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Synergistic Effects between Amoxicillin, Metronidazole, and the Hydroxymetabolite of Metronidazole against *Actinobacillus actinomycetemcomitans*

M. J. A. M. P. PAVIČIĆ, A. J. VAN WINKELHOFF, AND J. DE GRAAFF*

Department of Oral Microbiology, Academic Centre for Dentistry Amsterdam, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

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Interactions between metronidazole and amoxicillin, metronidazole and its hydroxymetabolite, and amoxicillin and the hydroxymetabolite of metronidazole were investigated with checkerboard titrations in combination with accurately determined MICs and MBCs. *Actinobacillus actinomycetemcomitans* was used as the test organism. Synergism was found for all three combinations. Fractional inhibitory concentration indices and fractional bactericidal concentration indices varied from 0.3 to 0.7. These synergistic interactions between these antibiotics may explain the efficacy of the combination of metronidazole and amoxicillin in various bacterial infections, including periodontal disease.

The efficacy of the combination of metronidazole and amoxicillin has been proven in a variety of mixed bacterial infections in humans (7, 8). *Helicobacter pylori* infections have been treated with the combination of metronidazole, amoxicillin, and bismuth salts, and eradication of this microorganism was observed in over 90% of the cases (1, 4a). Recently, the combination of metronidazole and amoxicillin was evaluated in patients with oral infections, e.g., periodontal disease (6, 9, 29). In some forms of periodontal disease, *Actinobacillus actinomycetemcomitans*, a capnophilic, gram-negative rod, seems to be of major etiological importance (18–20, 23, 25). In *A. actinomycetemcomitans*-associated periodontal disease, adjunct antibiotics are often necessary to achieve a satisfactory clinical treatment response. Tetracyclines have been used often in conjunction with subgingival mechanical debridement (21, 27). However, tetracyclines are not always successful in the elimination of *A. actinomycetemcomitans* (15, 21, 26, 27, 29). Unexpected clinical success was observed with the combination of metronidazole and amoxicillin in terms of the elimination of *A. actinomycetemcomitans* from periodontal lesions (6, 29). It is known that the hydroxymetabolite of metronidazole, which is produced in the human liver, is even more active against *A. actinomycetemcomitans* in vitro, and it has been suggested that the combination of metronidazole and its hydroxymetabolite acts synergistically against *A. actinomycetemcomitans* (13). It is therefore possible that the hydroxymetabolite of metronidazole plays an important role in the effectiveness of the combination of metronidazole and amoxicillin. It has also been suggested that metronidazole combined with penicillin has a potentiating effect against *Clostridium perfringens* (3). The aim of this study was to investigate the in vitro interactions between metronidazole, the hydroxymetabolite of metronidazole, and amoxicillin and the activities of combinations of these three compounds against *A. actinomycetemcomitans*.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. A total of 10 *A. actinomycetemcomitans* strains were included in the study; 6 strains had a rough appearance on the isolation medium (HG 1175, HG 1176, HG 1178, HG 1237, HG 1238, and HG 1239), and 4 strains had a smooth appearance on the isolation medium (HG 1174, HG 1177, HG 1183, and HG 1234). All strains were isolated from periodontal lesions of 10 patients with severe periodontitis by use of selective TSBV plates (22).

Determination of MICs and MBCs. MICs and MBCs were determined under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) by the microdilution method (14). Dilutions of metronidazole (Janssen Chimica, Beerse, Belgium), amoxicillin (Beecham Research Laboratories), and the hydroxymetabolite of metronidazole (kindly supplied by Rhône-Poulenc, Amstelveen, The Netherlands) were freshly prepared for each experiment. Stock solutions were made in anaerobically sterilized brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and filter sterilized. Bacterial cells were anaerobically grown overnight in brain heart infusion broth at 37°C. Inoculum suspensions containing approximately 5×10^5 CFU/ml were prepared. The MICs and MBCs of amoxicillin, metronidazole, and the hydroxymetabolite of metronidazole for the 10 *A. actinomycetemcomitans* strains were determined with twofold serial dilutions. For a more accurate determination of the MICs and MBCs, antibiotic dilutions were prepared by decreasing these concentrations by 10%, from 100% of the MIC or MBC, as determined with the twofold serial dilutions, to 10% of the initial strengths. The MICs and MBCs of metronidazole, the hydroxymetabolite of metronidazole, and amoxicillin could then be determined with more accuracy and did not deviate more than 10% from the theoretical MICs and MBCs. MIC and MBC determinations were performed in 96-well, flat-bottomed tissue culture clusters (Costar, Cambridge, Mass.). To a 100-μl dilution of antibiotic, a 100-μl inoculum suspension was added. After incubation of the cultures in an anaerobic glove box for 36 h at 37°C, the MICs of the three antibiotics were read. The MIC was defined as the lowest antibiotic concentration which allowed no visible growth. For determination of the MBCs, a 100-μl sample was taken from each well without visible growth and cultured on blood agar plates (blood agar

TABLE 1. MICs and MBCs for 10 *A. actinomycetemcomitans* strains

Strain	Concn (μg/ml) of:					
	Metronidazole		Hydroxymetabolite		Amoxicillin	
	MIC	MBC	MIC	MBC	MIC	MBC
HG 1174	40 ± 4	48 ± 4	10 ± 1	12 ± 1	0.8 ± 0.05	1.5 ± 0.1
HG 1175	10 ± 1	16 ± 1	3 ± 0.3	5 ± 0.3	0.8 ± 0.05	0.9 ± 0.1
HG 1176	20 ± 2	24 ± 2	7 ± 0.5	9 ± 0.5	1.1 ± 0.1	1.5 ± 0.1
HG 1177	16 ± 1	20 ± 1	5 ± 0.5	8 ± 0.5	1.2 ± 0.1	1.5 ± 0.1
HG 1178	20 ± 2	28 ± 2	5 ± 0.5	8 ± 0.5	1.6 ± 0.1	1.8 ± 0.1
HG 1183	36 ± 3	40 ± 4	16 ± 1	20 ± 1	1.8 ± 0.1	2.0 ± 0.1
HG 1234	30 ± 3	34 ± 3	10 ± 1	12 ± 1	5.2 ± 0.5	6.0 ± 0.5
HG 1237	28 ± 2	32 ± 3	8 ± 0.5	10 ± 1	1.5 ± 0.1	1.8 ± 0.1
HG 1238	12 ± 1	16 ± 1	6 ± 0.5	8 ± 0.5	1.8 ± 0.1	2.0 ± 0.1
HG 1239	24 ± 2	28 ± 3	8 ± 0.5	10 ± 1	0.9 ± 0.05	1.1 ± 0.1

base no. 2; Oxoid Ltd., Basingstoke, Hampshire, England). After anaerobic incubation for 48 h at 37°C, the MBC for each strain was determined and defined as the antibiotic concentration which allowed less than 0.1% of the original inoculum to survive. For each strain, the MIC and the MBC were determined three times in duplicate. In addition, the MICs of metronidazole and amoxicillin for the 10 *A. actinomycetemcomitans* strains were compared with the MICs determined by the agar dilution method with Wilkins-Chalgren agar plates (Oxoid Ltd.) (17).

Checkerboard titrations. In vitro interactions between antibiotics were studied in checkerboard titration experiments (2). The antibiotic dilutions used in the checkerboard titrations were prepared in the same way as described for the MIC and MBC determinations, from a final concentration of 100% of the MIC or MBC to 10% of the MIC or MBC of each antibiotic. Checkerboard titrations were also performed in 96-well, flat-bottomed tissue culture clusters. Fifty microliters of each of the two antibiotics used in the checkerboard titrations and 100 μl of an inoculum suspension were added to each of the wells. The clusters were wrapped in plastic and moisturized paper tissue to prevent vaporization and incubated in an anaerobic glove box at 37°C for 36 h. Checkerboard titrations were performed three times in duplicate for each *A. actinomycetemcomitans* strain.

Determination of FICs, FBCs, FICIs, and FBCIs. To specify the degree of interaction between the antibiotics, we calculated fractional inhibitory concentrations (FICs), fractional bactericidal concentrations (FBCs), fractional inhibitory concentration indices (FICIs), and fractional bactericidal concentration indices (FBCIs) from wells in which no visible growth could be detected and from wells with less than 0.1% survival of the original inoculum. The criteria on the basis of which one decides whether a combination of antibiotics acts synergistically, additively, or antagonistically are based on the accuracy of the MIC and MBC determinations (12). Synergism in a two-dimensional checkerboard test is defined by FICIs or FBCIs of ≤ 0.5 with twofold serial dilutions for the MIC or MBC determinations (2). A more exact understanding of the interactions between two antibiotics can be obtained by determining precise MICs and MBCs (12). Such a determination can be achieved with smaller dilution steps. In this study, the MICs and MBCs were determined with dilution steps of 10%. We deduced that synergism could be defined as FICIs and FBCIs of ≤ 0.7. This value was mathematically obtained by subtracting the sum of the maximal errors made in the MIC and MBC determinations (0.2) and the maximal errors made in pipetting and diluting (0.1) from the theoretical FICI and FBCI limits for synergism (1.0) (2).

Effect of the triple combination of metronidazole, the hydroxymetabolite of metronidazole, and amoxicillin against *A. actinomycetemcomitans*. For five *A. actinomycetemcomitans* strains, HG 1174, HG 1175, HG 1176, HG 1177, and HG 1178, the effect of the triple combination of metronidazole, the hydroxymetabolite of metronidazole, and amoxicillin, in concentrations of 0.1× the MICs as well as 0.2× the MICs, was studied. The experiment was carried out two times in duplicate. In a 96-well, flat-bottomed tissue culture cluster, 100 μl of a triple-combination solution (0.1× or 0.2× the MICs) was combined with 100 μl of an inoculum suspension. The cluster was incubated in an anaerobic glove box at 37°C for 36 h. Bacterial growth was examined. The experiment was performed two times in duplicate.

Inhibitory and bactericidal activities over time of the three double combinations. The inhibitory and bactericidal activities over time of the three double combinations (with minimal FICIs and FBCIs, respectively) were determined for two *A. actinomycetemcomitans* strains, HG 1174 and HG 1175. For each double combination, tubes containing 5 ml of anaerobically sterilized brain heart infusion broth with minimal FICIs and FBCIs of each antibiotic combination, as determined by checkerboard titration experiments, and control tubes with the highest FICs and FBCs of each antibiotic (all tubes contained approximately 5×10^5 CFU/ml) were incubated at 37°C in an anaerobic glove box in duplicate. At regular time intervals, between 0 and 26 h, 100-μl samples were taken from each tube. The samples were diluted 10 times in phosphate-buffered saline and plated with a spiral plater on blood agar plates. The plates were incubated in an anaerobic glove box for 48 h at 37°C. Viable counts were determined.

RESULTS

Accurately determined MICs and MBCs for the 10 *A. actinomycetemcomitans* strains are listed in Table 1. The MICs and MBCs for each strain were each determined three times in duplicate and showed little variation. The MICs determined with the microdilution method differed little from the MICs determined by the standard agar dilution method. A comparison of both methods resulted in correlation coefficients of 0.9688 for metronidazole and 0.9868 for amoxicillin. Between both methods linear equations were found: for metronidazole, $MIC_{\text{agar dilution method}} = (1.0 \pm 0.05) \times$

TABLE 2. FICIs and FBCIs of various drug combinations

Strain	FICI[a] of:		
	Metronidazole-hydroxy-metabolite	Amoxicillin-hydroxy-metabolite	Metronidazole-amoxicillin
HG 1174	0.5–0.6	0.5–0.6	0.6–0.7
HG 1175	0.3–0.4	0.5–0.6	0.4–0.5
HG 1176	0.5	0.5	0.5–0.6
HG 1177	0.4	0.4	0.4
HG 1178	0.5	0.5	0.5
HG 1183	0.4	0.5	0.5–0.6
HG 1234	0.4	0.4	0.3–0.4
HG 1237	0.5–0.6	0.5–0.6	0.6
HG 1238	0.5–0.6	0.5	0.6
HG 1239	0.5–0.6	0.6	0.6

[a] FBCIs were equal to FICIs.

$MIC_{\text{microdilution method}}$; for amoxicillin, $MIC_{\text{agar dilution method}} = (1.1 \pm 0.06) \times MIC_{\text{microdilution method}}$.

The MICs and MBCs of the hydroxymetabolite of metronidazole for each bacterial strain were two to four times lower than the MICs and MBCs of metronidazole. The results of the checkerboard titrations are listed in Table 2. The FICIs and FBCIs varied from 0.3 to 0.7. All checkerboard titrations showed a variation in FICIs and FBCIs of no more than 0.1 for each strain. The FICIs and FBCIs listed in Table 2 are the sums of the two lowest FICs and FBCs determined for each combination per strain. For strains HG 1175 and HG 1177, a triple combination of 0.1× the MIC of each antibiotic caused complete growth inhibition, resulting in an FICI of ≤0.3, while for strains HG 1174, HG 1176, and HG 1178, a triple combination of 0.2× the MIC of each antibiotic caused complete growth inhibition, resulting in an FICI of ≤0.6.

Figures 1 and 2 illustrate the inhibitory activity over time of the three antibiotic combinations with minimal FICIs against strains HG 1174 and HG 1175. Figures 3 and 4 illustrate the bactericidal activity over time of the three antibiotic combinations with minimal FBCIs against strains HG 1174 and HG 1175.

Double combinations of the antibiotics with minimal FICIs inhibited the growth of both strains over 24 h, while there was visible growth of both strains incubated with the FIC of each separate antibiotic (data not shown). The bactericidal activity of the antibiotic combinations with minimal FBCIs was as follows: total killing of strain HG 1174 in 12 h by the metronidazole-hydroxymetabolite combination, 16 h by the amoxicillin-hydroxymetabolite combination, and 21 ± 5 h by the metronidazole-amoxicillin combination and total killing of strain HG 1175 in 8 h by the metronidazole-amoxicillin combination, 10 h by the amoxicillin-hydroxymetabolite combination, and 12 h by the metronidazole-hydroxymetabolite combination. Both strains incubated with the FBC of each separate antibiotic showed visible growth, although with an increased generation time (data not shown).

DISCUSSION

The aim of this study was to investigate the interactions of metronidazole, the hydroxymetabolite of metronidazole, and amoxicillin and the activities of combinations of these three compounds against *A. actinomycetemcomitans*. The MICs of metronidazole, the hydroxymetabolite of metronidazole, and amoxicillin for the 10 *A. actinomycetemcomitans* strains tested agree with the MICs of all three antibiotics reported by other authors (11, 13, 16, 24, 26, 30). We also showed that the microdilution method is a reliable and reproducible method for the determination of accurate MICs. The results of this study are in accordance with those obtained with the agar dilution method.

The two- to fourfold lower MIC and MBC of the hydroxymetabolite of metronidazole for *A. actinomycetemcomitans* (13) as well as the suggested synergistic effect of

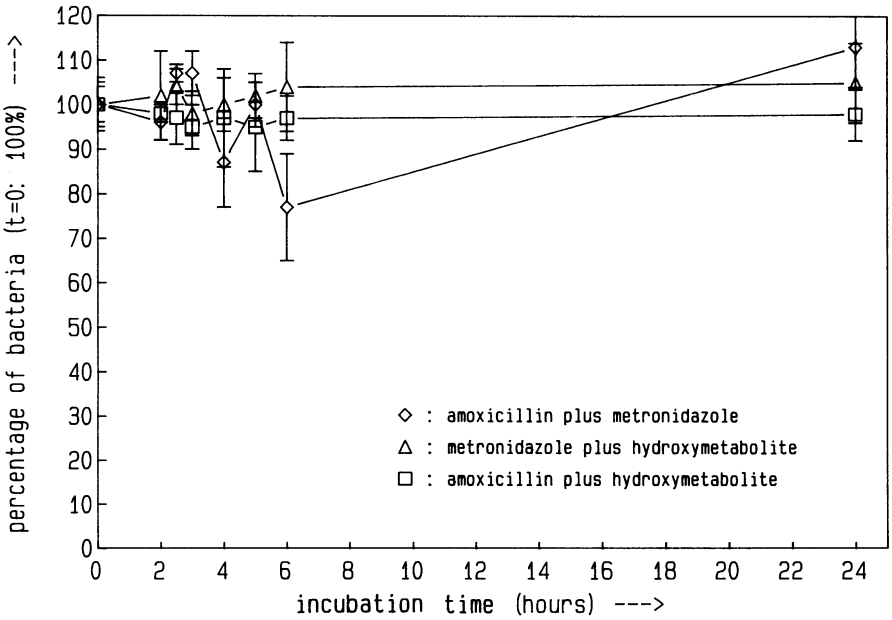

FIG. 1. Inhibitory activity over time against *A. actinomycetemcomitans* HG 1175 at minimal FICIs of various antibiotic combinations.

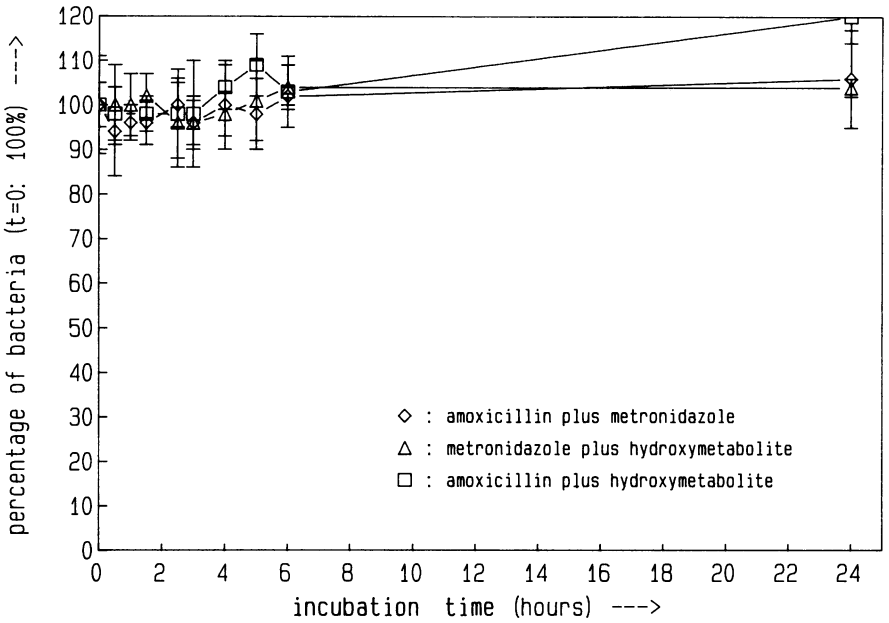

FIG. 2. Inhibitory activity over time against *A. actinomycetemcomitans* HG 1174 at minimal FICIs of various antibiotic combinations.

this compound with metronidazole (13) were confirmed in this study.

In addition, we found that both compounds act synergistically with amoxicillin. Because of this synergistic action, concentrations significantly lower than the MICs should be sufficient to be effective against *A. actinomycetemcomitans*. The concentrations of amoxicillin (4, 26), metronidazole (10, 26, 28), and the hydroxymetabolite of metronidazole (10) reported in the serum and crevicular fluid of humans should therefore be high enough to be effective against *A. actinomycetemcomitans*. The combination of metronidazole and amoxicillin is often used in the antimicrobial treatment of pelvic inflammatory disease (5) and other severe mixed anaerobic infections (7), but a synergistic action between both antibiotics against the facultative or anaerobic bacteria associated with these infections has not been described so far. Usually, the rationale for using metronidazole plus amoxicillin is the targeting of a broad spectrum of bacteria involved in an infection. Metronidazole covers most anaerobes and amoxicillin covers facultative and aerobic bacteria involved in an infection. However, the results of this study show that there is an obvious synergistic action between

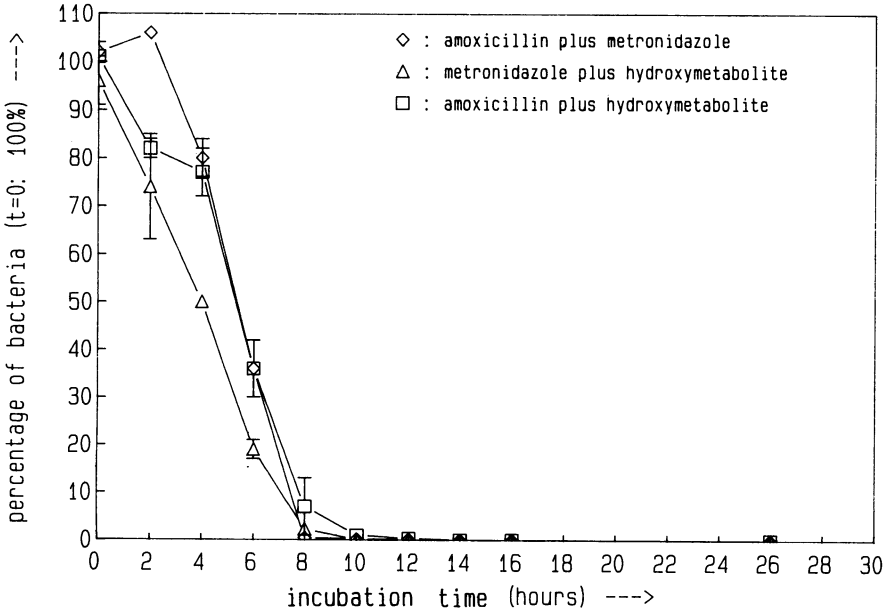

FIG. 3. Bactericidal activity over time against *A. actinomycetemcomitans* HG 1175 at minimal FBCIs of various antibiotic combinations.

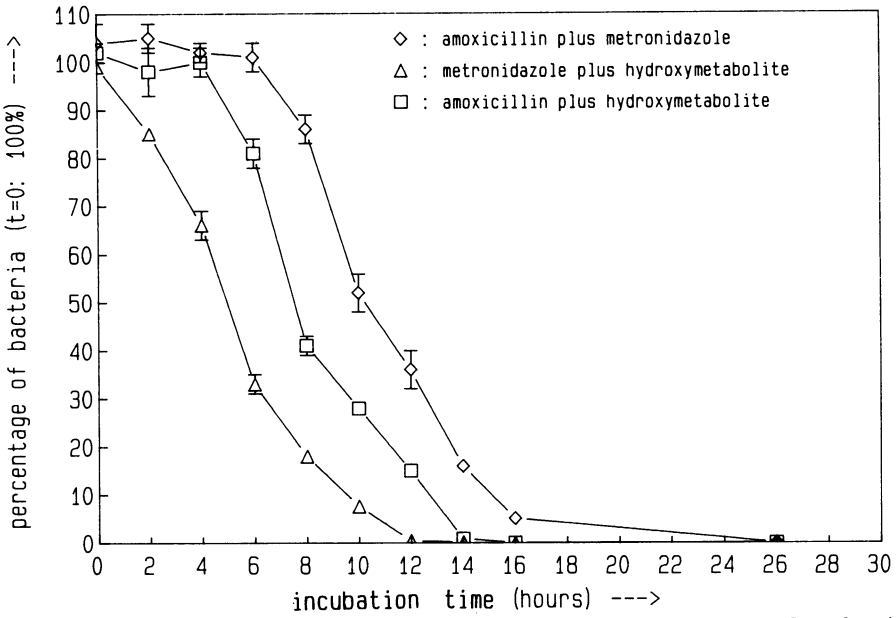

FIG. 4. Bactericidal activity over time against *A. actinomycetemcomitans* HG 1174 at minimal FBCIs of various antibiotic combinations.

both compounds against the target bacterium *A. actinomycetemcomitans*. Moreover, the hydroxymetabolite of metronidazole, which is produced in the human liver, also acts synergistically with both compounds. Therefore, it seems likely not only that separate actions of both compounds are responsible for the elimination of the microorganisms but also that synergistic actions between both compounds and the hydroxymetabolite contribute to the effectiveness of the metronidazole-amoxicillin combination in severe mixed anaerobic infections.

The degree of interaction between antibiotics was studied with checkerboard titrations in combination with accurately determined MICs and MBCs. This technique has proven to be a useful tool for the examination of the degree of interaction between antibiotics (2, 12). In the theoretical situation, an FICI or FBCI below 1.0 indicates synergism, a value of 1.0 indicates an additive effect of both antibiotics, and a value above 1.0 indicates antagonism. Since it is practically impossible to determine MICs and MBCs as precisely as in the theoretical situation, the FICI and FBCI limit for synergism depends upon the dilution steps which are used for the determination of the MICs and MBCs. In the case of twofold serial dilutions, an FICI or FBCI of ≤ 0.5 indicates synergism. The use of the more accurately determined MICs and MBCs, as in this study, consequently provides a higher FICI and FBCI limit for synergism and therefore reflects a more precise degree of interaction between the antibiotics studied. The FICI and FBCI limit for synergism in this study was mathematically fixed at 0.7. It is obvious that for all of the *A. actinomycetemcomitans* strains examined, the three antibiotic combinations had FICIs and FBCIs lower than or equal to 0.7, thereby indicating synergistic interactions. Moreover, tests of the two triple combinations of the three compounds indicated synergistic interactions against *A. actinomycetemcomitans*, with FICIs varying from 0.3 for the rough strains to 0.6 for the smooth strains. Killing curves showed that there was a clear difference in the killing rate between the smooth strain HG 1174 and the rough strain HG 1175 for each antibiotic combination tested, but both strains were killed within 26 h at minimal FBCIs. Because most *A. actinomycetemcomitans* strains are relatively nonsusceptible to metronidazole, the use of metronidazole alone may not be effective enough. However, in some patients, metronidazole alone has effectively eradicated susceptible *A. actinomycetemcomitans* strains (MIC < 10 μg/ml) from the periodontal pocket (unpublished results). This result may be explained by the synergistic action between metronidazole and its hydroxymetabolite against *A. actinomycetemcomitans*.

In conclusion, the efficacy of the combination of metronidazole and amoxicillin in several bacterial infections in humans can be explained by our in vitro observations. The nature of the observed synergistic action between amoxicillin, metronidazole, and the hydroxymetabolite of metronidazole is still not clear and will be a subject for further study.

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