

In Vitro Susceptibility of *Helicobacter pylori* to Several Antimicrobial Combinations

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Synergy between metronidazole and its hydroxymetabolite and between each compound and amoxicillin or tetracycline-HCl was determined against *Helicobacter pylori*. Metronidazole plus its hydroxymetabolite and either compound combined with amoxicillin showed synergism against all 10 strains of *H. pylori* tested. Metronidazole plus tetracycline-HCl or the hydroxymetabolite plus tetracycline-HCl acted synergistically against seven and six strains, respectively, acted additively against three strains, and had no additional effect against one strain. These results may help to explain the in vivo efficacies of metronidazole combinations in the treatment of *H. pylori*-associated gastritis.

Helicobacter pylori has been linked to various disorders of the upper gastrointestinal tract, particularly chronic gastritis (13) and peptic ulcer (10). Treatment with single antimicrobial agents has failed to eradicate *H. pylori* in these situations. The most successful treatment regimens for the eradication of *H. pylori* from the gastric mucosa have been obtained with two or three antimicrobial agents, namely, metronidazole and amoxicillin or tetracycline-HCl and a bismuth compound (8, 11). In vitro studies have shown that metronidazole plus amoxicillin act at least additively (6) and that metronidazole plus tetracycline-HCl act synergistically or additively against *H. pylori* (14). The hydroxymetabolite of metronidazole is a product of the oxidative metabolism of metronidazole in the liver (5). So far, attention has not been focused on the role of metronidazole's hydroxymetabolite or its interactions with metronidazole, amoxicillin, and tetracycline-HCl against *H. pylori* in vitro. Metronidazole and its hydroxymetabolite can act synergistically against both anaerobic and facultative anaerobic bacteria, such as *Bacteroides fragilis* and *Actinobacillus actinomycetemcomitans* (3, 9). Moreover, for the latter species, the MICs of the hydroxymetabolite are 2 to 4 times lower than those of metronidazole (4, 9). In addition, the concentrations of the hydroxymetabolite in serum and tissue samples are comparable to those of metronidazole (2). If interactions between metronidazole and other antimicrobial agents are studied, the contribution of the hydroxymetabolite can give additional insight into the potential of such combination activity. Metronidazole-amoxicillin and metronidazole-tetracycline combinations are commonly used in the treatment of *H. pylori* infections, with various results in eradicating the organism. We have investigated the effects of the combinations of metronidazole and its hydroxymetabolite with amoxicillin and tetracycline-HCl on *H. pylori* in vitro.

A total of 10 *H. pylori* strains were included in the study. All strains were isolated from endoscopic biopsy specimens of the gastric antrum in 10 patients suffering from active chronic gastritis.

The MICs were determined under microaerophilic condi-

tions (80% N₂, 10% CO₂, 5% H₂, and 5% O₂) by the standard microbroth dilution test (12). Quality control of the MIC determination was performed under anaerobic conditions (85% N₂, 10% CO₂, and 5% H₂), using *B. fragilis* (ATCC 25285) as the reference strain. The dilutions of metronidazole (Rhône-Poulenc, Amstelveen, The Netherlands), the hydroxymetabolite of metronidazole (kindly supplied by Rhône-Poulenc), amoxicillin (Sigma Chemicals Co., St. Louis, Mo.), and tetracycline-HCl (Sigma Chemicals) were freshly prepared for each experiment. Stock solutions were made in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% inactivated fetal calf serum (BBFCS) and filter sterilized. Bacterial cells were grown overnight in BBFCS in a microaerophilic atmosphere at 37°C on an orbit shaker. Inoculum suspensions containing approximately 10⁶ CFU/ml were prepared. The MICs of amoxicillin, tetracycline-HCl, and metronidazole and its hydroxymetabolite for the 10 *H. pylori* strains were determined by twofold serial dilutions of drug. MIC determinations were performed in 24-well, flat-bottom tissue culture plates (Costar, Cambridge, United Kingdom). To 1 ml of diluted antibiotic, 1 ml of inoculum was added, resulting in a final bacterial cell density of 5 × 10⁵ CFU/ml. After incubation of the cultures under microaerophilic conditions for 36 h on an orbit shaker

TABLE 1. MICs of metronidazole and its hydroxymetabolite, amoxicillin, and tetracycline-HCl against 10 *H. pylori* strains

Strain	MIC (µg/ml)			
	Metronidazole	Hydroxymetabolite of metronidazole	Amoxicillin	Tetracycline-HCl
VU1	1.25	0.61	0.015	0.61
VU2	2.5	1.25	0.015	0.61
VU3	40	20	0.015	0.15
VU4	2.5	1.25	0.50	0.15
VU5	5.0	2.5	0.007	0.030
VU6	2.5	2.5	0.007	0.015
VU7	2.5	0.61	0.015	0.030
VU8	40	5.0	0.030	0.15
VU9	40	20	0.007	0.030
VU10	2.5	1.25	0.060	0.030

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TABLE 2. FICs and FICIs for each antibiotic combination for 10 *H. pylori* strains

Strain	Metronidazole and amoxicillin		Metronidazole and tetracycline-HCl		Metronidazole and its hydroxymetabolite		Hydroxymetabolite and amoxicillin		Hydroxymetabolite and tetracycline-HCl	
	FICs	FICI	FICs	FICI	FICs	FICI	FICs	FICI	FICs	FICI
VU1	0.125, 0.125	0.25	0.25, 0.125	0.38	0.125, 0.0625	0.19	0.125, 0.125	0.25	0.375, 0.125	0.5
VU2	0.125, 0.125	0.25	0.125, 0.125	0.25	0.125, 0.125	0.25	0.125, 0.125	0.25	0.125, 0.125	0.25
VU3	0.25, 0.25	0.5	0.25, 0.25	0.5	0.25, 0.25	0.5	0.125, 0.125	0.25	0.375, 0.125	0.5
VU4	0.125, 0.125	0.25	0.5, 0.25	0.75	0.0625, 0.125	0.19	0.125, 0.0625	0.19	0.5, 0.5	1.0
VU5	0.125, 0.0625	0.19	0.125, 0.125	0.25	0.0625, 0.125	0.19	0.125, 0.125	0.25	0.125, 0.125	0.25
VU6	0.125, 0.125	0.25	0.25, 0.25	0.5	0.125, 0.0625	0.19	0.125, 0.125	0.25	0.25, 0.125	0.38
VU7	0.125, 0.0625	0.19	0.25, 0.25	0.5	0.125, 0.125	0.25	0.125, 0.125	0.25	0.75, 0.25	1.0
VU8	0.125, 0.0625	0.19	1.0, 1.0	2.0	0.125, 0.125	0.25	0.125, 0.125	0.25	1.0, 1.0	2.0
VU9	0.125, 0.0625	0.19	0.125, 0.125	0.25	0.125, 0.125	0.25	0.125, 0.125	0.25	0.375, 0.125	0.5
VU10	0.125, 0.0625	0.19	0.75, 0.25	1.0	0.125, 0.125	0.25	0.125, 0.125	0.25	0.25, 0.75	1.0

at 37°C, the MICs of the antibiotics were read. For each strain, the MIC was determined twice in duplicate.

In vitro interactions between the antibiotics were studied by checkerboard titration experiments (1). The checkerboard titrations were also performed in 24-well, flat-bottom tissue culture plates. Of each of the two antibiotics used in the checkerboard titrations, 0.5 ml of each antibiotic, diluted from the MIC to 1/32 of the MIC, was combined and 1.0 ml of the inoculum was added to each of the wells, resulting in a final bacterial cell density of 5×10^5 CFU/ml. The plates were wrapped in plastic together with a moistened paper tissue to prevent evaporation and then incubated in a microaerophilic atmosphere for 36 h on an orbit shaker at 37°C. The checkerboard titrations were performed twice in duplicate for each *H. pylori* strain.

The degree of interaction between the antibiotics was defined by calculating fractional inhibitory concentration indices (FICIs) from the sum of the fractional inhibitory concentrations (FICs) of each antibiotic (1). Synergism in a two-dimensional checkerboard test was defined by FICIs of ≤ 0.5 (1).

In Table 1, the MICs of metronidazole and its hydroxymetabolite, amoxicillin, and tetracycline-HCl are shown. The MIC of each antibiotic was highly reproducible. All strains were susceptible to both amoxicillin and tetracycline-HCl (amoxicillin MIC, 0.007 to 0.5 µg/ml; tetracycline-HCl MIC, 0.015 to 0.61 µg/ml). Metronidazole and its hydroxymetabolite showed strong activity against seven and eight *H. pylori* strains, respectively (metronidazole MIC, 1.25 to 5.0 µg/ml; hydroxymetabolite MIC, 0.61 to 5.0 µg/ml). Three strains were moderately susceptible to metronidazole (MIC, 40 µg/ml), and two strains were moderately susceptible to the hydroxymetabolite (MIC, 20 µg/ml). We found that the hydroxymetabolite MICs for nine *H. pylori* strains were 2 to 8 times lower than the corresponding metronidazole MICs. The MICs for metronidazole, amoxicillin, and tetracycline-HCl for the *B. fragilis* reference strain were in accordance with the reference values (7).

In Table 2, FICs and FICIs are listed for the combinations of metronidazole plus amoxicillin, metronidazole plus tetracycline-HCl, metronidazole plus its hydroxymetabolite, the hydroxymetabolite plus amoxicillin, and the hydroxymetabolite plus tetracycline-HCl against the 10 strains tested. Synergistic effects could be demonstrated for all 10 *H. pylori* strains for metronidazole plus its hydroxymetabolite, metronidazole plus amoxicillin, and the hydroxymetabolite plus amoxicillin. FICIs ranged from 0.19 to 0.5. Synergy was observed between metronidazole and tetracycline-HCl for

seven strains and between metronidazole's hydroxymetabolite and tetracycline-HCl for six strains (FICIs ranged from 0.25 to 0.5). Metronidazole plus tetracycline-HCl and the hydroxymetabolite plus tetracycline-HCl showed additive effects (FICIs ranged from 0.75 to 1.0) for two and three strains, respectively. For metronidazole and tetracycline-HCl or the hydroxymetabolite and tetracycline-HCl, no additional effects (FICI = 2.0) were observed for one strain.

On the basis of our observations, we conclude that the hydroxymetabolite of metronidazole could play an important role in the effectiveness of metronidazole-containing antimicrobial combinations which are currently used in the treatment of *H. pylori* infections. The variable in vitro results of the tetracycline combinations observed in this study might help to explain the various in vivo results seen in some clinical studies (8, 11). However, synergism between metronidazole and its hydroxymetabolite and between either analog and amoxicillin or tetracycline-HCl probably contributes to the efficacy of both the amoxicillin-metronidazole and tetracycline-metronidazole combinations.

REFERENCES

- Berenbaum, M. C. 1978. A method for testing synergy with any number of agents. *J. Infect. Dis.* 137:122-130.
- Gulaid, A., G. W. Houghton, O. R. W. Lewellen, J. Smith, and P. S. Thorne. 1978. Determination of metronidazole and its two major metabolites in biological fluids by high pressure liquid chromatography. *Br. J. Clin. Pharmacol.* 6:430-432.
- Haller, I. 1982. In vitro activity of the two principal oxidative metabolites of metronidazole against *Bacteroides fragilis* and related species. *Antimicrob. Agents Chemother.* 22:165-166.
- Jousimies-Somer, H., S. Asikainen, P. Suomala, and P. Summanen. 1983. Activity of metronidazole and its hydroxymetabolite against clinical isolates of *A. actinomycetemcomitans*. *Oral Microbiol. Immunol.* 3:31-34.
- Loft, S., S. V. Otton, M. S. Lennard, G. T. Tucker, and H. E. Poulsen. 1991. Characterization of metronidazole metabolism by human liver microsomes. *Biochem. Pharmacol.* 41:1127-1134.
- Mégraud, F., P. Trimoulet, H. Lamouliatte, and L. Boyanova. 1991. Bactericidal effect of amoxicillin on *Helicobacter pylori* in an in vitro model using epithelial cells. *Antimicrob. Agents Chemother.* 35:869-872.
- National Committee for Clinical Laboratory Standards. 1990. Methods for antimicrobial susceptibility testing of anaerobic bacteria, 2nd ed., vol. 9, no. 10. Approved standard M11-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
- O'Riordan, T., E. Mathai, E. Tobin, C. Keane, D. McKenna, E. Sweeney, and C. O'Morain. 1990. Adjuvant antibiotic therapy in duodenal ulcers treated with colloidal bismuth subcitrate. *Gut* 31:999-1002.

9. Pavičić, M. J. A. M. P., A. J. van Winkelhoff, and J. de Graaff. 1991. Synergistic effects between amoxicillin, metronidazole, and the hydroxymetabolite of metronidazole against *Actinobacillus actinomycetemcomitans*. *Antimicrob. Agents Chemother.* **35**:961-966.
10. Rathbone, B. Y., J. I. Wyatt, and R. V. Hentily. 1986. *Campylobacter pyloridis*: a new factor in peptic ulcer disease? *Gut* **27**:637-641.
11. Rauws, E. A. J., and G. N. J. Tytgat. 1990. Cure of duodenal ulcer associated with eradication of *Helicobacter pylori*. *Lancet* **335**:1233-1235.
12. Sahn, D. F., and J. A. Washington II. 1991. Antibacterial susceptibility tests: dilution methods, p. 1105-1116. In A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), *Manual of clinical microbiology*, 5th ed. American Society for Microbiology, Washington, D.C.
13. Warren, J. R., and B. Y. Marshall. 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **i**:1273-1275.
14. Xia, H. X., M. A. Draw, C. A. Keane, and C. A. O'Morain. 1991. Interaction between metronidazole and tetracycline against sensitive and resistant isolates of *Helicobacter pylori* *in vitro*. *Ital. J. Gastroenterol.* **9**:49, abstr. 124.