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Susceptibilities of Nontuberculosis Mycobacterial Species to Amoxicillin-Clavulanic Acid Alone and in Combination with Antimycobacterial Agents

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Neither amoxicillin nor clavulanic acid used alone was active at the highest level tested, i.e., 256.0 μg/ml, in vitro against 24 isolates of *Mycobacterium fortuitum*, *Mycobacterium kansasii*, and *Mycobacterium marinum*. However, the MIC of an amoxicillin-clavulanic acid combination of 2:1 was ≤8.0/4.0 μg/ml for 50 percent of the isolates tested, with all isolates being inhibited in the range of 4.0/2.0 to 32.0/16.0 μg/ml, respectively. Titration of amoxicillin-clavulanic acid with a fixed 2-μg/ml concentration of ethambutol resulted in synergistic activity against 3 of 9 isolates of *M. fortuitum*, 10 of 10 isolates of *M. kansasii*, and 5 of 5 isolates of *M. marinum*. This observation was confirmed in a checkerboard analysis in which fractional inhibitory concentrations were ≤0.5 for 20 of the 24 isolates. Synergistic activity was observed against the other four isolates in one of two trials. On the other hand, titration of amoxicillin-clavulanic acid in the presence of either one or two fixed concentrations of isoniazid, rifampin, cycloserine, tetracycline, or amikacin failed to result in synergism.

There has been a resurgence of mycobacterial infections in the United States mainly attributed to the spread of AIDS in large metropolitan areas (4). A significant number of the mycobacterial isolates causing disease are nontuberculosis mycobacteria (NTM) (29, 32). Of these organisms, *Mycobacterium avium* complex, which represents more than 60% of the NTM isolates, is by far the most common cause of opportunistic NTM infection in patients with AIDS (23, 32). Among other NTM isolates capable of causing infection are *M. fortuitum* complex (19%) and *M. kansasii* (10%), with the remaining 10% being other mycobacterial species (13, 21). In addition to disseminated infections in patients with AIDS, NTM cause pulmonary, postoperative, soft tissue, and bone and joint infections (29, 30).

NTM tend to be resistant to a wide variety of antimicrobial agents including many of the β-lactam antibiotics (2). Resistance to β-lactam antibiotics has been attributed to an interplay between β-lactamase activity and cell permeability as well as a low affinity to penicillin-binding proteins (12, 15). β-Lactamase production by mycobacteria is likely to be an important factor in the expression of resistance to β-lactam antibiotics (11, 12). Indeed, β-lactams in combination with either clavulanic acid or other β-lactamase inhibitors have in vitro activity against mycobacteria not observed with either agent alone (5, 7, 8, 31).

Amoxicillin-clavulanic acid in combination with antimycobacterial agents has been used successfully to treat two patients with multiple-drug-resistant *M. tuberculosis* infections (18). Preliminary data demonstrate that amoxicillin-clavulanic acid MICs for *M. tuberculosis* obtained with the BACTEC system were 8/4 or 16/8 μg/ml (17), and for 20 *M. avium* complex isolates screened, MICs were ≤ 8/4 μg/ml for 10 isolates, and MICs were ≤ 4/2 μg/ml for 8 isolates (28).

Since antimycobacterial agents are usually used in combination for the treatment of mycobacterial infections and many agents have synergistic activity when they are used together, it was decided to test for synergistic activity between amoxicillin-clavulanic acid and known antimycobacterial agents against 24 isolates of NTM, including *M. fortuitum*, *M. kansasii*, and *M. marinum.*

MATERIALS AND METHODS

Organisms. Nine *M. fortuitum*, 10 *M. kansasii*, and 5 *M. marinum* isolates were obtained from the Bureau of Laboratories, Pennsylvania Department of Health. Isolates were stored at −70°C in Trypticase soy broth with 20% glycerol. *M. fortuitum* ATCC 6841 and *Escherichia coli* ATCC 35218 were used as control organisms on each day that tests were performed. The amoxicillin-clavulanic acid (Augmentin) quality control ranges for strain ATCC 35218 were within the limits specified by the National Committee for Clinical Laboratory Standards (20).

Compounds. Amoxicillin sodium (BRL2333) and lithium clavulanate (BRL14151) were obtained as reference standards from SmithKline Beecham Pharmaceuticals, Philadelphia, Pa. Ethambutol (lot 61H0272), isoniazid (lot 41H0797), D-cycloserine (from the microbial source; lot 81H0596), amikacin (lot 11H0232), tetracycline (lot 23H0287), and rifampin (lot 81H3317) were obtained from Sigma Chemical Company, St. Louis, Mo.

Microdilution MIC testing. Determination of microdilution MICs for isolates of *M. fortuitum*, *M. kansasii*, and *M. marinum* was performed by a modification of a procedure reported earlier (24). *M. fortuitum* isolates were grown for 1 to 3 days at 35°C on 5% sheep blood Trypticase soy agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.). The *M. kansasii* and *M. marinum* isolates were grown for 5 to 7 days on Middlebrook 7H10 agar plates (Becton Dickinson Microbiology Systems) at 35°C. The medium of choice for these studies was Middlebrook 7H9 broth with 10% oleic acid, albumin, dextrose, and catalase (OADC) supplement (Becton Dickinson Microbiology Systems). When isoniazid was tested, Middlebrook 7H9 broth without the OADC supplement was used because literature supplied by Becton Dickinson Microbiology Systems noted that the OADC supplement may interfere with isoniazid susceptibility test results.

The final ranges of concentrations of antimycobacterial agents in the wells were as follows: amoxicillin-clavulanic acid, 0.25-0.125 to 128-64 μg/ml, respectively; ethambutol, 0.25 to 128 μg/ml; isoniazid, 0.015 to 8 μg/ml; rifampin, 0.015 to 8 μg/ml; cycloserine, 0.25 to 128 μg/ml; tetracycline, 0.25 to 128 μg/ml; and amikacin, 0.25 to 128 μg/ml. Amoxicillin-clavulanic acid and ethambutol MICs were obtained from seven independent trials with microtiter trays prepared in four different batches, whereas the ethambutol MICs were obtained from five independent trials with three batches of microtiter trays. The MICs of the other antimycobacterial agents listed above were obtained from two independent trials with one batch each of the microtiter trays.

Synergy testing. MICs were also determined in doubling dilutions of amoxi-

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TABLE 1. MIC_{50}s of antimycobacterial agents for 24 isolates of *Mycobacterium* species

Antibiotic[a]	MIC_{50} (range [μg/ml])		
	M. fortuitum	*M. kansasii*	*M. marinum*
Amox-clav[b]	8 (4–16)	8 (8–16)	8 (4–16)
Ethambutol	32 (16–128)	16 (8–16)	4 (4–8)
Isoniazid	2 (0.5–>8)	1 (0.5–8)	4 (4–8)
Rifampin	>8 (>8)	0.25 (0.125–0.5)	0.25 (0.25–0.5)
Cycloserine	>128 (>128)	32 (32)	8 (8)
Amikacin	2 (0.5–8)	8 (1–8)	1 (1–2)
Tetracycline	>64 (2–>128)	64 (32–128)	32 (32–64)

[a] Amoxicillin and clavulanic acid MICs were >256 μg/ml for all isolates.
[b] Amox-clav, amoxicillin-clavulanic acid at a 2:1 ratio (the MIC_{50} is expressed as the amoxicillin concentration).

cillin-clavulanic acid plus a constant 2-μg/ml concentration of ethambutol in three independent trials with two different batches of microtiter trays. MICs in doubling dilutions of amoxicillin-clavulanic acid in combination with constant concentrations of isoniazid (0.05 or 0.1 μg/ml), rifampin (0.05 μg/ml), cycloserine (4 μg/ml), amikacin (0.06 or 0.25 μg/ml), or tetracycline (2 or 4 μg/ml) were obtained from two independent trials prepared in one batch each of the microtiter trays. MICs were read as the lowest concentration of antimycobacterial agent required to inhibit visible growth of the organisms. For the constant amount of the antimycobacterial agents added to amoxicillin-clavulanic acid, synergy was defined as at least a 2-doubling-dilution decrease in the MIC between the MIC of the combination and that of its most active component.

Doubling dilution concentrations of amoxicillin-clavulanic acid and ethambutol also were tested by checkerboard analysis (10). Once the amoxicillin-clavulanic acid and ethambutol MICs for the atypical mycobacteria were obtained, combinations at doubling dilutions lower than the original MICs were tested. This was done in replicate independent trials with two batches of microtiter plates. The fractional inhibitory concentration (FIC), as described by Heifets (14), was calculated by the following formula:

$$\text{FIC} = \frac{\text{MIC of A-C in combination}}{\text{MIC of A-C alone}} + \frac{\text{MIC of E in combination}}{\text{MIC of E alone}}$$

where A-C is amoxicillin-clavulanic acid and E is ethambutol. Synergy is defined as an FIC of 0.5 or less, additive is an FIC of 1.0, and antagonism is an FIC of 2.0 or greater.

Determination of β-lactamase production. Isolates were grown in Middlebrook 7H9 broth with 10% OADC supplement for 4 to 10 days at 35°C. Following centrifugation (5,000 × *g* for 15 min at 5°C) the harvested cells were resuspended in water, chilled at 0°C, and disrupted (model 450 sonifier; Branson Ultrasonics Corp., Danbury, Conn.). The lysate was centrifuged (40,000 × *g* for 1 h at 5°C), and the supernatant was tested for the presence of β-lactamase with nitrocefin (50 μg/ml). A color change from yellow to red was considered positive. A strain of the *M. avium* complex (strain 8054) was used as a negative control, and *M. fortuitum* ATCC 6841 was used as a positive control.

RESULTS

The MICs of the test compounds that inhibited 50% of strains tested (MIC_{50}s) for the three species of *Mycobacterium* are given in Table 1. The amoxicillin-clavulanic acid MIC_{50}s for each of the species were 8/4 μg/ml, respectively. The ethambutol MIC_{50}s were 32 μg/ml for *M. fortuitum*, 16 μg/ml for *M. kansasii*, and 4 μg/ml for *M. marinum*. With the exception of isoniazid and amikacin, none of the other antimycobacterial agents displayed significant activity against *M. fortuitum*. Rifampin and amikacin showed potent activity against *M. kansasii* and *M. marinum*, whereas the other antimycobacterial agents had variable activities against strains of these microorganisms. Neither amoxicillin nor clavulanic acid was active against these isolates at concentrations of up to 256 μg/ml.

Amoxicillin-clavulanic acid MICs for the 15 isolates of *M. kansasii* and *M. marinum* decreased by at least 2 doubling dilutions in the presence of 2 μg/ml of ethambutol per ml, whereas the same combination had synergistic activity against only 3 of 9 *M. fortuitum* isolates (isolates 222, 225, and 1289).

When amoxicillin-clavulanic acid and ethambutol were tested alone and in combination against the 24 clinical isolates by the checkerboard methodology, synergistic activity was observed against the three mycobacterial species tested (Tables 2 to 4). The MIC_{50} of amoxicillin-clavulanic acid for *M. fortuitum*, which when tested alone was 8/4 μg/ml decreased to 0.5/0.25 to 2.0/1.0 μg/ml in the presence of sub-MICs of ethambutol (Table 2). In duplicate trials, the combination of amoxicillin-clavulanic acid failed to show a synergistic response against only one of nine *M. fortuitum* isolates tested (isolate 1088). Similar results were obtained against 10 strains of *M. kansasii* (Table 3). FICs were ≤0.5 for nine of these isolates. Synergistic activity was also observed against the other remaining isolate (isolate 1722), but it was not confirmed on repeat testing. Amoxicillin-clavulanic acid in combination with ethambutol also showed synergistic activity against the five isolates of *M. marinum* (Table 4). Although the results against this species were somewhat more variable, with the drug combination showing synergistic activity against two of the isolates in one of two trials, the data support the conclusion of synergistic activity with the combination.

When tested for the presence of β-lactamase activity, all of the *M. fortuitum* isolates and *M. kansasii* 258 showed an immediate color change from yellow to red. The remaining *M. kansasii* and *M. marinum* isolates produced β-lactamase only

TABLE 2. Activities of amoxicillin-clavulanic acid–ethambutol combinations against *M. fortuitum* isolates by the checkerboard methodology

Isolate	MIC (μg/ml)				Amoxicillin-clavulanic acid–ethambutol FIC[a]	
	Amoxicillin-clavulanic acid	Ethambutol	Amoxicillin-clavulanic acid–ethambutol			
			Trial 1	Trial 2	Trial 1	Trial 2
222	16/8	128	1/0.5–8	0.5/0.25–16	0.1	0.2
225	16/8	128	2/1–16	1/0.5–4	0.2	0.1
432	8/4	32	2/1–8	0.5/0.25–8	0.5	0.3
623	8/4	32	2/1–8	0.5/0.25–8	0.5	0.3
1044	8/4	32	2/1–8	0.5/0.25–8	0.5	0.3
1088	16/8	16	8/4–8	0.5/0.25–8	1.0	0.5
1178	4/2	32	1/0.5–8	0.25/0.125–8	0.5	0.3
1289	32/16	16	8/4–4	2/1–4	0.5	0.3
1293	8/4	32	2/1–8	0.5/0.25–8	0.5	0.3

[a] Synergy is defined as an FIC of ≤0.5.

TABLE 3. Activities of amoxicillin-clavulanic acid–ethambutol combination against *M. kansasii* isolates by the checkerboard methodology

Isolate	MIC (μg/ml)				Amoxicillin-clavulanic acid–ethambutol FIC[a]	
	Amoxicillin-clavulanic acid	Ethambutol	Amoxicillin-clavulanic acid–ethambutol			
			Trial 1	Trial 2	Trial 1	Trial 2
258	8/4	16	1/0.5–2	0.25/0.125–4	0.3	0.3
475	8/4	16	1/0.5–2	0.25/0.125–4	0.3	0.3
506	32/16	16	4/2–2	0.25/0.125–4	0.3	0.3
626	8/4	16	1/0.5–2	0.25/0.125–8	0.2	0.5
640	8/4	16	1/0.5–2	0.25/0.125–8	0.3	0.5
1220	8/4	8	1/0.5–1	1/0.5–1	0.3	0.3
1609	16/8	8	2/1–1	2/1–2	0.3	0.4
1610	16/8	16	2/1–2	2/1–2	0.3	0.3
1722	16/8	8	4/2–2	2/1–4	0.5	0.6
1870	8/4	16	1/0.5–2	1/0.5–4	0.3	0.4

[a] Synergy is defined as an FIC of ≤0.5.

TABLE 4. Activities of amoxicillin-clavulanic acid–ethambutol combinations against *M. marinum* isolates by the checkerboard methodology

Isolate	MIC (μg/ml)				Amoxicillin-clavulanic acid–ethambutol FIC[a]	
	Amoxicillin-clavulanic acid	Ethambutol	Amoxicillin-clavulanic acid–ethambutol			
			Trial 1	Trial 2	Trial 1	Trial 2
1580	16/8	8	2/1–1	0.25/0.125–2	0.3	0.3
2084	8/4	4	2/1–1	0.5/0.25–2	0.5	0.6
2162	8/4	4	2/1–1	0.25/0.125–2	0.5	0.5
4935	8/4	4	4/2–2	2/1–0.25	1.0	0.3
4939	16/8	8	4/2–2	1/0.5–1	0.5	0.2

[a] Synergy is defined as an FIC of ≤0.5.

after induction by growth in the presence of 10 μg of ampicillin per ml added daily.

DISCUSSION

All of the mycobacterial isolates used in the studies described here were shown to produce β-lactamase which apparently hydrolyzed amoxicillin, resulting in MICs of >256 μg/ml. However, when amoxicillin was tested in combination with the β-lactamase inhibitor clavulanic acid in a 2:1 ratio, amoxicillin-clavulanic acid MIC_{50} of 8.0/4.0 μg/ml for *M. fortuitum*, *M. kansasii*, or *M. marinum* were obtained (Table 1). These MIC results are in the same range reported previously for *M. fortuitum*, although they were obtained by another MIC method (8). Many clinical NTM isolates are resistant to the commonly used antitubercle drugs and the β-lactam antibiotics (2). The resistance to the β-lactams is, in part, attributable to the production of chromosomal β-lactamases which hydrolyze many penicillins and cephalosporins. β-Lactamases have been described for most isolates of pathogenic mycobacteria with the exception of members of the *M. avium* complex (1, 16, 19, 33). Use of the combination of β-lactam antibiotics with various β-lactamase inhibitors has been shown to be synergistic against a number of mycobacterial species (3, 6, 7, 27).

Preliminary studies were carried out by using microdilution titration of amoxicillin-clavulanic acid in the presence of a fixed fraction of the predetermined MIC of each of the various antimycobacterial agents. At the same time, the MIC of each test agent was also determined. Under the conditions of these tests, with synergy defined as at least a 2-doubling-dilution decrease in the MIC between the MIC of the combination and that of its most active component, isoniazid, rifampin, cycloserine, tetracycline, or amikacin showed neither synergy nor antagonism when used in combination with amoxicillin-clavulanic acid (data not shown). Only ethambutol (2 μg/ml) was found to be synergistic with amoxicillin-clavulanic acid against the three species of mycobacteria. It should be noted that the selection of only one or two fixed concentrations for testing drug combinations may not be adequate for demonstration of interactive drug activity if the concentration selected is outside the range needed to demonstrate synergy. Whereas 2 μg of ethambutol per ml was adequate to demonstrate synergistic activity against all of the *M. kansasii* and *M. marinum* isolates, it had synergistic activity against only three of nine isolates of *M. fortuitum*. The 2-μg/ml concentration was four- to eightfold less than the MICs for *M. kansasii* and *M. marinum* and 8 to 64 times less than the MIC for *M. fortuitum* (Table 1). In the checkerboard synergy studies, a concentration of at least 8 μg of ethambutol per ml with amoxicillin-clavulanic acid was required for synergistic activity against seven of the nine *M. fortuitum* isolates tested (Table 2). In the checkerboard protocol both amoxicillin-clavulanic acid and ethambutol were tested in combination over a broad range of concentrations, and synergistic activity was observed against almost all of the isolates tested (Tables 2 to 4).

The combination of two agents, amoxicillin and ethambutol, which act on different targets in the cell envelope, was shown to have synergistic activity against the three species of mycobacteria tested. Although the mode of action of ethambutol has not been fully elucidated, it does interfere with cell envelope synthesis in bacteria. It has been shown to inhibit mycolic acid transfer to the cell wall of *M. smegmatis* (25), and it was later suggested that this may result indirectly from inhibition of arabinogalactan synthesis (26). More recently, it was proposed that the drug inhibits an early step of glucose conversion into the monosaccharides used for the biosynthesis of cell wall polysaccharides: arabinogalactan, arabinomannan, and peptidoglycan (22). Deng et al. (9) have reported that ethambutol treatment resulted in the cleavage of the arabinosyl residues present in the mycobacterial cell wall by an arabinosyl-releasing enzyme which, in combination with inhibition of cell wall synthesis, results in cell wall disruption. They note that "ethambutol induced damage to the cell wall provides a ready molecular explanation for the known synergistic effects with ethambutol with other chemotherapeutic agents" (9). Thus, it would appear that ethambutol's effects on the cell wall promote the entry of amoxicillin and clavulanic acid and greater access to their respective targets, β-lactamase and penicillin-binding proteins.

The concentrations of amoxicillin-clavulanic acid alone or in combination with ethambutol required to inhibit mycobacterial growth in vitro are within the therapeutic ranges obtained in patients treated with these antimicrobial agents. Amoxicillin-clavulanic acid has been used to successfully treat two patients with multi-drug-resistant tuberculosis when it was administered along with second-line drugs (18). Further evaluation of the clinical usefulness of amoxicillin-clavulanic acid alone or in combination with ethambutol in the treatment of mycobacterial infections is warranted.

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