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In Vitro Susceptibility of *Mycobacterium fortuitum* to Amoxicillin or Cephalothin in Combination with Clavulanic Acid

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The comparative in vitro activity of cefoxitin, cephalothin, amoxicillin, and clavulanic acid in combination with the latter two agents against 13 isolates of *Mycobacterium fortuitum* was evaluated by agar dilution susceptibility testing. Amoxicillin was more active than cephalothin but less active than cefoxitin against the strains tested. Clavulanic acid in combination with these β-lactams usually improved the activity by one or two dilutions compared with the β-lactams alone.

Cefoxitin and cefmetazole, two cephamycins, have been shown to have good in vitro activity against *Mycobacterium fortuitum* (3). These organisms have been shown to have a β-lactamase which hydrolyzes benzylpenicillin, cephalothin, and cephaloridine but does not hydrolyze methicillin or oxacillin (4). Cephalosporins which are β-lactamase stable but do not have the 7-α-methoxy group have been found to be relatively inactive against *M. fortuitum* (2). The purpose of this study was to evaluate the activity of clavulanic acid, a β-lactamase inhibitor, in combination with the β-lactamase-labile β-lactams amoxicillin and cephalothin against *M. fortuitum* to better define the role of β-lactamase stability in the activity of the 7-α-methoxy derivatives. Temocillin (BRL 17421), a 6-α-methoxy derivative of ticarcillin with increased stability against β-lactamases, was included to study the effect of the 6-α-methoxy group on the penicillin nucleus (7).

The isolates of *M. fortuitum* were those used in our previous studies (2, 3). They all had β-lactamase activity when tested with nitrocefin (2). *Staphylococcus aureus* ATCC 25923 (Difco Laboratories, Detroit, Mich.), a β-lactamase-negative strain, and *S. aureus* Sbg, a β-lactamase-positive clinical isolate, were used as controls.

The antimicrobial agents evaluated in this study were provided as standard powders by the following: cefoxitin (potency, 948 μg/mg), Merck Institute for Research, Rahway, N.J.; amoxicillin (potency, 860 μg/mg), potassium clavulanate (potency, 826 μg/mg), and temocillin, Beecham Laboratories, Bristol, Tenn.; and cephalothin (potency, 955 μg/mg), Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, Ind. Nitrocefin was provided by Glaxo Research, Greenford, Middlesex, United Kingdom. Stock solutions of each antimicrobial agent were prepared immediately before use by hydrating a known weight of drug in 50 mM phosphate buffer (pH 6.0) and by filter sterilization through a GA-6 0.45-μm membrane filter (Gelman Sciences, Inc., Ann Arbor, Mich.). The appropriate concentration of antibiotic was added to Mueller-Hinton agar at 56°C before quadrant plates were prepared. The final concentrations of the antibiotics were as follows: cephalothin, cefoxitin, and amoxicillin, 2 to 256 μg/ml; potassium clavulanate and temocillin, 4 to 256 μg/ml; amoxicillin, 2 μg/ml, combined with clavulanic acid, 1 μg/ml; and cephalothin, 256 μg/ml, combined with clavulanic acid, 128 μg/ml.

The mycobacteria and *S. aureus* isolates were inoculated into tubes of Mueller-Hinton broth containing 0.05% Tween 80 (MHBT 80). The tubes were shaken at 37°C for 48 to 72 h (the *S. aureus* isolates were shaken overnight). The cultures were diluted with MHBT 80 to yield 10 Klett units per ml (approximately 5×10^6 CFU/ml). Quadrant plates were spotted in duplicate with 10 μl of culture, which yielded approximately 5×10^4 CFU (range, 1.1×10^4 to 9.6×10^4 CFU) per spot. The plates were incubated at 37°C for 72 h, and the minimum inhibitory concentration was defined as the lowest concentration of antibiotic at which there was no growth or a barely perceptible haze. The *S. aureus* plates were read after 24 h.

M. fortuitum 1045 and 1047 were inoculated into 300 ml of MHBT 80. The cultures were

TABLE 1. Activity of various β-lactams

Strain	Minimum inhibitory concn (μg/ml) of:				
	Cefoxitin	Cephalothin	Cephalothin-clavulanic acid	Amoxicillin	Amoxicillin-clavulanic acid
M. fortuitum					
AO5	32	>256	128–64	32	8–4
5–4	32	>256	128–64	32	8–4
914	4	32	8–4	8	4–2
1004	16	>256	128–64	32	16–8
1030	16	256	128–64	32	16–8
1045	32	128	256–128	>256	>256–128
1047	16	128	32–16	>256	64–32
1048	16	>256	256–128	64	16–8
1049	16	>256	128–64	64	16–8
1059	32	>256	>256–128	256	32–16
1260	8	>256	64–32	16	4–2
1261	16	>256	256–128	16	8–4
1263	16	>256	256–128	32	8–4
S. aureus					
Sbg	4	≤2	≤2–1	>256	≤2–1
ATCC 25923	≤2	≤2	≤2–1	≤2	≤2–1

grown for 48 h at 37°C in a rotary shaker. The cells were pelleted and washed two times with 50 mM Tris buffer (pH 7). The cells were broken in a Cell Homogenizer MSK (Braun Instruments, South San Francisco, Calif.) with 0.25- to 0.30-mm glass beads. After homogenization, the supernatant was subjected to a low-speed spin at 3,000 × *g* for 20 min, followed by a high-speed spin at 30,900 × *g* for 20 min. The supernatant was removed and stored at −20°C. The protein content of the extracts was determined using the Bio-Rad Protein Assay (1) with a bovine albumin standard. The protein content of the extract from strain 1045 was 8.4 mg/ml, and that from strain 1047 was 6.8 mg/ml.

The β-lactamase activity of the cell-free extract was determined by a modification of the method used by O'Callaghan et al. (6). A 1-cm cell, containing a total volume of 1 ml, was used. This consisted of nitrocefin (17.8 μg), cell-free extract (5 μl), and clavulanic acid (1 or 4 μg) in 50 mM Tris buffer (pH 7.0). A control cell without clavulanic acid was run simultaneously with the other cells. The rate of reaction was measured on a Gilford spectrophotometer 250 with a kinetic record system (Gilford Instrument Laboratories, Oberlin, Ohio) at 482 nm.

Temocillin was not active against *M. fortuitum* at 256 μg/ml, the highest concentration tested. Clavulanic acid inhibited only one strain (strain 914) at 256 μg/ml. Cephalothin inhibited 4 of 13 strains at 256 μg/ml or less (Table 1). Cefoxitin inhibited all of the strains at 32 μg/ml and 9 of 13 strains at 16 μg/ml or less. Cephalothin-clavulanic acid was usually more active than cephalothin alone, with a two-dilution decrease in the minimum inhibitory concentration for strains 914, 1047, and 1260. The minimum inhibitory concentration was decreased in the remaining strains, with the exceptions of 1045 and 1059. The cephalothin-clavulanic acid combination was not as active as cefoxitin.

Amoxicillin inhibited 8 of 13 strains at 32 μg/ml. Amoxicillin-clavulanic acid usually resulted in a severalfold-greater activity compared with amoxicillin alone. The combination was more active against strains 1047 and 1059 than against strain 1045.

The β-lactamase activity in crude cell-free extracts from strains 1045 and 1047 was partially inhibited by clavulanic acid at a concentration of 4 μg/ml or less (Fig. 1). There was substantially greater inhibition at 4 μg/ml than at 1 μg/ml.

The absence of activity of temocillin is curious since its 6-α-methoxy group might be expected to be analogous to the 7-α-methoxy group of cefoxitin. It would be interesting to evaluate the 6-α-methoxy analog of amoxicillin.

Cefoxitin and cephalothin are structurally

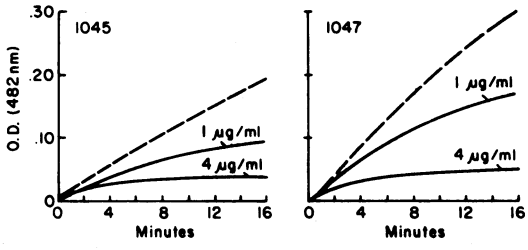

FIG. 1. β-Lactamase activity of *M. fortuitum* cell-free extract. (– – –) Reaction mixture without clavulanic acid; (——) reaction mixture with clavulanic acid. O.D., Optical density.

similar except for the 7-α-methoxy group and the substituent at position 3 of the heterocyclic nucleus. The latter difference is believed to determine the pharmacokinetic properties but to have little effect on the antimicrobial activity (5). Clavulanic acid inhibits the β-lactamase activity in cell-free extracts from strains 1045 and 1047; however, the activity of the β-lactam was increased by the addition of clavulanic acid only with strain 1047. The increased activity of clavulanic acid combined with cephalothin or amoxicillin provides indirect evidence that β-lactamase stability has a role in the susceptibility of *M. fortuitum* to β-lactam antibiotics. The role of β-lactamase does not appear to be major, since cephalothin, a good substrate for *M. fortuitum* β-lactamase, had relatively little increase in activity when it was combined with clavulanic acid. It seems likely that the presence of the 7-α-methoxy group has significance beyond its role in conferring β-lactamase stability on cefoxitin. It is possible that the 7-α-methoxy group improves interaction with the receptor site(s) or improves penetration of the β-lactam into the bacterial cell. Further studies are needed to distinguish between these two mechanisms.

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