and DPI is 0.03125 to 32 μg/mL. The MIC range for metronidazole is 0.5 to 128 μg/mL. The standard *H. pylori* ATCC 43504 strain was included in these susceptibility tests as a control. The resistance breakpoints for amoxicillin, clarithromycin, metronidazole, and levofloxacin were defined as ≥1.0, >1.0, ≥8 and >1.0 μg/mL, respectively. The MIC results were obtained from two experiments.

RESULTS

1. Anti-*H. pylori* activities of DPI

More than 50 chemical compounds from the small-molecule LOPAC library prevented any visible *H. pylori* growth, and were regarded as having an inhibitory effect on *H. pylori*. Based on currently known pharmacological applications, we excluded antibacterial and antifungal agents that are currently used in clinical practice. Anticancer drugs acting on the cell cycle, apoptosis, DNA metabolism, and phosphorylation were also excluded. We also excluded antipsychotics and antidepressants that could affect the central nervous system through unexpected

mechanisms of action. Calcium channel blockers and calcium channel activators were also excluded due to the possibility of cardiac toxicity. Finally, we selected a promising candidate, DPI, for further analysis (Fig. 1). DPI is a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor that reduces the production of reactive oxygen species (ROS) and may reverse atherosclerosis. DPI is presently under investigation for its possible neuroprotective effects against focal cerebral ischemia, but there are no reports on its antimicrobial activity. The MIC value of DPI against the *H. pylori* ATCC 43504 strain was <0.03 μg/mL.

We further evaluated the possible anti-*H. pylori* activities of DPI using multidrug-resistant *H. pylori* strains. The MIC values of DPI for the ATCC 43504 strain and 23 clinical isolates are listed in Table 1. The antibiotic resistance of each strain is also stated in Table 1. The MIC value of DPI was <0.03 μg/mL against all of the tested *H. pylori* strains, indicating strong anti-*H. pylori* activity.

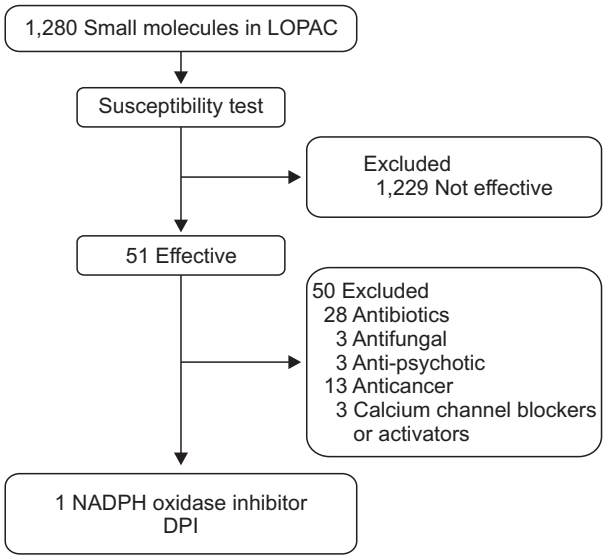

Fig. 1. Flow chart showing the selection of anti-*Helicobacter pylori* agents.
LOPAC, library of pharmacologically active compounds; NADPH, nicotinamide adenine dinucleotide phosphate; DPI, diphenyleneiodonium.

Table 1. Antimicrobial Susceptibilities of the Tested *Helicobacter pylori* Strains

Strain	Resistance	MIC, μg/mL				
		CLA	AMO	MET	LEV	DPI
HP43504	MET	0.03	<0.03	128	0.25	<0.03
CS_S1	-	0.03	0.125	4	0.25	<0.03
CS_S2	-	0.03	0.125	4	0.25	<0.03
CS_S3	-	0.03	<0.03	2	0.25	<0.03
CS_C1	CLA	64	0.25	4	0.5	<0.03
CS_C2	CLA	32	0.125	4	0.5	<0.03
CS_C3	CLA	64	<0.03	2	0.25	<0.03
CS_M1	MET	0.06	<0.03	128	0.5	<0.03
CS_M2	MET	0.06	<0.03	128	0.5	<0.03
CS_M3	MET	0.06	0.125	128	0.5	<0.03
CS_L1	LEV	0.03	0.25	2	16	<0.03
CS_L2	LEV	0.06	0.125	2	32	<0.03
CS_L3	LEV	0.06	<0.03	4	8	<0.03
CS_A1	AMO	0.06	1	4	0.25	<0.03
CS_A2	AMO	0.06	2	4	0.25	<0.03
CS_A3	AMO	0.03	4	4	0.25	<0.03
CS_CM1	CLA/MET	32	<0.03	128	0.5	<0.03
CS_CM2	CLA/MET	64	<0.03	16	0.25	<0.03
CS_CM3	CLA/MET	32	0.03	64	0.25	<0.03
CS_CA1	CLA/AMO	64	1	2	0.25	<0.03
CS_CA2	CLA/AMO	64	1	4	0.25	<0.03
CS_CA3	CLA/AMO	32	2	1	0.25	<0.03
CS_CML	CLA/MET/LEV	128	0.06	128	64	<0.03
CS_CMA	CLA/MET/AMO	64	1	64	0.5	<0.03

MIC, minimal inhibitory concentration; CLA, clarithromycin; AMO, amoxicillin; MET, metronidazole; LEV, levofloxacin; DPI, diphenyleneiodonium; HP, *Helicobacter pylori*; CS, clinical strain.

Table 2. Antimicrobial Susceptibility of Gram-Negative and Gram-Positive Pathogenic Bacteria to DPI and Vancomycin

	Strain	MIC, μg/mL	
		DPI	Vancomycin
Gram-positive	*Staphylococcus aureus* 29213 (MSSA)	1	1
	Staphylococcus aureus 33591 (MRSA)	1	1
	Enterococcus faecalis 29212	1	4
	Staphylococcus epidermidis 12228	1	2
Gram-negative	*Escherichia coli* 25922	0.5	>32
	Pseudomonas aeruginosa 27853	2	>32
	Acinetobacter baumannii 19606	1	>32
	Salmonella enteritidis 13076	1	>32
	Salmonella typhimurium 13311	1	>32
	Enterobacter cloacae 13049	1	>32

DPI, diphenyleneiodonium; MIC, minimal inhibitory concentration; MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*.

2. Antibacterial activity of DPI against common pathogenic bacteria

The activity of DPI against common gram-negative and -positive pathogenic bacteria was also assessed. The MIC results are indicated in Table 2 and ranged between 0.5 and 2 μg/mL against gram-negative bacteria. The MIC of DPI against all of the tested gram-positive bacteria was 1 μg/mL. These MIC values are comparable to those of vancomycin, which is a commonly used antibiotic against gram-positive bacteria including methicillin-resistant *Staphylococcus aureus*.

DISCUSSION

We demonstrate that the NADPH oxidase inhibitor DPI has inhibitory activities against reference and resistant *H. pylori* strains and could be a candidate for further studies and future drug development. Importantly, the MIC value of DPI is lower than previously reported chemical compounds.[22] The direct mechanism by which DPI inhibits *H. pylori* has not been clearly evaluated, but our indirect *in vitro* evidence suggests that DPI noncompetitively inhibits NADPH oxidase via covalent binding to flavin adenine dinucleotide. Although DPI is a nonspecific inhibitor of flavoenzymes, a decrease in ROS production has been reported in DPI-treated cells.[23] Based on this decrease in ROS production, it has been speculated that DPI may reverse atherosclerosis and this compound is now under investigation to determine its neuroprotective effects in focal cerebral ischemia, a neurodegenerative disease.[24] Micromolar concentrations of DPI are highly toxic, but sub-picomolar concentrations of DPI inhibit NADPH oxidase activation with high specificity.[25] In another study, DPI was reported to inhibit *H. pylori*-induced increases in ROS, NADPH oxidase activity, MCP-1 expression, and the activation of MAPKs (mitogen-activated protein kinases), including the extracellular signal-regulated kinases, p38, and Jun N-terminal kinases, in AGS cells.[26]

DPI is likely to be a novel agent in the future for two reasons. First, a NADPH oxidase inhibitor is a new substance that has never been used to treat *H. pylori* infection. Previous studies have shown that some chemicals inhibit activation of NADPH oxidase, suggesting that they may alleviate cell inflammation in *H. pylori*-infected gastric epithelial cells.[27,28] DPI may also suppress unnatural apoptosis in gastric epithelial cells infected with *H. pylori*.[29] However, whether DPI can directly eradicate *H. pylori* has not been reported. In our *in vitro* study, we demonstrated that DPI effectively eradicated *H. pylori*. This is the first study to investigate the efficacy of DPI for eradicating *H. pylori*. This novel agent may be free from current antibiotic resistance, so it may help to overcome antibiotic resistance. Second, NADPH oxidase exists in gastric mucosal tissue, and DPI is a nonselective inhibitor of NADPH oxidase.[30,31] *H. pylori* infections increase ROS in infected cells using NADPH oxidase. This increase in ROS causes oxidative DNA damage in infected cells and may provoke *H. pylori*-infected carcinogenesis.[32] Therefore, a substance with a NADPH oxidase inhibitor mechanism, such as DPI, may inhibit carcinogenesis and eradicate *H. pylori*. Finally, in this study, DPI was effective against all *H. pylori* strains regardless of antibiotic resistance. Considering these findings, we expect that DPI may help to overcome multidrug-resistant *H. pylori* in the future.

As mentioned earlier, the rate of resistance to commonly used antibiotics such as clarithromycin, metronidazole, and fluoroquinolone is the main cause of the decrease in the eradication rate of certain bacteria. In addition, the number of multidrug-resistant bacterial strains has increased. This highlights a critical need to develop selective antibacterial agents with novel target sites and establish an effective drug-resistance management strategy. In contrast, the development of new antimicrobial

agents is somewhat out of proportion. Rifabutin, furazolidone, sitafloxacin, and nitazoxanide have been introduced but are not always available in some countries. In addition, rifabutin demonstrates serious side effects, such as myelosuppression, and should be reserved to treat mycobacterial infections. Furazolidone is a nitrofuran antibiotic that has demonstrated efficacy in some trials. However, it has been recognized by the U.S. Food and Drug Administration as a carcinogenic agent and thus is no longer used, except in a few developing countries.[33] Sitafloxacin seems to be effective, but clinical data are still limited in Japan.[34,35] Nitazoxanide is an antiprotozoal agent that has demonstrated efficacy in a restricted study, but it is a somewhat expensive agent.[36,37] Further studies of these antibiotics are needed.

In addition to these antimicrobial approaches, therapeutic alternatives beyond antibiotics have been investigated in recent years, including natural phytotherapy and probiotics.[22,38] Representative agents include *Lactobacillus reuteri*, Korean red ginseng, and sulforaphane. Micro- and nano-technologies have also been used to develop gastric drug delivery systems.[39,40]

Acid stability is an important factor for antibiotics used to treat *H. pylori*. Some studies have reported that major antibiotics (clarithromycin, amoxicillin, metronidazole) for *H. pylori* treatment degrade in human gastric acid at different pH values. Metronidazole is stable in an acidic environment (half-life >800 hours); however, clarithromycin is unstable in an acidic environment (half-life <1 hour).[41,42] The quick destruction of antibiotics in gastric acid makes them less effective at eradicating *H. pylori*. Therefore, it is important that this problem is solved. No study has reported on the influence of gastric juice on antibacterial efficacy and acid stability of DPI. Additionally, DPI has not been reported as an antibiotic in humans. However, DPI has excellent antibiotic effects *in vitro*, and it has an antimicrobial effect on *H. pylori* as well as other gram-negative and -positive pathogenic bacteria. DPI is also expected to maintain acid stability using these methods, but more studies are needed.

We demonstrated that DPI has the ability to strongly eradicate *H. pylori*. Although DPI is relatively highly toxic, some studies have reported that ultra-low dose DPI is not overtly toxic in mice and has potent neuroprotective efficacy.[24,43] Therefore, we could produce a substance that strongly inhibits *H. pylori* infection without toxic side effects by identifying the proper dose of DPI to treat *H. pylori*. Additionally, DPI has excellent antibacterial effects even in multidrug-resistant *H. pylori*. These advantages could help DPI serve as a novel agent in the future. Although we identified a novel *H. pylori* chemical inhibitor, additional steps are needed to develop such a substance into a viable drug because of several limitations. First, DPI is a non-specific NADPH inhibitor, so drug development is somewhat limited. Second, the interactions between DPI and other medications (PPIs and other antibiotics) for treating *H. pylori* have not been elucidated. Finally, the effects of an acidic environment on the activities of anti-*H. pylori* agent have not yet been considered. The high environmental pH of the stomach may affect the susceptibility of these bacteria to antibiotics. Further research is needed to examine the effects of pH on anti-*H. pylori* activity in different agents. Animal experiments will also be needed to determine whether substances such as DPI are effective under *in vivo* conditions.

In conclusion, DPI shows a potent MIC value against *H. pylori*, suggesting that it might be a promising new agent for that could be used to significantly improve anti-*H. pylori* treatment success and eradicate this pathogen.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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