Effects of Azlocillin in Combination with Clavulanic Acid, Sulbactam, and N-Formimidoyl Thienamycin Against β-Lactamase-Producing, Carbenicillin-Resistant Pseudomonas aeruginosa

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We investigated the effects of the combination of azlocillin with the β-lactamase inhibitors clavulanic acid and sulbactam and with N-formimidoyl thienamycin against strains of Pseudomonas aeruginosa with R-factor-mediated carbenicillin resistance. The 10 strains tested (1 R−, 9 R+) were isogenic, except for the presence of individual plasmids determining each of nine plasmid-mediated β-lactamases found in P. aeruginosa. We utilized a checkerboard technique for testing antibiotic combinations. Low concentrations of clavulanic acid produced synergy with azlocillin against the strains producing the TEM-1, TEM-2, PSE-1, PSE-3, and PSE-4 β-lactamases; for the strains producing the OXA-1, OXA-2, OXA-3, and PSE-2 β-lactamases, such synergy was not found. With sulbactam, synergy was demonstrated in all strains except that producing PSE-2 β-lactamase; for several strains, however, the concentration of sulbactam required to produce synergy was substantially higher than that for clavulanic acid. N-Formimidoyl thienamycin was highly active as a single agent against all of the strains, regardless of β-lactamase production. The combination of N-formimidoyl thienamycin and azlocillin produced synergy against only two of the strains tested.

Pseudomonas aeruginosa characteristically produces a chromosomally mediated, inducible β-lactamase which hydrolyzes penicillin G, ampicillin, and cephaloridine (19). Carbenicillin and certain other new β-lactam antibiotics are not hydrolyzed by this enzyme and so inhibit the majority of strains of P. aeruginosa (19). Resistance to carbenicillin may result from the presence of R plasmids that code for the production of constitutive β-lactamases capable of hydrolyzing these drugs (8, 9). Such R plasmids confer resistance not only to carbenicillin, but also to several other β-lactams, such as ticarcillin, azlocillin, piperacillin, and some of the cephalosporins. At least 11 different types of such plasmid-mediated β-lactamases have been described in gram-negative bacteria (12); plasmids determining nine of these enzymes have been found in P. aeruginosa (7, 9; A. Philippeon and G. Jacoby, unpublished data).

Attempts to develop effective chemotherapy for such antibiotic-resistant strains have involved two different strategies. One approach has been the development of β-lactam antibiotics intrinsically resistant to hydrolysis by the plasmid-mediated enzymes. Examples of such compounds include cefotaxime, moxalactam, and N-formimidoyl thienamycin (15). The second approach has been to combine a β-lactam, such as carbenicillin (or one of its newer relatives), with a specific inhibitor of the plasmid-mediated β-lactamases. Two such inhibitors have been previously described: sulbactam (CP-45,899) and clavulanic acid (3, 14).

In this study, we examined the effects of the combination of azlocillin with either of two β-lactamase inhibitors (clavulanic acid or sulbactam) against strains of P. aeruginosa which were carbenicillin resistant by virtue of a plasmid-mediated β-lactamase. Azlocillin was chosen because it is intrinsically more active against P. aeruginosa than either carbenicillin or ticarcillin (4). In addition, we investigated the combination of azlocillin and N-formimidoyl thienamycin against these same strains. This combination was chosen because N-formimidoyl thienamycin has marked intrinsic activity of its own against strains of P. aeruginosa (6, 10, 20), as well as being an inhibitor of a wide variety of plasmid and chromosomally mediated β-lactamases (21).
We tested the antibiotic combinations against a series of 10 *P. aeruginosa* strains (1 R<sup>−</sup>, 9 R<sup>+</sup>) which were isogenic except for the presence of individual plasmids encoding each of nine plasmid-mediated β-lactamases found in *P. aeruginosa*. This approach allowed us to evaluate the effect of the β-lactamase on antibiotic resistance, independent of other factors operative in the host strain. Our results therefore should be generalizable to all *P. aeruginosa* strains that are carbenicillin resistant because of these R-plasmid-mediated β-lactamases.

**MATERIALS AND METHODS**

**Strains.** *P. aeruginosa* PU21, an R<sup>−</sup> strain, was used as a recipient to construct a series of transconjugants, each of which carries a plasmid determining a different β-lactamase. Techniques for constructing these strains have been described (7). In addition to eight plasmids mediating β-lactamases previously documented in *P. aeruginosa*, we also included pMG90, a plasmid determining an OXA-1 β-lactamase recently found in *P. aeruginosa* (A. Philippon and G. Jacoby, unpublished data).

**Antibiotics.** Antimicrobial reference powders from the manufacturers were used for susceptibility testing. Antibiotics were supplied by the following sources: azlocillin (Miles Laboratories, Inc.); carbenicillin and clavulanic acid (Beecham Laboratories); N-formimidoyl thienamycin (Merck Institute for Therapeutic Research); and sulbactam (Pfizer, Inc.). Azlocillin, carbenicillin, and sulbactam were reconstituted in distilled water, as recommended. Clavulanic acid was reconstituted in 100 mM potassium phosphate buffer (pH 7.0), to a final concentration not exceeding 1 mg/ml. N-Formimidoyl thienamycin was reconstituted in 10 mM potassium phosphate buffer (pH 7.2), to a final concentration not exceeding 2 mg/ml. All antibiotic solutions were prepared fresh each day.

**Media.** Susceptibility testing was done in cation-supplemented Mueller-Hinton broth, pH 7.4 (BBL Microbiology Systems). Stock solutions of magnesium chloride and calcium chloride were sterilized by membrane filtration and stored at 4°C. Mueller-Hinton broth was supplemented with 50 mg of elemental calcium per liter and 25 mg of elemental magnesium per liter.

**Susceptibility testing.** The minimal inhibitory concentration (MIC) of carbenicillin for each strain was determined by the standard broth dilution technique (22). Serial twofold dilutions of carbenicillin were made in Mueller-Hinton broth (1 ml), and a standardized inoculum was added (1 ml) to produce a final volume of 2 ml in each tube; the final carbenicillin concentrations ranged from 16,384 to 32 μg/ml. Tubes were incubated at 35°C for 16 to 20 h, and the lowest concentration of antibiotic preventing visible growth was taken as the MIC. Tubes that showed no visible growth were subcultured onto brucella agar plates containing 5% horse blood, utilizing a calibrated 10-μl loop. These plates were incubated at 35°C for 16 to 20 h, and the minimal bactericidal concentration (MBC) was taken as the lowest concentration of antibiotic producing greater than 99.99% killing of the original inoculum (16). Standardization of the inoculum was achieved by diluting an overnight culture of the test strain into Mueller-Hinton broth and reincubating at 35°C. When the turbidity of the actively growing broth culture was visually comparable to the 0.5 McFarland standard, it was further diluted 1:200 in Mueller-Hinton broth and used as the inoculum. The final inoculum was 10<sup>6</sup> to 10<sup>8</sup> colony-forming units.

The MICs of azlocillin, clavulanic acid, sulbactam, and N-formimidoyl thienamycin, as well as the interactions of azlocillin with each of the other three antibiotics, were determined in a checkerboard broth dilution matrix (13 by 13). The concentrations of azlocillin used in each matrix ranged from 2.048 to 1 μg/ml; the concentrations of the second antibiotic ranged from 128 to 0.06 μg/ml. In the first column of each matrix, sterile broth was used in place of azlocillin to allow determination of the MIC of the other antibiotic by itself. Similarly, in the first row of each matrix, sterile broth was added in place of the second antibiotic to allow determination of the azlocillin MIC. Each tube in the matrix was constructed by adding 0.5 ml of an appropriate concentration of azlocillin in Mueller-Hinton broth to 0.5 ml of an appropriate concentration of the second antibiotic; 1 ml of the standardized inoculum was then added to all tubes for a final volume of 2 ml. The inoculum size for all of the tubes in a given checkerboard matrix was therefore identical. Recordings of MICs and MBCs were done as described above. Azlocillin was tested in combination with each of the three other antibiotics against each of the 10 strains to be evaluated.

**Definition of synergy and antagonism.** Synergy in the checkerboard matrix was said to be present if the MICs of the drugs in combination were fourfold or more lower than the MICs of each drug tested individually. Antagonism of drug A by drug B was said to be present if the MIC of drug A was fourfold or more higher in the presence of drug B than in its absence. Mutual antagonism was said to be present if both drug A and drug B antagonized each other. A neutral or indifferent effect was said to be present if the combination produced neither synergy nor antagonism.

**RESULTS**

**MICs.** The MICs of the individual antibiotics against the 10 strains tested are shown in Table 1. The R<sup>−</sup> PU21 strain was carbenicillin susceptible. The plasmid-containing strains were carbenicillin resistant. Strains producing OXA-1, OXA-2, OXA-3, and PSE-2 β-lactamases had lower levels of carbenicillin resistance than strains producing the other enzymes. All of the carbenicillin-resistant strains were also resistant to azlocillin, but the level of azlocillin resistance was substantially lower. Neither clavulanic acid nor sulbactam as single agents inhibited any strains at concentrations of <128 μg/ml. N-Formimidoyl thienamycin, in contrast, was highly effective against all of the strains; susceptibility to this agent was not affected by the presence of plasmid-mediated β-lactamases.

**MBCs.** The MBCs of the antibiotics tested are also shown in Table 1. The MBC for carbenicillin was generally two to four times higher than
TABLE 1. Antimicrobial susceptibility of plasmid-containing strains of *P. aeruginosa* PU21

| Plasmid | β-Lactamase type | MIC (MBC) (μg/ml) |
|---------|------------------|------------------|
|         | Carbenicillin    | Azlocillin       | Clavulanic acid | Sulbactam | N-Formimidoyl thienamycin |
| R⁻      | 64 (128)         | 32 (>2,048)      | 128            | >128      | 2 (64) |
| R2      | 16,384 (>16,384) | 512 (>2,048)     | 128            | >128      | 2 (64) |
| RP1     | 16,384 (>16,384) | 512 (>2,048)     | 128            | >128      | 2 (16) |
| pMG90   | OXA-1            | 1,024 (2,048)    | 128            | >128      | 4 (ND)*a |
| pMG40   | OXA-2            | 512 (1,024)      | 256 (>2,048)   | >128      | 2 (64) |
| RJP64   | OXA-3            | 4,096 (>16,384)  | 512 (>2,048)   | >128      | 2 (16) |
| RPL11   | PSE-1            | 16,384 (>16,384) | 256 (>2,048)   | >128      | 4 (32) |
| R151    | PSE-2            | 1,024 (1,024)    | 128 (>2,048)   | >128      | 2 (64) |
| Rms149  | PSE-3            | 16,384 (>16,384) | 128 (>2,048)   | >128      | 2 (64) |
| pMG19   | PSE-4            | 16,384 (>16,384) | 512 (>2,048)   | >128      | 2 (64) |

*ND* Not done.

the corresponding MIC. For azlocillin, however, the MBCs were all in excess of the highest concentration tested. The MBCs of N-formimidoyl thienamycin ranged from 16 to 64 μg/ml.

**Effects of azlocillin in combination with three other antibiotics.** The interactions of azlocillin with the other antibiotics are shown in Table 2. Clavulanic acid produced antagonism when combined with azlocillin against the R⁻ PU21 strain. In contrast, against the plasmid-containing strains, low concentrations of clavulanic acid produced synergy with azlocillin in the presence of TEM-1, TEM-2, PSE-1, PSE-3, and PSE-4 β-lactamases. Synergy resulted with the OXA-3-producing strain only at high concentrations of clavulanic acid, and the remainder of the plasmid-containing strains showed no synergy. Interestingly, the synergism seen between azlocillin and low concentrations of clavulanic acid against the PSE-1, PSE-3, and PSE-4 strains disappeared as higher concentrations of clavulanic acid were used.

Sulbactam produced a neutral effect when combined with azlocillin against the R⁻ PU21 strain. Against the plasmid-containing strains, however, sulbactam produced synergy with azlocillin against all of the strains tested, with the exception of the PSE-2-producing strain. For the TEM-1-, TEM-2-, PSE-1-, PSE-3-, and PSE-4-producing strains, the concentration of sulbactam required to produce synergy with azlocillin was substantially higher than that for clavulanic acid. For the OXA-1-, OXA-2-, and OXA-3-producing strains, however, sulbactam was more effective in producing synergy with azlocillin than was clavulanic acid. No paradoxical loss of synergism at higher concentrations of sulbactam was observed.

*N*-Formimidoyl thienamycin demonstrated striking antagonism when combined with azlocillin against the R⁻ PU21 strain; even at the lowest concentration of *N*-formimidoyl thienamycin tested (0.06 μg/ml), the MIC of azlocillin in the combination was increased eightfold over that with azlocillin alone. Against the plasmid-containing strains, *N*-formimidoyl thienamycin was highly active as a single agent. When tested together with azlocillin, the combination of antibiotics generally produced a neutral effect, although the interactions for two strains just met our definition of synergy, and in another strain, there was antagonism of azlocillin by *N*-formimidoyl thienamycin. In no circumstance did azlocillin produce antagonism of the thienamycin derivative.

The MBC of azlocillin by itself for each of the 10 strains tested was in excess of 2,048 μg/ml. The MBC of azlocillin in the presence of each of the three other antibiotics still remained in excess of 2,048 μg/ml for all strains, even with strains for which azlocillin and the second antibiotic were synergistic. Synergy in this study, therefore, was apparent in the MICs of the antibiotics, but not in the MBCs.

**DISCUSSION**

Strains of *P. aeruginosa* produce a chromosomally mediated inducible β-lactamase capable of hydrolyzing a variety of β-lactam antibiotics (19). Carbenicillin resists hydrolysis by this enzyme and is active against most isolates of *P. aeruginosa*. Resistance to carbenicillin, however, is not uncommon in *P. aeruginosa* and occurs in as many as 15% of hospital isolates (11). This resistance may result from the presence of an R plasmid encoding a constitutive β-lactamase capable of inactivating carbenicillin (8, 9).

Azlocillin is a newer semisynthetic penicillin which is intrinsically more active than carbenicillin or ticarcillin against strains of *P. aeruginosa* (4). As with carbenicillin, however, azlocillin is susceptible to hydrolysis by the plasmid-
mediated β-lactamases found in *P. aeruginosa* (Table 1), and such plasmids may mediate azlocillin resistance. In addition, azlocillin is partially susceptible to hydrolysis by the chromosomally determined inducible β-lactamase (1). This observation may explain, in part, the marked inoculum effect seen when testing strains of *P. aeruginosa* for azlocillin susceptibility (1, 23).

Clavulanic acid and sulbactam have very little intrinsic activity against strains of *P. aeruginosa*, but are potent inhibitors of a variety of plasmid-mediated β-lactamases (3, 14). Hence, these compounds might be expected to enhance the activity of carbenicillin or azlocillin against strains possessing β-lactamase-producing R plasmids. The chromosomal β-lactamase of *P. aeruginosa*, on the other hand, is little affected by either clavulanic acid or sulbactam (3, 14). Hence these compounds should not be expected to enhance the activity of β-lactams, such as penicillin G, ampicillin, and cephaloridine, which are inactivated by this chromosomal enzyme.

Clavulanic acid has been shown to enhance the activity of carbenicillin against strains of *P. aeruginosa* producing TEM-1, PSE-1, and PSE-4 β-lactamases (17). In addition, clavulanic acid enhanced the activity of ticarcillin and azlocillin against these same strains (A. Philippon, G. C. Paul, S. Benredjeb, R. Labia, and P. A. Nevot, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no. 603, 1980). It did not, however, enhance the activity of these antibiotics against strains containing OXA-1 or OXA-3 enzymes.

In the present study, we tested a series of strains of *P. aeruginosa*, isogenic except for the presence of R plasmids encoding each of nine β-lactamases. In accord with previous studies, we found that low concentrations of clavulanic acid produced synergy with azlocillin against strains producing the TEM-1, TEM-2, PSE-1, PSE-3, and PSE-4 enzymes (Table 2). For these strains, increasing concentrations of clavulanic acid lowered the MIC of azlocillin to that of the R" host; in contrast, clavulanic acid had no effect on the MBC of azlocillin. For the strain producing the OXA-3 enzyme, a minimum of 16 μg of clavulanic acid per ml was necessary to produce synergy with azlocillin; for the strains producing the remaining β-lactamases (OXA-1, OXA-2, and PSE-2), no synergy was demonstrated.

The observation that clavulanic acid produced antagonism of azlocillin in the R" strain is intriguing. The antagonism of cefamandole by cefoxitin in *Enterobacteriaceae* has been attributed to the induction by cefoxitin of β-lactamases capable of hydrolyzing cefamandole (18). Clavulanic acid has been shown to be a potent inducer of the β-lactamase of *Enterobacter cloacae* (13). Clavulanic acid may also be an inducer of the chromosomal β-lactamase of *P. aeruginosa*; enhanced hydrolysis of azlocillin by this enzyme might, therefore, account for the antagonism that we observed. Further study will be necessary to clarify this point. A similar phenomenon may be operative for the PSE-1-, PSE-3-, and PSE-4-producing strains, which showed striking synergy between azlocillin and low concentrations of clavulanic acid, but disappearance of such synergy when higher concentrations of clavulanic acid were used. These particular strains were the most susceptible to clavulanic acid-azlocillin synergy, and perhaps
low concentrations of clavulanic acid totally neutralized the presence of the plasmid-mediated β-lactamase. Higher concentrations of clavulanic acid, therefore, might produce the same antagonism as seen in the R' strain.

Subbactam produced synergy with azlocillin against all of the plasmid-containing strains of *P. aeruginosa* tested, with the exception of the PSE-2-producing strain. For several of the strains, however, relatively high concentrations of the inhibitor were necessary to produce such synergy. The lowered potency of subbactam as a β-lactamase inhibitor in comparison to clavulanic acid is in accord with previous studies (3, 5). As with clavulanic acid, subbactam did not affect the MBC of azlocillin, even for strains showing synergy on MIC testing.

*N*-Formimidoyl thienamycin proved to be highly active against the 10 strains of *P. aeruginosa* tested, in agreement with several previous studies (2, 6, 10, 20). The activity of this antibiotic was not affected by the presence of any of the plasmid-mediated β-lactamases. This is also in agreement with previous studies (21). The MBC of *N*-formimidoyl thienamycin ranged from 16 to 64 μg/ml and was similarly unaffected by the presence of the R plasmids.

When combined with azlocillin against the R− PU21 strain, *N*-formimidoyl thienamycin produced a striking antagonism of azlocillin inhibition. As discussed for clavulanic acid, this may represent the induction of the chromosomal β-lactamase by the thienamycin derivative or a different mechanism yet to be defined. For the R' strains, no consistent synergy could be demonstrated between azlocillin and *N*-formimidoyl thienamycin.

Based on the results of this study, *N*-formimidoyl thienamycin appears to be a promising agent in the treatment of carbenicillin-resistant *P. aeruginosa* infections. Clavulanic acid and subbactam might have selective uses, combined with azlocillin or a similar agent, but in vitro testing would be necessary to ensure their efficacy. Further work is needed to elucidate the mechanism of antagonism found in this study between azlocillin and both clavulanic acid and *N*-formimidoyl thienamycin.

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