In Vitro Activity of Piperacillin, Ticarcillin, and Mezlocillin Alone and in Combination with Aminoglycosides against Pseudomonas aeruginosa

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A total of 103 isolates of Pseudomonas aeruginosa were studied to compare the in vitro effectiveness of three beta-lactam antibiotics (piperacillin, ticarcillin, and mezlocillin) when used alone and in combination with four aminoglycosides (tobramycin, gentamicin, amikacin, and netilmicin). All drugs were tested as single agents against a standard inoculum (5 × 10^8 CFU/ml). The three antipseudomonal penicillins were also tested against the isolates at a higher inoculum concentration (10^7 CFU/ml). Synergy testing was performed by the two-dimensional checkerboard method and was defined by (i) a fractional bactericidal index of ≤0.5 and (ii) bacterial killing accomplished at antibiotic concentrations no greater than those achievable in serum. All combinations were assessed for synergy. The degree of synergy was further analyzed by dividing the isolates into groups based on their susceptibility and resistance to the individual agents in the combination. The overall effectiveness of the various aminoglycoside-antipseudomonal penicillin combinations was assessed regarding their ability to kill the isolates either as single agents or through synergy. Piperacillin was the most active antipseudomonal penicillin, and tobramycin and amikacin were the most active aminoglycosides when used as single agents. When tested against isolates at a higher inoculum concentration, ticarcillin was significantly more active than the other beta-lactams. The highest degree of overall synergy was noted with gentamicin-ticarcillin (78.2% of strains) and amikacin-piperacillin (77% of strains). When assessed for overall effectiveness, all combinations containing amikacin were the most active. The combination of amikacin-piperacillin was the most effective, with activity against 96% of all isolates.

The newer semisynthetic penicillins have been shown by previous investigators to demonstrate increased antibacterial activity against a wide range of organisms (3, 17, 26, 29), including strains of members of the family Enterobacteriaceae and Pseudomonas aeruginosa that are multiply resistant to other antimicrobial agents (3, 10). Moreover, these antimicrobial agents have been found to be especially useful when combined with an aminoglycoside (AMG) in the treatment of severe infections with gram-negative bacilli (1, 2), perhaps reflecting their in vitro synergistic activity (10, 11, 13, 15, 16, 19, 24).

The purpose of this study was to compare the in vitro synergistic activity of the four most commonly used AMGs with that of three of the newer semisynthetic antipseudomonal penicillins (APP) against 103 hospital strains of P. aeruginosa. Only results demonstrating synergy at clinically attainable concentrations in serum of both antimicrobial agents were considered to be synergistic. In addition, we examined each drug combination for overall effectiveness in vitro. Analyses of these results took into consideration synergistic responses as well as single-drug activities.

MATeRIALS AND METHODS

Organisms. Altogether, 103 P. aeruginosa strains were obtained from the clinical microbiology laboratories of the University of Alabama at Birmingham Medical Center and the Birmingham Veterans Administration Medical Center. Before being tested, each Pseudomonas isolate was streaked onto a sheep blood agar plate and incubated overnight at 35°C.

To assess the epidemiology of the P. aeruginosa strains used in this study, we examined the hospital records of each patient from whom an isolate was obtained and assigned the underlying condition of the patient to a specific category. Ten patients had a malignant disorder, either hematologic or solid tumor, and four patients had undergone renal transplantation. There were 33 patients with a moderately severe underlying medical condition, including serious trauma in 29 (12 of whom were paraplegic), chronic obstructive pulmonary disease in 12, severe heart failure in 5, renal failure in 3, liver disease in 1, and cerebrovascular disease in 3. There were 6 patients with burns, 18 with miscellaneous conditions, and 3 with apparently unrelated disorders. In only two patients from whom a P. aeruginosa isolate was obtained was there no underlying disorder.

Antibiotics and media. Laboratory standard powders of piperacillin (Lederle Laboratories, Pearl River, N.Y.), mezlocillin (Miles Pharmaceuticals, West Haven, Conn.), ticarcillin (Beecham Laboratories, Bristol, Tenn.), gentamicin and netilmicin (Schering Corp., Kenilworth, N.J.), tobramycin (Eli Lilly & Co., Indianapolis, Ind.), and amikacin (Bristol Laboratories, Syracuse, N.Y.) were obtained. Stock solutions of piperacillin, mezlocillin, and ticarcillin were prepared in concentrations of 10,000 μg/ml, and stock solutions of gentamicin, netilmicin, tobramycin, and amikacin were prepared in concentrations of 1,000 μg/ml. All stock solutions were stored frozen in 10-ml aliquots at −70°C. The potency of these antibiotics was checked before use with reference cultures of known susceptibility (P. aeruginosa ATCC 27853, Staphylococcus aureus ATCC 29213, and Escherichia coli ATCC 25922). Final working concentrations of the antibiotics were as follows: piperacillin, mezlocillin,
and ticarcillin at 1 to 512 µg/ml; gentamicin, tobramycin, and netilmicin at 0.5 to 32 µg/ml; and amikacin at 1 to 64 µg/ml.

All testing was done with cation-supplemented Mueller-Hinton (CSMH) broth obtained from GIBCO Laboratories (Lawrence, Mass.). The calcium content ranged from 41 to 51 mg/liter; the magnesium concentration ranged from 25 to 28 mg/liter.

Methods. Synergy testing was performed by the two-dimensional checkerboard method with the standard microdilution broth technique (21). Microdilution plates (Belloco Glass, Inc., Vineland, N.J.) were prepared with a Minispense II automatic dispenser (Sandy Springs Instrument Co., Ijamsville, Md.). A total volume of 0.1 ml of each antibiotic solution, antibiotic combination, or broth control was dispensed into each well of a 96-well microdilution plate. Plates were stored frozen at –70°C until ready for use.

For testing purposes, portions of four to five colonies of each Pseudomonas isolate were inoculated into 5 ml of CSMH broth and incubated at 35°C until the turbidity was that of a 0.5 McFarland of CSMH broth and incubated at 35°C for 18 to 20 h before reading.

After incubation, the microdilution plates were examined for growth with a mirror reader (Dynatech Laboratories, Inc., Alexandria, Va.). All growth was recorded with a template, and the APP and AMG MICs were recorded.

We determined bactericidal results by using a semiautomatic pipette (Rainin Instrument Co., Woburn, Mass.) to remove 0.01 ml from each well showing no growth and from the final well in each row showing visible growth and then streaking the samples onto antibiotic-free CSMH agar.

The following steps were taken to eliminate the possibility of antibiotic carry-over in MBC testing. (i) Mueller-Hinton agar at pH 5.5 was used to inactivate any AMG carry-over (25). (ii) A broad-spectrum beta-lactamase solution (0.5 µl; International Enzymes, Inc., Fallbrook, Calif.) was streaked on the surface of a Mueller-Hinton agar (pH 5.5) plate (100 by 15 mm) and allowed to dry before inoculation for MBC testing to eliminate beta-lactam antibiotic carry-over. All plates for bactericidal determinations were incubated 18 to 20 h, and growth was recorded with a template; the MBCs were noted and recorded for the specific APPs and AMGs.

The MBC was defined as the lowest concentration of an antimicrobial agent which resulted in at least a 99.9% killing of the original inoculum. With Pearson’s rejection value at an initial inoculum size of 5 × 10^8 CFU/ml, a pipette error of ±5%, test sensitivity at 96%, and test specificity at 99%, a colony count of ≤11 (rejection value) would represent a 99.9% kill (22).

The definition of synergy was established with the following criteria: (i) a fractional bactericidal index (ΣFBC) of ≤0.5 when calculated by the formula ΣFBC = (MBC combination A/MBC alone A) + (MBC combination B/MBC alone B), in which A is APP and B is AMG (4, 14); (ii) bactericidal results of antibiotic combinations used in determining the ΣFBC required that the concentrations be within the range of a clinically attainable concentration in serum for each antibiotic in the pair tested (15). For the purpose of this study, antibiotic concentrations approximating clinically achievable ranges were mezlocillin, ≤64 µg/ml; piperacillin, ≤64 µg/ml; ticarcillin, ≤64 µg/ml; amikacin ≥16 µg/ml; gentamicin, ≤4 µg/ml; netilmicin, ≤8 µg/ml; and tobramycin, ≤4 µg/ml (21).

High-inoculum-concentration testing for MICs and MBCs. MICs and MBCs were determined for all 103 Pseudomonas isolates against piperacillin, mezlocillin, and ticarcillin with an initial inoculum concentration of approximately 10^7 CFU/ml.

Microdilution plates were prepared for single-agent testing by dispensing antibiotic dilutions as described above, except that for higher-inoculum-concentration testing, 0.05 ml of antibiotic solution was dispensed into each well of the 96-well plate.

Each organism to be tested was prepared as follows. Portions of four to five colonies of each Pseudomonas isolate were inoculated into 5 ml of CSMH broth and incubated overnight at 35°C to achieve an inoculum of approximately 10^7 CFU/ml. A dilution of 1:50 was then made. A sample (50 µl) of the adjusted inoculum was added to each appropriate well of the thawed microdilution plate with a calibrated pipette dropper (Dynatech Laboratories), resulting in a final well volume of 0.1 ml and a final inoculum size of approximately 10^6 CFU/ml. Inoculum verification was performed as described above.

MBC testing was also performed as described above, with the MBC defined by 99.9% killing of the original inoculum. With the rejection value of Pearson et al. (22) at an inoculum size of 10^7 CFU/ml, a pipette error of ±5%, test sensitivity at >99%, and test specificity at >99%, a count of ≤227 CFU would represent a 99.9% kill.

Statistical methodology. Comparisons of the inhibitory and bactericidal activities of the three APPs and four AMGs were made with both an overall chi-square (χ^2) test (9) and an analysis of variance of the dichotomous data (7, 18). Because the results of each test were essentially the same, the mean square error from the analysis of variance was used in Duncan’s multiple range test to make separate comparisons among the agents. Similar analyses were made for all subsequent multiple comparisons of the synergistic killing data. In all of these comparisons, the results of the overall χ^2 test and analysis of variance remained essentially equal.

RESULTS

A total of 103 strains of P. aeruginosa were recovered from 96 patients. There were no duplicate isolates from the same patients. The age range of the patients varied from the same patients. The age range of the patients varied from 18 to 88 years, with a mean of 50.7 and a median of 53 years. Approximately equal numbers of strains were recovered from the respiratory tract (38.8%) and from blood cultures (1.9%), intravenous lines (1.0%), wound infections (11.7%), stools (2.9%), burn wound sepsis (1.9%), other body fluids (1.0%), and miscellaneous cultures (3.9%). Two-thirds of the patients had received antimicrobial agents before the date on which the culture for P. aeruginosa was collected. Some patients were still receiving antibiotics at the time the cultures were obtained.

The MBC of the four AMGs and the three APPs against the 103 strains of P. aeruginosa are shown in Table 1. Piperacillin was the most active APP, while tobramycin was the most active AMG by weight; however, because the peak levels of amikacin in serum are generally three times those of

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The other AMGs tested, amikacin was essentially as active as tobramycin. As a group, piperacillin, amikacin, and tobramycin used as single agents were significantly (P < 0.05) more effective than netilmicin and gentamicin.

Among the APPs, piperacillin had the lowest MBC for 50% of the strains tested, but the MBC for 90% of the strains tested was greater than that of ticarcillin (Table 1). Among the AMGs, tobramycin was bactericidal at the lowest concentration but again was not superior to amikacin when relative drug levels in serum were considered.

The data from tests evaluating the APPs against a high inoculum concentration (10^5 CFU/ml) of bacteria are shown in Table 2. There was a striking increase in the MICs for 50% of the strains tested with mezlocillin and piperacillin that was not evident in results with ticarcillin (P < 0.05), although the MIC for 50% of the strains tested with ticarcillin was still somewhat higher than that with an inoculum of 5 x 10^5 CFU/ml. The MBC data also demonstrated killing at lower concentrations (P < 0.05) by ticarcillin than by mezlocillin or piperacillin at the higher inoculum concentration.

The results of tests for synergistic killing with combinations of APPs and AMGs against the 103 isolates of P. aeruginosa are shown in Table 3. A few Pseudomonas strains with bactericidal endpoints too low to be used in the SBFCV determination were excluded from the analysis. Concentrations of antimicrobial agents were chosen in accordance with what can reasonably be attained in human serum. The combinations of gentamicin-ticarcillin and amikacin-piperacillin, with 78.2 and 77.0% killing, respectively, demonstrated the highest degree of synergy, while all combinations that included tobramycin generated the lowest amount of synergistic activity (see Discussion).

The overall susceptibility of the Pseudomonas isolates to individual drugs or their synergistic combinations is also shown in Table 3. When analyzed in this fashion, amikacin-piperacillin, amikacin-mezlocillin, and amikacin-ticarcillin were clearly superior to netilmicin-mezlocillin and gentamicin-mezlocillin (P < 0.05). The effect of the inherent antimicrobial activity of single agents on the ability of the combinations to achieve synergy is assessed in Table 4. Four categories were used in this analysis: (i) susceptible to both the APP and the AMG, (ii) susceptible to the AMG but resistant to the APP, (iii) susceptible to the APP but resistant to the AMG, and (iv) resistant to both the APP and the AMG. Differences of statistical significance were achieved in only two of the four categories. In category i (susceptible to both APP and AMG), the netilmicin-mezlocillin combination demonstrated significantly more synergy than did tobramycin-ticarcillin (96.0% versus 56.3%; P < 0.05). In categories ii and iii, no significant differences were revealed among the combinations. The degree of synergy achieved in category iv, in which the isolates were resistant to both agents alone, demonstrated significantly more activity for amikacin-piperacillin, netilmicin-mezlocillin, netilmicin-ticarcillin, and gentamicin-ticarcillin than for netilmicin-piperacillin, tobramycin-mezlocillin, and tobramycin-ticarcillin (P < 0.05).

**DISCUSSION**

The patients from whom these isolates were obtained are representative of our current hospital population. Only 2 patients among the entire 96 had no underlying medical problems, and a number of patients had severe disorders such as chronic obstructive pulmonary disease, malignancies, or cardiovascular disease. There were approximately an equal number of men and women.

We found piperacillin to be the most active APP when tested for both MIC and MBC, and these data agree with those of other investigators (3, 10, 17, 24, 26, 29). Tobramycin appeared to be the most active AMG by weight against
The strains of *P. aeruginosa* tested. However, amikacin was equally as effective as tobramycin when attainable drug levels in serum were used in the evaluation.

When a high inoculum concentration was used, ticarcillin was strikingly superior to mezlocillin and piperacillin. The inoculum effect of the newer beta-lactams also has been noted by other authors (6, 8, 17, 24; C. C. Sanders, W. E. Sanders, Jr., and R. V. Goering, Proc. 13th Int. Congr. Chemother., 1983). At 10^7 CFU/ml, MIC determinations were difficult; endpoints were not always distinct, although there was a definite increase in turbidity after 18 to 20 h of incubation. This phenomenon was noted also by Eng et al. (8) and was found to be associated with the formation of giant aberrant and filamentous metabolically active bacillary forms and thus with increased cell mass when the organisms were exposed to antibiotics such as piperacillin that were not rapidly bactericidal.

When the two-dimensional checkerboard technique was used for the determination of synergistic killing, gentamicin-ticarcillin and amikacin-piperacillin were found to be the most synergistic. Other combinations were less active in this test, but only the combinations containing tobramycin were statistically different from gentamicin-ticarcillin and amikacin-piperacillin. This is most likely due to the high level of bactericidal activity of tobramycin when used as a single agent, thereby making the criteria for synergistic killing more difficult to achieve. Indeed, when analyzed in terms of overall susceptibility to single agents or their synergy with others, combinations containing tobramycin were almost as effective as the statistically superior combinations containing amikacin.

There have been previous studies purporting to show synergistic killing by combinations of AMGs and beta-lactams for strains which were resistant to one or the other agent of the combination or, in some cases, to both agents when used alone (19). Our data indicate that synergistic killing was more likely when the isolate was susceptible to one or both of the agents and was less likely when it was resistant to both antimicrobial agents. However, since these drugs are often used as empiric therapy in patients who are likely to be infected with potentially resistant organisms acquired in a hospital setting, the combinations that exhibit synergy when the isolates are resistant to both antimicrobial agents individually appear to be the most important. In that category in our study, the combinations killing the most strains were amikacin-piperacillin (67.7%), netilmicin-mezlocillin (58.1%), netilmicin-ticarcillin (57.1%), and gentamicin-ticarcillin (55.2%).

Our study did not allow us to predict with any amount of confidence whether a combination of an APP and an AMG would exhibit synergistic killing with a given *Pseudomonas* strain when the isolate was susceptible to one or both of the agents or resistant to both. However, by assessing the data

### TABLE 3. Comparative synergistic killing activity of 12 antibiotic combinations against *P. aeruginosa*

<table>
<thead>
<tr>
<th>Antibiotic combination</th>
<th>No. of strains</th>
<th>Strains killed synergistically</th>
<th>Strains susceptible to either drug alone or to synergistic effects of combination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of strains</td>
<td>%</td>
<td>No. of strains</td>
</tr>
<tr>
<td>Amikacin-mezlocillin</td>
<td>100</td>
<td>74</td>
<td>74.0</td>
</tr>
<tr>
<td>Amikacin-piperacillin</td>
<td>100</td>
<td>77</td>
<td>77.0</td>
</tr>
<tr>
<td>Gentamicin-mezlocillin</td>
<td>101</td>
<td>69</td>
<td>68.3</td>
</tr>
<tr>
<td>Gentamicin-piperacillin</td>
<td>101</td>
<td>72</td>
<td>71.3</td>
</tr>
<tr>
<td>Gentamicin-ticarcillin</td>
<td>101</td>
<td>79</td>
<td>78.2</td>
</tr>
<tr>
<td>Netilmicin-mezlocillin</td>
<td>102</td>
<td>76</td>
<td>74.5</td>
</tr>
<tr>
<td>Netilmicin-piperacillin</td>
<td>100</td>
<td>70</td>
<td>70.0</td>
</tr>
<tr>
<td>Tobramycin-mezlocillin</td>
<td>100</td>
<td>74</td>
<td>74.0</td>
</tr>
<tr>
<td>Tobramycin-piperacillin</td>
<td>97</td>
<td>63</td>
<td>64.9</td>
</tr>
<tr>
<td>Tobramycin-ticarcillin</td>
<td>102</td>
<td>67</td>
<td>67.5</td>
</tr>
<tr>
<td>Tobramycin-ticarcillin</td>
<td>95</td>
<td>50</td>
<td>52.6</td>
</tr>
</tbody>
</table>

* Excluding strains in each combination with bactericidal endpoints too low to evaluate the ΣFBC.
* Total number of strains tested: 103.
* Overall P < 0.02; all combinations with amikacin were significantly better than netilmicin-mezlocillin and gentamicin-mezlocillin (P < 0.05) by analysis of variance and Duncan's multiple range test.

### TABLE 4. Susceptibility and resistance of isolates to single agents versus APP and AMG combinations

<table>
<thead>
<tr>
<th>Antibiotic combination</th>
<th>No. (%) of strains affected synergistically of those:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible to both APP and AMG individually</td>
</tr>
<tr>
<td>Amikacin-mezlocillin</td>
<td>44 (80.0)</td>
</tr>
<tr>
<td>Amikacin-piperacillin</td>
<td>55 (79.7)</td>
</tr>
<tr>
<td>Gentamicin-mezlocillin</td>
<td>49 (74.2)</td>
</tr>
<tr>
<td>Gentamicin-ticarcillin</td>
<td>10 (69.6)</td>
</tr>
<tr>
<td>Gentamicin-ticarcillin</td>
<td>35 (77.8)</td>
</tr>
<tr>
<td>Netilmicin-mezlocillin</td>
<td>30 (85.7)</td>
</tr>
<tr>
<td>Netilmicin-piperacillin</td>
<td>24 (96.0)</td>
</tr>
<tr>
<td>Netilmicin-ticarcillin</td>
<td>37 (82.2)</td>
</tr>
<tr>
<td>Tobramycin-mezlocillin</td>
<td>26 (76.5)</td>
</tr>
<tr>
<td>Tobramycin-piperacillin</td>
<td>33 (71.7)</td>
</tr>
<tr>
<td>Tobramycin-ticarcillin</td>
<td>46 (66.7)</td>
</tr>
<tr>
<td>Tobramycin-ticarcillin</td>
<td>56 (56.3)</td>
</tr>
</tbody>
</table>

* All results for synergy are based on bactericidal data.
* No significant differences by analysis of variance and Duncan's multiple range test (P > 0.05).
* Overall P < 0.002; amikacin-piperacillin, netilmicin-mezlocillin, netilmicin-ticarcillin, and gentamicin-ticarcillin were significantly better than netilmicin-piperacillin, tobramycin-mezlocillin, and tobramycin-ticarcillin (P < 0.05) by analysis of variance and Duncan's multiple range test.
* Overall P < 0.06; netilmicin-mezlocillin was significantly better than tobramycin-ticarcillin (P < 0.05) by analysis of variance and Duncan's multiple range test.
obtained in our study, it was interesting to note that piperacillin and amikacin were consistently the most active, both as single antimicrobial agents and in combination. Any comment on the apparent superiority of piperacillin should be tempered with the fact that azlocillin, another APP, was not tested in this trial.

The excellent in vitro activity of the amikacin-piperacillin combination may have interesting consequences in vivo. It has been shown that APPs interact chemically with AMGs, resulting in inactivation of both agents (5, 12, 23, 28). This can be especially significant in patients with severe renal failure, since accumulation of APPs, as well as AMGs, can occur in serum, resulting in significant inactivation of both agents (5, 23). However, amikacin demonstrates the greatest stability in vitro when combined with various concentrations of beta-lactams (12), as well as resistance to inactivation in vivo (5). In addition, piperacillin has been shown to have an increased rate of nonrenal elimination in renal failure patients (27, 28). The superiority of the combination in vitro, as well as the stability of amikacin and the pharmacokinetics of piperacillin, could be clinically significant in the treatment of patients with renal failure with severe gram-negative infections.

Although true clinical efficacy is assessable only in vivo, it is useful to know in a given patient whether a drug combination will provide bactericidal activity in serum when the combination is given in the usual dosages. Even though our studies were not done with serum, we anticipate that when the combination is synergistic in broth, it probably will demonstrate activity in serum as well.

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LITERATURE CITED

27. Thompson, M. I. B., M. E. Russo, J. M. Matsen, and E.
