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A Specific Anti-*Helicobacter pylori* Agent NE2001: Synthesis and Its Effect on the Growth of *H. pylori*

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Abstract—The synthesis and anti-*Helicobacter pylori* activity of a novel agent NE2001, 4-(4-methylbenzyl)-4′-[guanidinomethylbenzoyloxy] biphenyl-4-carboxylate hydrochloride, are described. NE2001 had a specific inhibitory effect on the growth of *H. pylori* preceded by the suppression DNA synthesis in the cell. The effects of NE2001 on RNA and protein syntheses in *H. pylori* were also examined.

It has been widely accepted that *Helicobacter pylori*, a Gram negative spiral bacterium is an important bacterial pathogen which induces chronic gastritis and is associated with gastroduodenal ulcer, adenocarcinoma of the distal stomach, and gastric lymphoma in humans.[1] Recently, the World Health Organization classified *H. pylori* as a group 1 carcinogen responsible for its leading role in the development of gastric cancer.[2] The most recent guidelines proposed in the Maastricht 2-2000 Consensus Report recommend the use of proton pump inhibitors (PPI) along with antibacterial agent to eradicate *H. pylori*.[3] Since oral administration of metronidazole, PPI, and clarithromycin and amoxicillin was put to use, the infection in up to 80–90% of the cases have been cured. However, the application of antibacterial agents causes a serious problem which induces the resistant strain of *H. pylori* to the reagent. Actually, the resistant strains to metronidazole, clarithromycin and amoxicillin have already been reported.[4] Another severe problem is caused by the administration of PPI and antibacterial agent, that is, PPI can induce indigestion and large amounts of antibacterial agent can result in severe destruction of the bacterial flora in digestive tract. Therefore, it is important to find out new types of active compounds for the eradication of *H. pylori*. When we were screening various compounds, we discovered that a novel compound 4-(4-methylbenzyl)-4′-[guanidinomethylbenzoyloxy] biphenyl-4-carboxylate hydrochloride, NE-2001, specifically inhibited the growth of *H. pylori* in vitro.

The synthetic route for preparing NE2001 is shown in Scheme 1. The minimum inhibitory concentrations (MIC, μg/mL) of NE2001 and several related compounds against *H. pylori*, *Staphylococcus aureus* and *Escherichia coli* are shown in Table 1. The results indicate that no intermediates showed any anti-bacterial activity (MIC > 64 μg/mL), except intermediate **6** that had weak activity against *H. pylori*, while NE2001 showed potent anti-*H. pylori* activity (MIC 0.8 μg/mL). The structure–activity relationships (SAR) of NE2001 were also studied and the results are shown in Table 1. NE2001 showed about 10-fold improved activity compared to **B**, which indicated when compound **A** and **B** linked by an ester bond, the anti-*H. pylori* activity significantly increased. Compound **G** linked by an ester bond showed some anti-*H. pylori* activity while compound **H** linked by an amide bond had none anti-*H. pylori* efficiency. These results indicated that the ester bond may play an important role in the activity of NE2001. The MICs for NE2001, amoxicillin and metronidazole against 12 clinical isolates of *H. pylori* and other common bacteria are shown in Table 2. NE-2001 inhibited the growth of all the tested *H. pylori* strains with the MIC ranging from 0.4 to 1.6 μg/mL. No strain resistant against NE2001 was found among the 12 clinical isolates of *H. pylori*,

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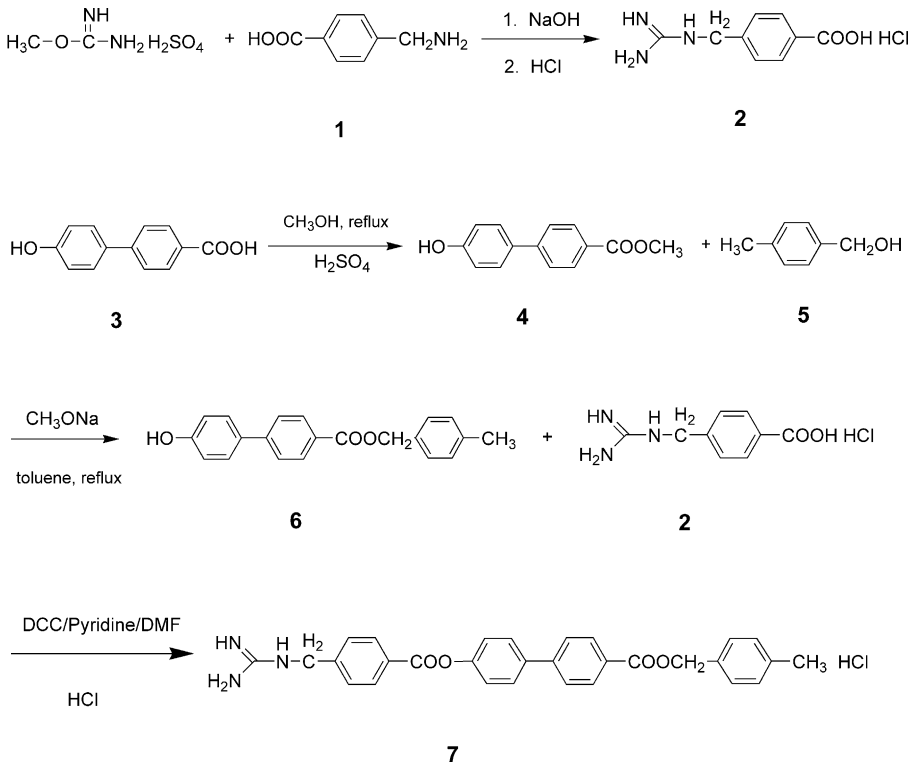
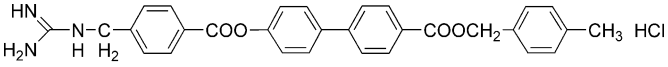
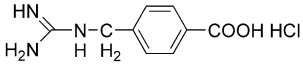
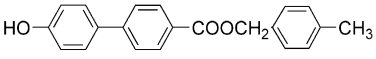
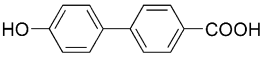
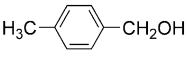
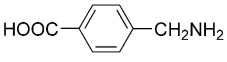
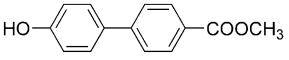
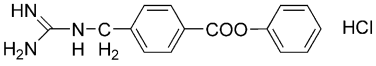
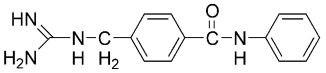


Scheme 1.

Table 1. Antibacterial spectrum of NE2001 and related compounds

Compd	Chemical structure	MIC (μg/mL)[a]		
		H. pylori ATCC43504	*S. aureus* 209P JC	*E. coli* K-12
NE2001		0.8	> 64	> 64
A		> 64	> 64	> 64
B		8	> 64	> 64
C		> 64	> 64	> 64
D		> 64	> 64	> 64
E		> 64	> 64	> 64
F		> 64	> 64	> 64
G		16	> 64	> 64
H		> 64	> 64	> 64

[a]MIC (μg/mL): Columbia agar + 7% defibrinated sheep blood for *H. pylori*, 37 °C, 72 h, agar dilution method; Mueller–Hinton agar for other bacteria.

and NE2001 showed no antibacterial activity against the selected bacteria as compared to amoxicillin. The selective anti-*H. pylori* activity of NE2001 may be conferred by its preferential penetrability into the outer membrane of *H. pylori* cells, as shown for hydrophobic compounds of small molecular size.[5] As the antibacterial spectrum of NE2001 is mainly restricted to *H. pylori*, its oral use will not cause the disturbance of normal gastrointestinal microflora.

The effects of NE2001 on the growth of *H. pylori*, DNA, RNA, and protein syntheses were examined. The

Table 2. Antibacterial activity of NE2001, amoxicillin and metronidazole against *Helicobacter pylori* and other common bacteria

Organism	Strain	MIC (μg/mL)[a]		
		NE2001	Amoxicillin	Metronidazole
Helicobacter pylori	ATCC43504	0.8	0.05	64
	Clinical isolates			
	HP001	0.4	0.025	4
	HP003	0.8	0.05	2
	HP004	0.8	0.05	8
	HP007	0.8	0.05	16
	HP008	0.8	0.05	32
	HP016	0.8	0.05	32
	HP017	1.6	0.025	64
	HP018	0.4	0.05	8
	HP019	1.6	0.05	32
	HP020	0.8	0.05	128
	HP021	0.8	0.025	32
	HP022	0.8	0.025	16
Staphylococcus aureus	209P JC	>64	0.2	>100
Staphylococcus epidermidis	ATCC1228	>64	0.78	>100
Enterococcus faecalis	ATCC29212	>64	0.78	>100
Escherichia coli	K12	>64	1.6	>100
Klebsiella pneumoniae	NCTC9632	>64	50	>100
Morganella morganii	KONO	>64	>100	>100

[a]MIC (μg/mL): Columbia agar + 7% defibrinated sheep blood for *H. pylori*, 37 °C, 72 h, agar dilution method; Mueller–Hinton agar for other bacteria.

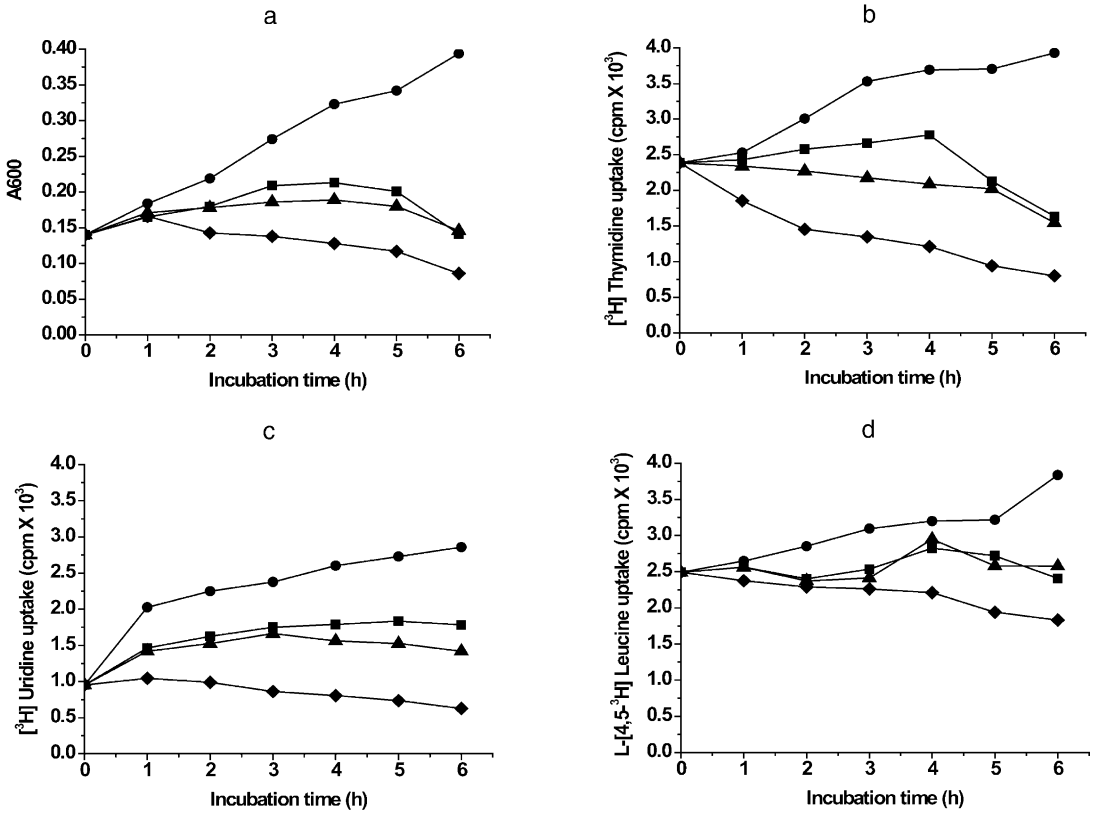


Figure 1. Inhibitory effects of NE2001 on the growth of *H. pylori* (a) and on the uptake of [^{3}H] thymidine; (b) [5-^{3}H] uridine; (c) or L-[4,5-^{3}H] leucine; (d) into DNA, RNA or protein without (●) or with 1.6 μg/mL (■), 3.2 μg/mL (▲) or 6.4 μg/mL (◆) NE2001.

growth of *H. pylori* was determined by an increase in turbidity at 600 nm. NE2001 dose-dependently inhibited the growth of *H. pylori* after 2 h of incubation, and the cell growth was completely suppressed at a concentration of 6.4 μg/mL (Fig. 1a). Fig. 1b shows the effect of NE2001 on the uptake of [^{3}H] thymidine into DNA. Without NE2001 the incorporation increased time-dependently and NE2001 dose-dependently suppressed the uptake of [^{3}H] thymidine into DNA after an hour of incubation. The addition of the above 3.2 μg/mL of NE2001 immediately reduced the uptake. Figure 1c and d shows the effects of NE2001 on RNA and protein synthesis respectively. NE2001 also showed an inhibitory effect on RNA and protein synthese in the cells performed in a dose-dependent manner similar to the patterns observed in the growth of *H. pylori* (Fig. 1a). The results shown in Figure 1 suggest that the suppression of the cell growth by NE2001 is due to the inhibition of DNA replication, because the suppression of DNA replication by the inhibitor precedes that of cell growth. But the exact target of NE2001 action remains to be investigated.

Experimental

Synthesis

4-Guanidinomethyl benzoic acid hydrochloride 2. 2N NaOH solution 72 mL was added to a solution of methyl isothiourea disulfate 20.0 g (0.14 mol) in 36 mL water cooled in ice batch, and stirred. Then 21.0 g (0.138 mol) 4-aminomethylbenzoic acid in 140 mL 2N NaOH solution was added drop wise. The mixture was left to stand overnight at room temperature and then chilled in ice water for 1 h. The precipitated white crystals were filtered off and washed with cold water. The filtrate was dissolved in warm 1N HCL and insoluble material was removed by filtration. The solution was concentrated in vacuum to crystallize. The colorless prisms crystallized when the solution was cooled, and then was filtered and dried, giving 4-guanidinomethyl benzoic acid hydrochloride 22.1 g (yield 70%) LC/MS = 194(M + H)

4-Methyl-4′-hydroxybiphenyl-4-carboxylate 4. A solution of 4-(4-hydroxyphenyl) benzoic acid 21.4 g (0.1 mol) in 500 mL absolute methanol in a flask equipped Soxhlet apparatus filled with A4 molecular sieve. Then 2.0 mL concentrated sulfuric acid was dropped. The mixture refluxed for 72 h. After the removal the solvent by vacuum, the residual oil was dissolved in 100 mL toluene and washed to pH = 7 with water. The organic layer was dried by $MgSO_4$ and filtered. The obtained filter liquor was added to a certain quantity of activated charcoal heated to reflux for 10–15 min and filtered. The solvent was removed to obtain a white crystal, 4-methyl-4′-hydroxybiphenyl-4-carboxylate 18.2 g (yield 80%).

4-(4-Methylbenzyl)-4′-hydroxybiphenyl-4-carboxylate 6. A suspension of 4-methyl-4-hydroxybiphenyl-4-carboxylate 9.0 g (40.0 mmol), 4-methylbenzylalcohol 24.4 g (200.0 mmol), sodium methoxide 1.0 g (4.0 mmol), toluene 20.0 mL, under N_2 prevention, were heated to reflux 2.5 h. During the reaction, additional 20.0 mL toluene was added to bring out the resultant methanol under reflux. Then it was cooled to room temperature, and 10 mL acetic acid and ice 40 g were added to adjust the pH = 5. The obtained organic layer was concentrated under reduced pressure to remove the solvent and the excess 4-methylbenzylalcohol. Then it was cooled to obtain brownish oil and left to stand to produce the crude crystals slowly. The crude crystals were recrystallized from toluene/n-hexane. A white crystal, 4-(4-methylbenzyl)-4′-hydroxybiphenyl-4-carboxylate 7.3 g was obtained (yield 71%) ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.35 (s, 3H), 5.35 (s, 2H), 6.90 (d, 2H), 7.15 (d, 2H), 7.35 (d, 2H), 7.50 (d, 2H), 7.60 (d, 2H), 8.10 (d, 2H).

4-(4-methylbenzyl)-4′-[guanidinomethylbenzoyloxy] biphenyl-4-carboxylate hydrochloride 7. A suspension of 4-(4-methylbenzyl)-4′-hydroxybiphenyl-4-carboxylate, 2.42 g (0.010 mol), 4-guanidinomethyl benzoic acid hydrochloride 2.3 g (0.010 mol) and dicyclohexylcabondiimide 4.1 g (0.020 mol) in pyridine/DMF 150 mL was stirred at room temperature for 48 h, after the removal of insoluble materials by filtration. The filtrate was evaporated to dryness and residue solid was treated with 0.1N hydrochloric acid (50 mL) and ether (50 mL). The aqueous layer was washed with ether again and concentrated to 20 mL, the resulting crystals were recrystallized in ethanol/hexane, giving 4-(methylbenzyl)-4′[guanidinomethylbenzoyloxy] biphnyl-4-carboxylate hydrochloride, 2.9 g (yield 55%) LC/MS = 494 (M + H).

Antimicrobial Activity

Bacterial strains

Twelve clinical isolates of *H. pylori* and strain ATCC 43504 were used. The identification of clinical isolates was based on microaerophilic growth requirement, morphology, Gram stain and oxidase, catalase, and rapid urease reactions.[6] Six reference strains of different species were also used. All strains were stored at −80 °C until susceptibility testing.

Susceptibility testing

The MICs of NE2001 and other compounds for *H. pylori* were determined by an agar dilution method using Columbia agar (Difco Co.), which was added with 7% defibrinated sheep blood. In brief, all the compounds were dissolved in dimethylsulphoxide (DMSO) and serial double dilutions were performed. *H. pylori* cultured in Brucella broth medium containing 5% fetal bovine serum under microaerophilic condition (5% O_2, 10% CO_2 and 85% N_2) at 37 °C for 24 h, and the number of bacteria thereof was adjusted to about 10^8CFU/mL. This bacteria solution was painted in each square about 2 cm with inoculating line loop. The plates were cultured under microaerophilic condition for three days at 37 °C. Minimum concentration which completely inhibit the growth of the bacteria was judged

as the value of MIC. The MICs for other common bacteria were also determined by the agar dilution method using Mueller–hinton agar (Difco Co.).

Determination of DNA, RNA and protein syntheses in *H. pylori*

H. pylori cells were grown in brucella broth medium containing 5% fetal bovine serum with or without different concentration of NE2001 under microaerophilic conditions at 37 °C. The uptake of [^{3}H] thymidine, [5-^{3}H] uridine or L-[4,5-^{3}H] leucine (Amersham Pharmacia Biotech, UK) into DNA, RNA or protein was determined in a way described by Joselean-Petit.[7] In brief, a volume of 0.4 mL of the growing *H. pylori* suspension was withdrawn and incubated with 20 μL radiolabelled thymidine, uridine or leucine (100 μCi/mL) for 30 min under the same condition. Adding 0.4 mL of 10% trichloroacetic acid stopped the reaction. After 30 min, precipitates were collected on a 0.45 μm Millipore filter and rinsed with 5% trichloroacetic acid. Filter were dried and counted by liquid scintillation on a 1400 WinSpectral scintillation counter (Wallac).

Acknowledgements

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