ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2005, p. 3468–3473 Vol. 49, No. 8
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.8.3468–3473.2005

Bactericidal and Morphological Effects of NE-2001, a Novel Synthetic Agent Directed against *Helicobacter pylori*

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Received 3 July 2004/Returned for modification 5 September 2004/Accepted 5 May 2005

The antibacterial activities of NE-2001 were tested against 24 clinical isolates of *Helicobacter pylori* and compared with those of amoxicillin, clarithromycin, metronidazole, and furazolidone. The MIC_{50} and MIC_{90} of this synthetic compound on the isolates were 8 and 16 μg/ml, respectively. This action was highly selective against *Helicobacter pylori*; there was a >4-fold difference between the concentration of NE-2001 required to inhibit the growth of *Helicobacter pylori* and that required to inhibit the growth of common aerobic and anaerobic bacteria. Exposure of *Helicobacter pylori* (ATCC43504) to NE-2001 at the MIC (4 μg/ml), or at a greater concentration, resulted in an extensive loss of viability. The phenomenon was also observed at pH levels between 3.0 and 7.0. When two clinical *Helicobacter pylori* strains were successively cultured at subinhibitory concentrations of NE-2001, no significant changes in the bactericidal effects were found. The morphological alterations of *Helicobacter pylori* cells (ATCC43504), exposed to NE-2001 at various concentrations for 6 h, were observed using transmission electron microcopy. The bacterium displayed features such as swelling, vacuole-like structures in the cytoplasm, and cell destruction following exposure to NE-2001. The efficacy of NE-2001 was maintained when evaluated in eight clinical isolates resistant to metronidazole and five isolates resistant to both metronidazole and clarithromycin (MIC ranging between 4 and 16 μg/ml). The above-described results suggest that NE-2001 may have the potential to be developed as a candidate agent for the treatment of *Helicobacter pylori* infection.

Helicobacter pylori (*H. pylori*) is a ubiquitous gram-negative, microaerophilic spiral bacterium infecting half the world's population and causing chronic active gastritis in virtually all infected individuals (5). The majority of patients who acquire chronic *H. pylori* infection exhibit mild gastritis (7). Epidemiological, laboratory, and interventional human studies strongly suggest that *H. pylori* plays a pathogenic role in the development of adenocarcinoma of the distal stomach (6). The mechanisms by which *H. pylori* may cause gastroduodenal disease and contribute to gastric carcinogenesis are still hypothetical. However, the production of specific virulence factors by the bacterium, the inflammatory response of the host, and the association with environmental contributors may all be responsible (3).

Treatment regimens for *H. pylori* infection have been evolving since the early 1990s, when monotherapy was first recommended. Antimicrobial therapy for this infection is a complex issue, and the following drugs are currently used in combination regimens: proton-pump inhibitors and/or bismuth, metronidazole, clarithromycin, and amoxicillin (14). Tetracycline is used in the rescue therapy (8). Although optimal first-line treatment is associated with high cure rates, the rising prevalence of resistance to the antibiotic component of current eradication regimens increasingly threatens to compromise the efficacy of these regimens. Strains resistant to metronidazole (9) and clarithromycin (18) have been well documented, while resistance to amoxicillin (23) and tetracycline was mainly reported in Asia (11). Therapeutic regimens directed against *H. pylori* infection will continue to evolve. What is required is a simpler and more efficacious strategy for the treatment of *H. pylori* infection. New antibiotics with the following characteristics have been sought among many synthetic compounds and secondary metabolites of microorganisms: (i) high specificity for *H. pylori*; (ii) stability in 0.1 N HCl; and (iii) lower frequency of natural resistance. Following vigorous screening of various compound libraries, NE-2001, a small synthetic molecule with the novel structure 4-(4-methylbenzyl)-4′-[guanidinomethylbenzoyloxy]biphenyl-4-carboxylate hydrochloride (Fig. 1), was discovered to demonstrate a specific inhibitory effect on the growth of *H. pylori* in vitro (24). It was proposed that the mechanism of action by which NE-2001 exerts its anti-*H. pylori* activity may relate to suppression of bacterial DNA synthesis (4). In the present study, we investigated the effects of NE-2001 on the viability, urease activity, and morphology of *H. pylori* in vitro, in conjunction with resistance development following repeated exposure and its ability to

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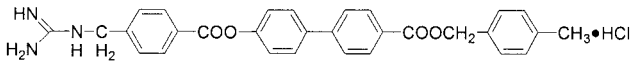

FIG. 1. Chemical structure of NE-2001.

inhibit the growth of metronidazole- and clarithromycin-resistant strains of the bacterium.

MATERIALS AND METHODS

Bacterial strains. Clinical isolates of *H. pylori* were randomly collected from 50 patients (aged between 30 and 61 years), consulted at Renji Hospital in Shanghai and Jiangsu People's Hospital in Nanjing, China, who suffered from duodenal ulcer, reflux esophagitis, chronic superficial gastritis, chronic atrophic gastritis, and chronic erosive gastritis diagnosed by endoscopy and/or histology. Gastric biopsy specimens were collected from antrum of the stomach, before or after first-line treatment, and identified for *H. pylori* infection according to morphology by Gram staining and oxidase, catalase, and urease reactions (19). These isolates were maintained frozen at −70°C before experimentation. The standard strain ATCC43504 was used for experimental and quality control purposes. The laboratory standard strains of common aerobic and anaerobic bacteria were obtained from our culture collection.

Antibacterial agents. NE-2001 was prepared using the method described previously (24). Amoxicillin, metronidazole, and furazolidone were commercially available (Sigma Chemical Co., St. Louis, MO). Clarithromycin was obtained from Livzon Pharmaceutical Group Inc. (Zhuhai, Guangdong, China). Amoxicillin and furazolidone were dissolved in dimethyl sulfoxide (DMSO), metronidazole in water, and clarithromycin in acetone. NE-2001 was dissolved in a 2-hydroxypropyl-β-cyclodextrin (Sigma) solution (molar ratio = 1:10; prepared at 50°C for 30 min). These stock solutions were serially diluted in sterile water to give final concentrations on the day of use.

Susceptibility testing. The MICs for *H. pylori* were determined by an agar dilution method (16) with minor modification. Briefly, Mueller-Hinton agar (Oxoid, Basingstoke, U.K.) plates (10 ml/each) were prepared containing 7% lysed horse blood (Shanghai Institute of Biological Products, China) and twofold serial dilutions of the test compounds. They were inoculated with 5 μl of each bacterial suspension (10^7 CFU/ml) by use of a multipoint inoculator (Sakuma, Tokyo, Japan) and incubated at 37°C for 3 days in an incubator in a microaerobic atmosphere consisting of 5% O_2, 10% CO_2, and 85% N_2 with 98% humidity (Napco Co., Winchester, VA). An antibiotics-free plate and plates with corresponding dilutions of DMSO or 2-hydroxypropyl-β-cyclodextrin were used as negative controls to ensure bacteria viability and no contaminants in the inoculums. The MICs for other common bacteria were also determined by the agar dilution method using Mueller-Hinton agar inoculated with respective bacteria suspensions.

Bactericidal activity. Portions of Brucella broth with 10% fetal bovine serum (FBS) (10 ml) and 0.1% β-cyclodextrin (Sigma) containing various concentrations of NE-2001 were inoculated with the bacteria from an overnight culture to yield an initial cell concentration of approximately 10^6 CFU/ml. The cultures were shaken at 37°C in a microaerobic atmosphere, and 100 μl were removed at various time points (0, 3,6, 24, and 48 h). Viable bacteria were counted following 10-fold serial dilutions in Brucella broth with 10% FBS, and each strain was inoculated in triplicate onto Columbia agar (Difco Co., Sparks, MD) supplemented with 8% defibrinated sheep blood. Colonies were counted after 3 days of incubation in a microaerobic atmosphere. Bactericidal activities of NE-2001 under various pH conditions were also measured by altering the medium pH levels.

Assay for resistance development. Two clinical isolates of *H. pylori*, adjusted to a cell density of approximately 10^6 CFU/ml in Brucella broth supplemented with 10% FBS, were exposed to serial twofold dilutions of NE-2001 and metronidazole, respectively. Following incubation at 37°C for 24 h, the MICs were recorded. The culture that attained turbidity comparable to that of the untreated culture in the presence of the highest level of the test agents was further exposed to increasing concentrations of NE-2001 or metronidazole. These procedures were repeated for up to 10 cycles, and fluctuations in MICs during the course of continued exposure were determined.

Urease activity measurement. Three types of urease were used. Crude urease from *H. pylori* (ATCC43504) was prepared from the whole cell according to the method described by Dunn and colleagues (5) with modifications. Briefly, bacterial cells, cultured overnight in Brucella broth supplemented with 10% FBS, were collected and suspended to reach a concentration of 10^7 cells/ml. The cell suspension was vortex mixed for 10 min and centrifuged at 1,500 × *g* for 15 min at 4°C to extract urease. The supernatant was frozen and stored at −80°C until use. The stock solution was diluted with purified water after thawing, and 25-μl volumes containing 0.5 to 1.0 μg of protein were incubated with different concentrations of the test compounds for 60 min at 37°C. This was followed by the addition of 100 μl phosphate-buffered saline (PBS) buffer (pH 6.8) containing 500 mM urea, 0.02% phenol red, and 0.1 mM dithiothreitol in each sample. The ureases from *Bacillus pasteurii* and jack beans were purchased from Sigma and used as controls. Color development was monitored at 560 nm (25°C) during the 60-min incubation period.

Transmission electron microscopy. *H. pylori* cells, after exposure to NE-2001 at 0, 2, 4, and 8 μg/ml for 6 h at 37°C under microaerobic conditions, were collected by centrifugation and treated with Karnovsky's fixative at 4°C for 24 h. The samples were then rinsed with 0.1 M PBS and stained with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 2 h. Following a wash with 0.1 M PBS, they were dehydrated for multiple times (15 min each) in escalating concentrations of ethanol (70%, 80%, 90%, 95%, and 100% [vol/vol]) and embedded in a Quetol mixture. Sections were cut with a diamond knife on a Porter-Blum MT6000 ultra microtone (RMC, Tucson, AZ) and stained with both 1% uranyl acetate and lead citrate. The sections were examined with a transmission electron microscope (Hitachi H-600; Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV.

Reversal of drug resistance by NE-2001. A total of 27 of the above-described clinical isolates (including two from the same patient) were cultured on Columbia agar (Difco) supplemented with 7% lysed horse blood and then submitted to metronidazole and clarithromycin susceptibility testing, respectively, using the above-described agar dilution method. Eight strains resistant to metronidazole (breakpoint MIC, >8 μg/ml) (23) and five strains resistant to both metronidazole and clarithromycin (breakpoint MIC, >2 μg/ml) (21) were identified thereafter (from 12 patients). They were subcultured once to ascertain reliable growth before measurement of MICs for NE-2001.

TABLE 1. Antibacterial activities of NE-2001, amoxicillin, clarithromycin, metronidazole, and furazolidone against 24 clinical isolates of *H. pylori*

Compound	MIC (μg/ml)[a]		
	Range	MIC_{50}	MIC_{90}
NE-2001	4–16	8	16
Amoxicillin	0.0156–0.125	0.03125	0.0625
Clarithromycin	0.03125–4	0.03125	1
Metronidazole	2–128	4	32
Furazolidone	0.03125–0.5	0.125	0.5

[a] Data represent MIC values observed in three independent experiments.

RESULTS

Antibacterial activity. The ranges of MICs for NE-2001, amoxicillin, clarithromycin, metronidazole, and furazolidone against 24 clinical isolates of *H. pylori* and the minimal concentrations required to inhibit the growth of 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates are shown in Table 1. NE-2001 inhibited the growth of all the *H. pylori* strains tested, with MICs ranging between 4 and 16 μg/ml. No strain resistant to NE-2001 was found among the 24 clinical isolates. Both NE-2001 and metronidazole were shown to be inactive (MICs > 64 μg/ml) against a collection of four gram-positive (*Staphylococcus aureus* 209P JC, *Staphylococcus epidermidis* ATCC12228, *Enterococcus faecalis* ATCC29212, and *Bacillus subtilis* ATCC6633) and five gram-negative (*Escherichia coli* K12, *Providencia rettgeri* NIH96, *Pseudomonas aeruginosa* PAO-1, *Klebsiella pneumoniae* NCTC9632, and *Morganella morganii* KONO) bacteria compared to the results seen with amoxicillin or clarithromycin. Amoxicillin was unable to inhibit *Pseudomonas aeruginosa* PAO-1 and *Morganella morganii* KONO

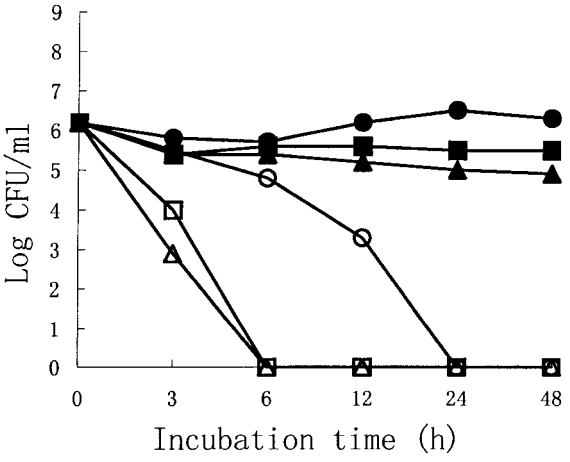

FIG. 2. Bactericidal effects of NE-2001 on *H. pylori* ATCC43504 (MIC = 4 μg/ml). NE-2001 concentrations used were 0 (●), 0.8 μg/ml (■), 1.6 μg/ml (▲), 3.2 μg/ml (○), 6.4 μg/ml (□), and 12.8 μg/ml (Δ).

(MICs > 100 μg/ml), while clarithromycin had no effect on the growth of *Providencia rettgeri* NIH96, *Pseudomonas aeruginosa* PAO-1, and *Morganella morganii* KONO (MICs > 100 μg/ml).

Bactericidal activity. The killing kinetics of NE-2001 for *H. pylori* (ATCC43504) is summarized in Fig. 2. NE-2001 displayed a concentration-dependent bactericidal activity against *H. pylori*, and the number of viable organisms decreased progressively following exposure to 3.2 μg/ml or greater concentrations.

Effect of NE-2001 on the viability of *H. pylori* at various pH levels. As shown in Fig. 3, following exposure to NE-2001 at pH levels between 3.0 and 7.0, *H. pylori* (ATCC43504) lost its viability. NE-2001 displayed concentration-dependent bactericidal effects at all pH values tested. In particular, no cell growth was observed at 6 h after exposure to 12.8 μg/ml at pH 3.0.

Effect of NE-2001 on resistance development. There was no significant alteration in the susceptibilities of the *H. pylori* strains tested to NE-2001 following repeated exposure. Met-

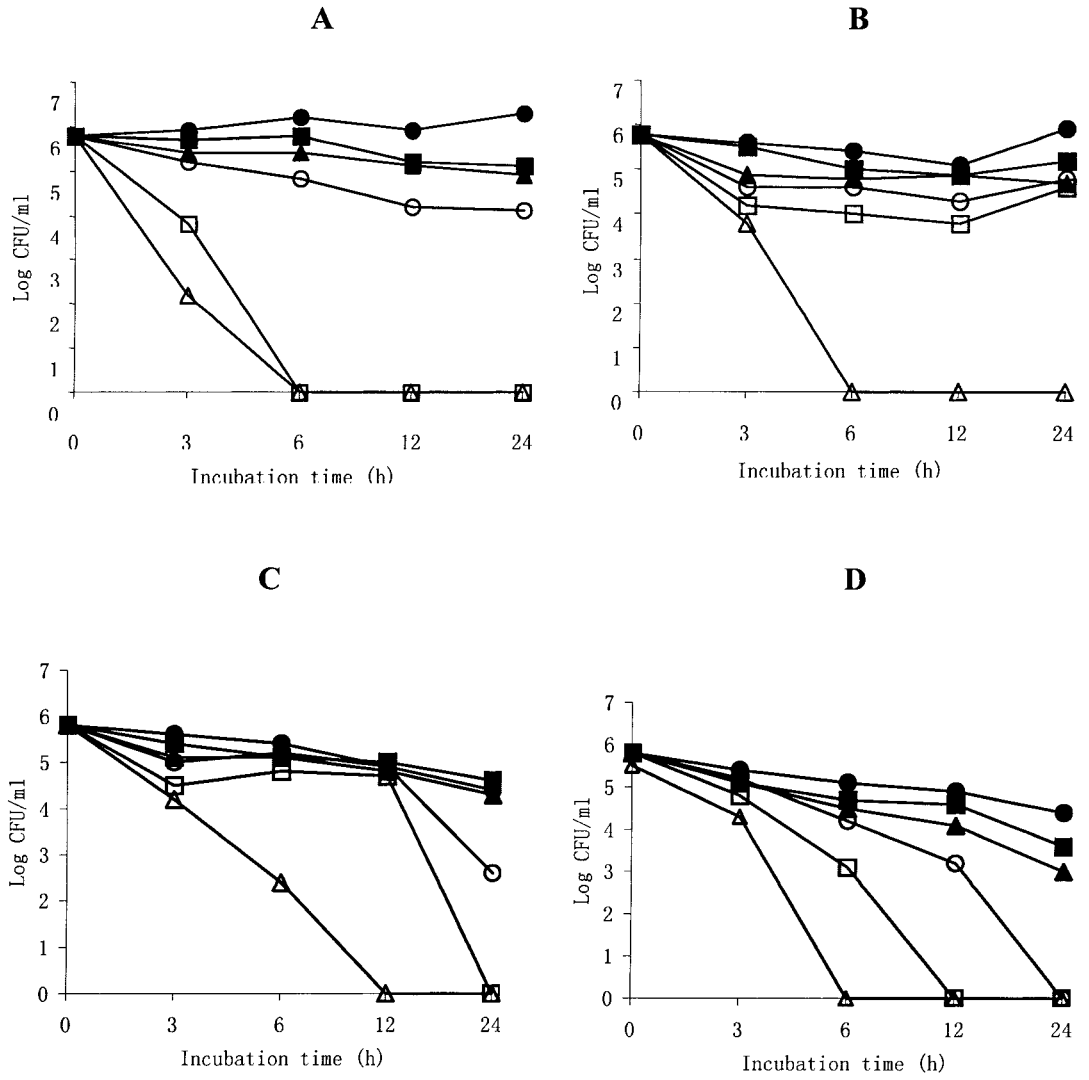

FIG. 3. Bactericidal effects of NE-2001 on *H. pylori* ATCC43504 (MIC = 4 μg/ml) at pH 7 (A), 6 (B), 5 (C), and 3 plus 10 mM urea (D). NE-2001 concentrations used were 0 (●), 0.8 μg/ml (■), 1.6 μg/ml (▲), 3.2 μg/ml (○), 6.4 μg/ml (□), and 12.8 μg/ml (Δ).

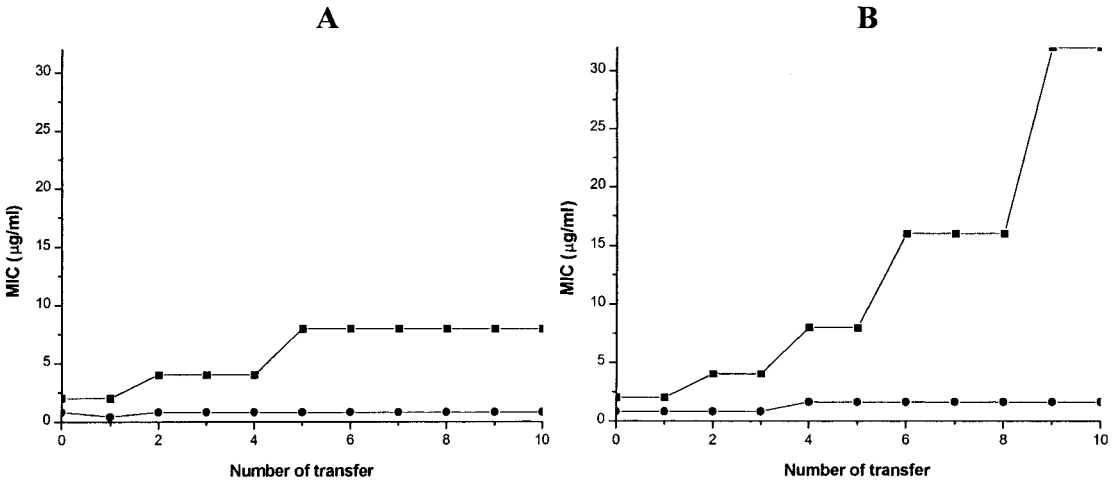

FIG. 4. Development of resistance to NE-2001(●) and metronidazole (■) in *H. pylori* strains HP003 (A) and HP032 (B).

ronidazole, on the other hand, induced a rapid emergence of drug resistance (Fig. 4).

Inhibition of urease activity. The comparative inhibitory effects of NE-2001 and acetohydroxamic acid on the urease activities of *H. pylori* (ATCC43504) and two other sources are shown in Table 2. Acetohydroxamic acid significantly inhibited urease activities from all three sources when preincubated for 1 h at 37°C, while at a concentration equal to eightfold of the MIC NE-2001 did not show any inhibitory effect on the three ureases in vitro.

Effect on *H. pylori* morphology. The morphological alterations of *H. pylori* cells (ATCC43504) exposed to 2 μg/ml, 4 μg/ml, and 8 μg/ml of NE-2001 for 6 h are shown in Fig. 5. Transmission electron microscopy demonstrated that NE-2001 treatment induced swelling and vacuole-like structures in the cytoplasm of *H. pylori* cells. The phenomenon was concentration dependent, and after exposure to 2 (Fig. 5B) or 4 (Fig. 5C) μg/ml of NE-2001, the organism changed its appearance from bacilliform to doughnut-shaped form. The bacterium lost its structure and displayed destructive features at 8 μg/ml (Fig. 5D). Moreover, the outer envelope of an atypically shaped organism was detached from the inner side of the bend.

Effect on drug-resistant strains of *H. pylori*. Of the 27 clinical isolates subjected to metronidazole and clarithromycin susceptibility testing, 8 strains were found to be resistant to metronidazole and 5 to be resistant to both metronidazole and clarithromycin. When further exposed to various concentrations of amoxicillin, furazolidone, or NE-2001, the growth of these drug-resistant strains of *H. pylori* was significantly inhibited, with MICs similar to those reported previously (12, 20) or observed with the standard strain (ATCC43504) (Table 3). No effect of DMSO or 2-hydroxypropyl-β-cyclodextrin was noted in agar culture plates.

TABLE 2. Inhibitory effects of NE-2001 and acetohydroxamic acid on various ureases

Urease source	IC_{50}[a]	
	NE-2001(μg/ml)	Acetohydroxamic acid (μM)
H. pylori ATCC43504	>32	3.7
Bacillus pasteurii	>32	9.8
Jack beans	>32	4.5

[a] IC_{50}, 50% inhibitory concentration.

DISCUSSION

Following the recognition of the important pathogenic role of *H. pylori* infection in the development of gastroduodenal diseases, there has been a continuous search for improved eradication therapy, especially for small molecules with novel mechanism(s) of action. Based on the initial discovery of NE-2001 (4), we further tested the antimicrobial activities of this compound against 24 clinical isolates of *H. pylori*, as presented in this paper. The MIC_{90} of NE-2001 was 16 μg/ml, lower than metronidazole and higher than amoxicillin, clarithromycin, and furazolidone. Time-to-kill studies revealed that the anti-*H. pylori* activity of NE-2001 is of a bactericidal nature, resulting in cell lysis after 6 h of exposure at a concentration of 6.4 μg/ml. In contrast to conventional antibiotics, the effect of NE-2001 is *H. pylori* specific, with little impact on common aerobic and anaerobic bacteria. This unique selectivity may be attributed to its preferential penetrability to the target site, as has been shown by other hydrophobic compounds of low molecular weight (1). The antibiotics currently used in *H. pylori* eradication all have a broad antibacterial spectrum, and their use would therefore affect the normal gut flora, leading to a series of gastrointestinal side effects. It is expected that NE-2001 may have less liabilities due to its high specificity for *H. pylori*.

There have been discrepancies between in vitro bioactivities and clinical efficacies of several antibacterial agents in the clearance of *H. pylori* from the stomach (22). In the Mongolian gerbil model, eradication efficacy was significantly improved by addition of a proton-pump inhibitor (10) to clarithromycin or by use of mucoadhesive microspheres containing amoxicillin (15). Such augmentation was achieved either through neutralization of the low pH environment or extension of exposure time to the treatment regimen. Obviously, NE-2001 may overcome this deficiency, as it is stable and remains efficacious under acidic conditions.

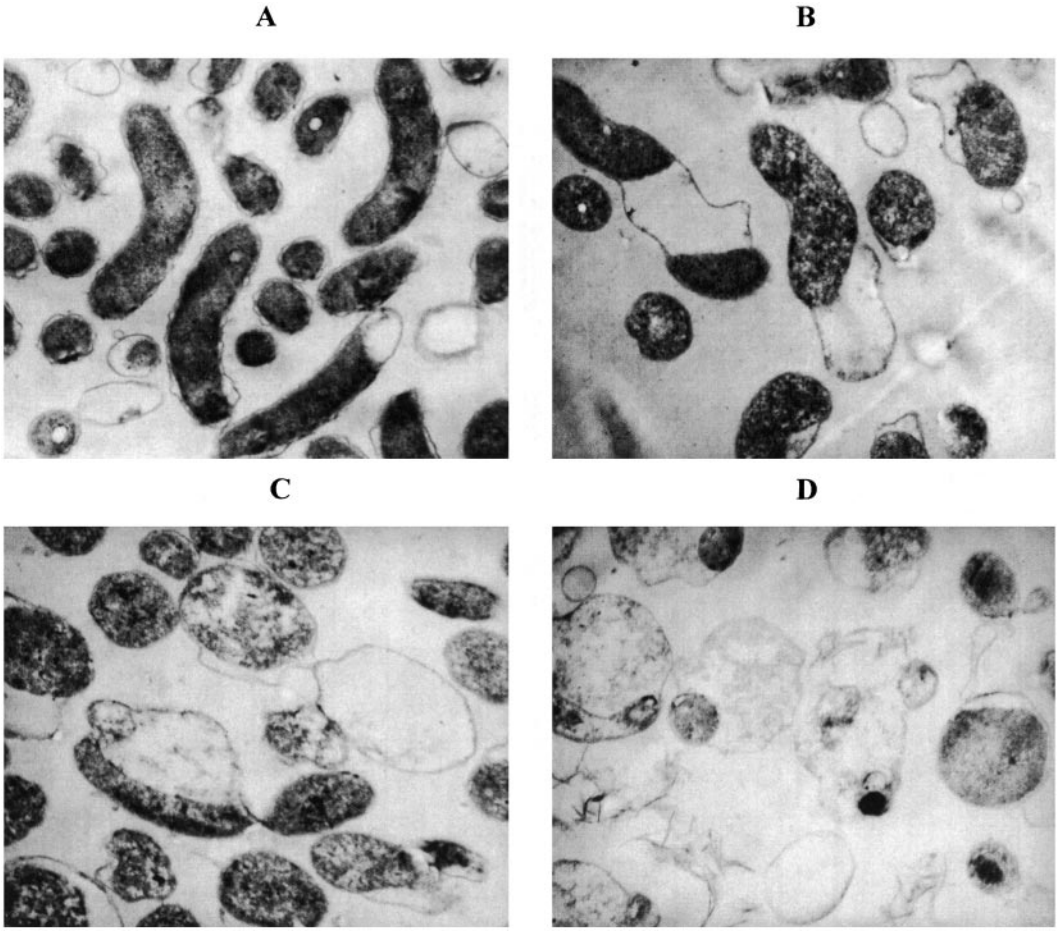

FIG. 5. Transmission electron micrographs of *H. pylori* exposed to NE-2001. *H. pylori* ATCC43504 cells were treated with NE-2001 for 6 h at 0 μg/ml (A), 2 μg/ml (B), 4 μg/ml (C), and 8 μg/ml (D).

TABLE 3. Effect of NE-2001 on drug-resistant strains of *H. pylori*

H. pylori strain group	Diagnosis[b]	Gender[c]	Age (yr)	MIC[a] (μg/ml)				
				Metronidazole	Clarithromycin	Amoxicillin	Furazolidone	NE-2001
Non-drug-resistant strain								
Standard strain (ATCC43504)				128	0.03125	0.03125	0.03125	4
Drug-resistant strains								
O-81	RE-CSG	F	48	64	8	0.125	0.25	16
O-59	CAG	F	61	128	8	0.03125	1	16
O-60	CAG	M	56	64	8	0.03125	0.25	16
O-60-2				64	4	0.03125	0.0625	4
O-79	CSG-CAG	F	52	16	4	0.125	0.25	4
O-69	CSG-CAG	F	40	128	0.03125	0.03125	1	16
O-146	DU	M	34	16	0.125	0.03125	0.25	8
O-130	DU	M	40	64	0.0625	0.03125	0.125	8
O-3	CAG	M	46	64	0.03125	0.25	1	8
O-157	CAG	M	44	32	0.0625	0.0625	0.5	16
O-80	CEG	F	43	16	0.03125	0.03125	0.25	8
O-72	CSG-CAG	M	32	128	0.03125	0.03125	0.25	16
O-78	DU (H_2)	M	33	64	0.03125	0.03125	0.25	8

[a] Each value represents the median observed in three independent experiments.
[b] RE, reflux esophagitis; CSG, chronic superficial gastritis; CAG, chronic atrophic gastritis; DU, duodenal ulcer; CEG, chronic erosive gastritis; H_2, healing stage II.
[c] M, male; F, female.

The increasing prevalence of *H. pylori* strains resistant to some of the most commonly used antibacterial agents is the major cause of failure to eradicate the infection (8). Some investigators have suggested that secondary resistance to metronidazole and clarithromycin develops very rapidly and thereby limits the usefulness of a number of potentially effective agents (2). It was reported previously that the resistance rates of *H. pylori* to metronidazole and clarithromycin found in randomly collected clinical isolates in Shanghai were 49.7% and 7.3%, respectively (13). In addition to confirmation of the above-described observations, we have demonstrated in this study that the clinical strains resistant to metronidazole and clarithromycin were all susceptible to NE-2001 treatment in vitro. It is worth noting that unlike metronidazole, repeated exposure of *H. pylori* to NE-2001 in vitro did not lead to selection of any resistant mutants. The data, taken together, point to the potential of developing NE-2001 as a novel candidate agent against *H. pylori* with high sensitivity to certain drug-resistant strains of the bacterium and low frequency of natural resistance.

It has been shown that urease is an important virulence factor of *H. pylori* for the development of gastric infection and induction of damages to the gastric mucosa (5). However, NE-2001 did not display any inhibitory effect on *H. pylori* urease activity compared to acetohydroxamic acid, a widely used urease inhibitor. This result indicates that the inhibitory action of NE-2001 on the growth of *H. pylori* is independent of urease. Our previous study demonstrated that the effect of NE-2001 is mediated through an inhibition of the bacterial DNA replication mechanism (4), but the exact molecular target for NE-2001 remains to be investigated.

The marked morphological changes of *H. pylori* cells following exposure to NE-2001 include swelling of the bacilliforms, development of numerous blebs on cell surface, and emergence of vacuole-like structures in the cytoplasm. These observations suggest that the target whereby NE-2001 exerts its biological effect may be located on the cell surface that functions as a permeability barrier. Conceivably, the bactericidal mechanism of NE-2001 against *H. pylori* may be the result of its perturbation of the permeability barrier within cell membranes. Nevertheless, other mechanisms of action could not be ruled out, including interruption of *H. pylori* colonization (17).

In conclusion, the new chemical entity, NE-2001, is highly selective in inhibiting the growth of *H. pylori* with moderate concentrations at neutral pH and under acidic conditions. This in vitro effect of NE-2001 may have the potential when given orally to decrease the viability of *H. pylori* in the stomach or gastric mucus, thereby relieving pathological damages caused by the bacterium. Further studies will be directed towards the exploration of NE-2001 to become a new and locally acting therapeutic agent to treat *H. pylori* infection.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China Grant (30271555 to D.X.Z.) and grants from the Shanghai Municipality Science & Technology Development Fund (014319238 and 044319220 to M.W.W.), the Ministry of Science and Technology of China (2002AA2Z343A to M.W.W.), and the Chinese Academy of Sciences (KSCX1-SW-11-2 to M.W.W.).

We are indebted to Weiwen Xu for technical assistance, Qing Zheng for patient specimen and data collection, and Dale E. Mais for valuable comments and critical review of the manuscript.

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