Activity of Mecillinam Alone and in Combination with Other β-Lactam Antibiotics

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The in vitro activities of mecillinam, ticarcillin, cefamandole, and cefoxitin, singly and in all possible combinations, against 53 clinical isolates were studied by a checkerboard method of determining minimal inhibitory concentrations. For selected representative strains, bactericidal activity was determined by minimal bactericidal concentrations and killing curves. Mecillinam was the least active antibiotic against gram-positive cocci, Pseudomonas aeruginosa, and Bacteroides fragilis and the most active against Enterobacteriaceae. Reproducibility of mecillinam minimal inhibitory concentrations for susceptible Enterobacteriaceae was often poor, however, due to minor variations in inoculum size. When mecillinam resistance was observed with Enterobacteriaceae, partial inhibition could be demonstrated at concentrations below minimal inhibitory concentrations, and bacterial cells were consistently ovoid or round; under those conditions the addition of a second study antibiotic resulted in marked synergistic inhibition and killing which was independent of inoculum size and susceptibility to the second antibiotic. In contrast, synergy with mecillinam against mecillinam-susceptible strains or with other antibiotic combinations against any species was not consistently observed.

Mecillinam (formerly called FL 1060) is the prototype of a relatively new class of β-lactam antibiotics. Unlike the penicillins, cephalosporins, and cephamycins, it has a 6-amidino rather than a 6-acylamino side chain. Its spectrum of activity is different, with marked activity against some Enterobacteriaceae but little activity against gram-positive organisms (6). Its effects on Escherichia coli, and presumably other Enterobacteriaceae, are also different than those of other β-lactam antibiotics. None of the known enzymes of cell wall synthesis is inhibited, and the formation of filaments or spheroplasts which rapidly lyse is not observed. Over a wide range of concentrations, ovoid, osmotically stable round cells are formed which divide slowly and eventually lyse (9). The unique biochemical and morphological effects of mecillinam on E. coli have been attributed to its affinity for interacting with penicillin-binding protein 2 and thus affecting cell shape. It does not interact with penicillin-binding proteins 1 or 3, the preferential targets of other β-lactam antibiotics (11). Because mecillinam has a unique mechanism of action among β-lactam antibiotics against bacterial cells, it was postulated and subsequently shown, both in vitro (15) and in animal models (5), that it may act synergistically with those drugs.

This paper reports on the in vitro activity of mecillinam alone and in combination with a penicillin (ticarcillin), a cephalosporin (cefamandole), and a cephamycin (cefoxitin) against a variety of clinical isolates. For comparison, the activities of ticarcillin, cefamandole, and cefoxitin, singly and in all possible combinations, were studied simultaneously.

MATERIALS AND METHODS

Organisms. Fifty-three bacterial strains which were isolated from patients hospitalized in The Ohio State University Hospitals were studied. They were selected because they represented a broad spectrum of common pathogens and demonstrated varied, but typical, patterns of susceptibility to the four study antibiotics. Included were strains of Staphylococcus aureus, Streptococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Enterobacter aerogenes, Serratia marcescens, Proteus mirabilis, Pseudomonas aeruginosa and Bacteroides fragilis. All isolates, except the strains of B. fragilis, were blood isolates.

Antibiotics. The antibiotics studied included mecillinam obtained from Hoffmann-LaRoche Inc., Nutley, N.J.; ticarcillin obtained from Beecham Products, Pittsburgh, Pa.; cefamandole obtained from Eli Lilly and Co., Indianapolis, Ind.; and cefoxitin obtained from Merck Sharp and Dohme, West Point, Pa. Laboratory standards were diluted as recommended by manufacturers to stock concentrations of 1,000 μg/ml and used immediately or frozen at −20°C, for up to 1 month, until used.

Susceptibility tests. Minimal inhibitory concentrations (MICs) of the four study antibiotics were...
determined singly and in combinations by the checkerboard method with microdilution techniques previously described (4). The media used were Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for *S. aureus* and *S. faecalis*, Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with CaCl₂ and MgCl₂ to contain 50 mg of calcium per liter and 25 mg of magnesium per liter for *Enterobacteriaceae* and *P. aeruginosa* and Schaedler broth (Difco) for *B. fragilis*. Antibiotic concentrations consisted of log, dilutions which ranged from 128 to 0.13 µg/ml or 16 to 0.01 µg/ml. Inoculums sizes of approximately 2 × 10⁴ and 2 × 10⁵ colony-forming units (CFU) per ml were tested. Incubation was for 18 to 20 h at 37°C in a room-air incubator, except for *B. fragilis* which was incubated in an anaerobic glove box containing 85% N₂, 10% H₂, and 5% CO₂ for 48 h.

MICs were read as the lowest concentration of each antibiotic which completely inhibited visible growth. Antibiotics were considered to be synergistic when there was a fourfold or greater reduction in MICs of both drugs in the combination as compared with that of each drug alone. Antibiotics were considered to be antagonistic when there was a fourfold or greater increase in MICs of both drugs in the combination or when the addition of an inactive antibiotic to an active antibiotic increased the MIC of the latter by fourfold or more. Antibiotics were considered to be indifferent when changes in MICs were intermediate.

With three selected strains of *Enterobacteriaceae* (*E. coli* 1924, *K. pneumoniae* 1864, and *S. marcescens* 1923) which showed typical patterns of inhibition by mecillinam (see Results), MICs of mecillinam and one or more of the other study antibiotics, singly and in combinations, were repeated with inocula of approximately 2 × 10⁶, 2 × 10⁷, 2 × 10⁸, and 2 × 10⁹ CFU/ml prepared from the same culture. Bactericidal activity of the antibiotics was studied by determining minimal bactericidal concentrations (MBCs) and performing killing curves (1) with the four inoculum sizes. To determine MBCs, MIC plates which had been incubated for 20 h were subcultured to microdilution plates containing sterile broth by using disposable inoculators (Dynatech Laboratories, Alexandria, Va.). The subcultures were incubated overnight. The absence of growth in the subcultures from a given well of an MIC plate indicated a reduction in the original number of CFU to <10² CFU/ml (>99.9% kill with an original inoculum of 2 × 10⁵ CFU/ml) in that well. MBCs were read as the lowest concentrations of antibiotics which yielded no growth in subcultures after overnight incubation. To perform killing curves, the same cultures tested above were used. The appropriate number of organisms was incubated with clinically achievable concentrations of the test antibiotics, singly and in combinations, in 50 ml of broth for 20 h. Organisms inoculated into antibiotic-free broth served as growth controls. After 0, 4, and 20 h of incubation, pour plate colony counts on portions of cultures were performed.

**RESULTS**

The MICs of the four study antibiotics for the 53 bacterial strains tested (with an inoculum of 2 × 10⁸ CFU/ml) are shown in Table 1. When there was more than one pattern of susceptibilit-
Table 1. Susceptibilities of bacterial isolates to β-lactam antibiotics\(^a\) singly and in combinations

| Organism          | No. | Range of MICs (µg/ml) | S, I, A\(^a\) (no.) |
|-------------------|-----|-----------------------|----------------------|
|                   |     | MCL | TIC | CMD | CXT | MCL/TIC | MCL/CMD | MCL/CXT | TIC/CMD | TIC/CXT | CMD/CXT |
| S. aureus         | 4   | 64–128 | 2–8 | 0.25–0.5 | 4 | 0.4, 0.4 | 0.4, 0.4 | 0.4, 0.4 | 0.4, 0.4 | 1.3, 0.4 | 2.2, 0.4 |
| S. faecalis       | 4   | >128  | 32–64 | 32–64 | >128 | 1.3, 0.4 | 0.4, 0.4 | 0.4, 0.4 | 0.4, 0.4 | 1.5, 0.4 | 2.4, 0.4 |
| E. coli           | 6   | 0.03–0.06 | 1–2 | 0.25–1 | 2–4 | 0.6, 0.6 | 0.6, 0.6 | 4.2, 0.4 | 1.5, 0.4 | 2.6, 0.4 |
| K. pneumoniae     | 8   | 0.06–2 | >128  | 0.25–4 | 1–4 | 0.8, 0.6 | 0.8, 0.6 | 0.8, 0.6 | 0.8, 0.6 | 1.7, 0.6 | 2.6, 0.6 |
| E. cloacae        | 2   | 0.06–0.13 | 64 | 0.5 | 2 | 0.2, 0.2 | 0.2, 0.2 | 0.2, 0.2 | 0.2, 0.2 | 1.1, 0.2 | 2.0, 0.2 |
| E. aerogenes      | 2   | 0.06–0.13 | 2–8 | 2–4 | ≥128 | 0.2, 0.2 | 0.2, 0.2 | 0.2, 0.2 | 0.2, 0.2 | 0.2, 0.2 | 0.2, 0.2 |
| S. marcescens     | 1   | 0.13–0.25 | 2 | 1–2 | ≥128 | 0.2, 1.1 | 0.2, 1.1 | 0.2, 1.1 | 0.2, 1.1 | 0.2, 1.1 | 0.2, 1.1 |
| P. mirabilis      | 2   | 0.1–8–128 | 32–64 | 64–>128 | >128 | 2.0, 2.0 | 2.0, 2.0 | 2.0, 2.0 | 0.1, 0.1 | 0.1, 0.1 | 0.1, 0.1 |
| P. aeruginosa     | 6   | 0.13–0.5 | 0.5 | 0.5–1 | 2–4 | 1.3, 1.3 | 0.4, 0.4 | 4.0, 0.4 | 2.2, 0.4 | 2.6, 0.4 |
| B. fragilis       | 2   | 0.13–0.5 | 16–32 | 16–>128 | 16–32 | 6.0, 0.0 | 3.3, 0.0 | 6.0, 0.0 | 0.6, 0.6 | 0.6, 0.6 |
|                   |     | 0.13–0.5 | 0.5 | 0.5–1 | 2–4 | 1.3, 1.3 | 0.4, 0.4 | 4.0, 0.4 | 2.2, 0.4 | 2.6, 0.4 |
|                   |     | 0.13–0.5 | 0.5 | 0.5–1 | 2–4 | 1.3, 1.3 | 0.4, 0.4 | 4.0, 0.4 | 2.2, 0.4 | 2.6, 0.4 |

\(^a\) MCL, mecillinam; TIC, ticarcillin; CMD, cefamandole; CXT, cefoxitin.

\(^b\) S, Synergy; I, indifference; A, antagonism.

\(^c\) Partial inhibition observed at lower concentrations: 0.25 to 2 µg/ml for E. aerogenes and 0.5 to 8 µg/ml for S. marcescens.
antibiotics, singly and in combinations, for three selected strains of *Enterobacteriaceae* are shown in Table 2. With *E. coli* 1924 (which typified mecillinam-susceptible *Enterobacteriaceae* whose MICs were unaffected by inoculum size in the above experiments), inoculum size only affected mecillinam MBCs; combinations of mecillinam and cefamandole were synergistic when mecillinam MBCs were high. With *K. pneumoniae* 1864 (which typified *Enterobacteriaceae* whose MICs were markedly affected by inoculum size in the above experiments), inoculum size affected mecillinam MICs as well as MBCs; combinations of mecillinam and cefamandole or cefoxitin were synergistic when mecillinam MICs and MBCs were high. With *S. marcescens* 1923 (which typified mecillinam-resistant *Enterobacteriaceae* whose MICs were unaffected by inoculum size in the above experiments), combinations of mecillinam and ticarcillin or cefamandole were synergistic in both inhibitory and bactericidal activities.

After MIC plates for *E. coli* 1924, *K. pneumoniae* 1864, and *S. marcescens* 1923 were subcultured for determination of MBCs, the contents of mecillinam-containing wells which had visible growth were examined by phase-contrast microscopy and Gram stains. Organisms from wells below MICs were ovoid or round regardless of strain, inoculum size, or antibiotic concentration.

The killing curves for mecillinam and comparative study antibiotics, singly and in combinations, with the three selected strains of *Enterobacteriaceae* were performed simultaneously with the MICs and MBCs shown in Table 2 and are illustrated in Fig. 1 and 2. (Mecillinam and cefamandole against *K. pneumoniae* are not included because the curves were identical to those observed with mecillinam and cefoxitin). With all four antibiotics, concentrations which exceeded MICs with a given inoculum size usually resulted in a reduction of viable organisms by 10^2 to 10^3 CFU/ml at 4 h.

### Table 2. Inoculum effect on MICs and MBCs of study antibiotics singly and in combinations against selected bacterial strains

| Strain and inoculum size (CFU/ml) | Susceptibilities (μg/ml) |
|----------------------------------|--------------------------|
|                                  | Mecillinam | Control antibiotic | Combination |
|                                  | MIC       | MBC     | MIC | MBC | MIC | MBC |
| **E. coli 1924**                  |           |         |     |     |     |     |
| 2 × 10^3                          | 0.25      | 0.25    | 2   | 4   | 0.13/0.06 | 0.13/0.06 |
| 2 × 10^4                          | 0.5       | 2       | 4   | 4   | 0.13/0.03 | 0.13/0.13 |
| 2 × 10^5                          | 0.25      | 16      | 4   | 4   | 0.13/0.13 | 0.25/0.25 |
| 2 × 10^6                          | 0.25      | 16      | 4   | 8   | 0.13/0.13 | 0.25/0.5  |
| **K. pneumoniae 1864**            |           |         |     |     |     |     |
| 2 × 10^3                          | 0.06      | 0.06    | 0.25 | 0.25 | 0.13/≤0.01 | 0.13/≤0.01 |
| 2 × 10^4                          | 0.13      | 0.13    | 0.5  | 0.5  | 0.13/≤0.01 | 0.13/≤0.01 |
| 2 × 10^5                          | 0.06      | 0.06    | 0.25 | 0.5  | 0.06/≤0.01 | 0.06/≤0.01 |
| 2 × 10^6                          | >16(0.25)^b | >16    | 1   | 1   | 0.13/≤0.01 | 0.13/≤0.01 |
| **K. pneumoniae 1864**            |           |         |     |     |     |     |
| 2 × 10^3                          | 0.03      | 0.03    | 1   | 1   | 0.03/0.25 | 0.03/0.25 |
| 2 × 10^4                          | 0.06      | 0.06    | 1   | 1   | 0.06/0.13 | 0.06/0.13 |
| 2 × 10^5                          | 0.13      | 0.25    | 1   | 2   | 0.06/0.25 | 0.25/0.25 |
| 2 × 10^6                          | 16(0.25)^c | 16    | 1   | 2   | 0.06/0.13 | 0.25/0.25 |
| **S. marcescens 1923**            |           |         |     |     |     |     |
| 2 × 10^3                          | >16(1)    | >16     | 8   | 8   | 0.5/0.13  | 0.5/0.25  |
| 2 × 10^4                          | >16(1)^e  | >16     | 8   | 16  | 0.5/1     | 1/1       |
| 2 × 10^5                          | >16(2)    | >16     | 16  | >128 | 1/2      | 2/2       |
| 2 × 10^6                          | >16       | >16     | 16  | >128 | 1/2      | 2/4       |
| **S. marcescens 1923**            |           |         |     |     |     |     |
| 2 × 10^3                          | 1         | >16     | >16 | >16 | 1/≤0.01  | 2/0.25    |
| 2 × 10^4                          | >16(1)    | >16     | >16 | >16 | 1/1      | 4/4       |
| 2 × 10^5                          | >16(1)    | >16     | >16 | >16 | 2/1      | 8/8       |
| 2 × 10^6                          | >16(1)    | >16     | >16 | >16 | 2/4      | >16/16    |

> *a* Cefamandole, cefamandole, cefoxitin, ticarcillin, and cefamandole, respectively.

> *b* Parentheses indicate lowest concentration at which partial inhibition was observed.

> *c* MIC >128 μg/ml in previous experiment (Table 1).
Fig. 1. In vitro killing of E. coli 1924 (top) by (A) 16 μg of mecinillam per ml, (B) 16 μg of cefamandole per ml, and (C) both and of K. pneumoniae 1864 (bottom) by (A) 16 μg of mecinillam per ml, (B) 16 μg of cefoxitin per ml, and (C) both. Broken line represents growth controls.

Fig. 2. In vitro killing of S. marcescens 1923 (top) by (A) 16 μg of mecinillam per ml, (B) 128 μg of ticarcillin per ml and (C) both and (bottom) by (A) 16 μg of mecinillam per ml, (B) 16 μg of cefamandole per ml, and (C) both. Broken line represents growth controls.
which caused partial inhibition with a given inoculum size (observed only with mecillinam and \textit{K. pneumoniae} and \textit{S. marcescens}) resulted in little or no reduction in the number of CFU per milliliter at 4 h. Concentrations which caused no inhibition (observed only with cefamandole and \textit{S. marcescens}) resulted in an increase in CFU per milliliter. There was poor correlation of reduction in CFU per milliliter at 4 h with MBCs and of reduction in CFU per milliliter at 20 h with either MICs or MBCs. With all five mecillinam combinations, reductions in CFU per milliliter at 4 or 20 h or both were greater than with either drug alone, and the number of viable organisms was always reduced regardless of susceptibilities to the individual antibiotics.

**DISCUSSION**

Previous studies have indicated that mecillinam has little in vitro activity against gram-positive bacteria, \textit{P. aeruginosa}, and other non-fermentative gram-negative bacilli or anaerobic bacteria (2, 3, 6, 7). In one study (8), combinations with other \beta-lactam antibiotics against gram-positive organisms and anaerobes were indifferent, but synergy was observed with some strains of \textit{P. aeruginosa}. In another study (13), mecillinam antagonized the activity of ampicillin but was synergistic with carbenicillin against some strains of \textit{Bacteroides}. In the present study, mecillinam had little activity against those organisms, and combinations with other \beta-lactam antibiotics were usually indifferent. The occasional synergy which was observed occurred no more frequently than with nonmecillinam \beta-lactam antibiotic combinations.

Ever since the original report of mecillinam (6), it has been clear that it has a broad spectrum of in vitro activity against \textit{Enterobacteriaceae} (2, 7, 14) and that it is often synergistic with other \beta-lactam antibiotics against those organisms (8, 15). The interpretation of susceptibility tests has been difficult, however, because of variations in media and inoculum size which markedly affected results. To solve the problem of inconsistency of results, the use of NIH medium (7) was recommended for dilution susceptibility tests because of its "proper" osmolality and conductivity, but acceptance of that recommendation is not practical because it is unknown whether MICs determined in NIH broth are better predictors of in vivo efficacy than MICs determined in other media and because cation-supplemented Mueller-Hinton broth has become the standard medium for the in vitro susceptibility testing of \textit{Enterobacteriaceae} (12). In the present study, which used cation-supplemented Mueller-Hinton broth, \textit{Enterobacteriaceae} fit into three categories of susceptibility to mecillinam. Some, typified by \textit{E. coli} 1924, were highly susceptible regardless of inoculum size, and marked synergy with other \beta-lactam antibiotics was not observed. Others, typified by \textit{K. pneumoniae} 1864, were variably susceptible, depending on inoculum size, and some, typified by \textit{S. marcescens} 1923, were resistant regardless of inoculum size. Under conditions when mecillinam MICs and MBCs were high, there was partial inhibition of growth, morphologically abnormal cells and marked synergy with other \beta-lactam antibiotics at concentrations below MICs, indicating that mecillinam had some effect on all strains of \textit{Enterobacteriaceae} studied.

The only antagonism among \beta-lactam antibiotics observed in the present study was the antagonism of cefamandole and ticarcillin by cefoxitin against cefoxitin-resistant strains of \textit{Enterobacter}. Presumably, cefoxitin induced \beta-lactamase production by those strains (10) which interfered with the activity of cefamandole and ticarcillin.

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