Semisynthetic Penicillin 6-[D(--)-α-Carboxy-3-Thienylacetamido] Penicillanic Acid Active Against Pseudomonas In Vitro

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The activity of 6-[D(--)-α-carboxy-3-thienylacetamido] penicillanic acid, BRL2288, was determined against Pseudomonas aeruginosa and various gram-negative bacilli. The majority of Pseudomonas strains (89%) were inhibited by 100 μg of the antibiotic per ml. BRL2288 is twofold more active than carbenicillin against Pseudomonas at 100 μg/ml or less. Among Enterobacteriaceae tested, 87% Enterobacter and 87% of Proteus mirabilis strains were inhibited by 25 μg/ml or less. Indole-positive Proteus were inhibited by 10 μg/ml or less. Fifty-five per cent of ampicillin-resistant Escherichia coli were inhibited by 100 μg/ml. Klebsiella were uniformly resistant. BRL2288 is not hydrolyzed by most resistant Pseudomonas, but it is destroyed by the β-lactamases of E. coli and P. mirabilis. The antibiotic shows synergy with gentamicin but not with penicillinase-resistant penicillins such as cloxacillin. Activity of BRL2288 against gram-positive organisms is two- to eightfold less than that of ampicillin or benzylpenicillin G.

Infections caused by gram-negative bacilli have assumed the major role in causing hospital infections, replacing the staphylococcus. Debilitated patients have been particularly susceptible to Pseudomonas aeruginosa infections. Polymyxins (B and E) and gentamicin have had restricted usefulness because of serious renal and otic toxicity. Recently, a new semisynthetic penicillin, carbenicillin, has been found to be active against Pseudomonas strains and indole-positive Proteus strains (1). Carbenicillin has been administered in high doses to seriously ill patients with Pseudomonas infections with excellent clinical results (3). However, reports of resistance to carbenicillin have begun to appear (2). We have now examined the in vitro activity of 6-[D(--)-α-carboxy-3-thienylacetamido] penicillanic acid, BRL2288, against a variety of gram-negative bacilli.

MATERIALS AND METHODS

6-[D(--)-α-Carboxy-3-thienylacetamido] penicillanic acid, BRL2288 (Fig. 1), was supplied by Beecham Pharmaceuticals as the dry powder and was stored in a dessicator at 4 C. Fresh solutions were prepared each day in sterile Penassay Broth (Difco) or saline.

The bacteria were isolated from hospitalized patients in the Presbyterian Hospital except for a few selected laboratory strains.

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Susceptibility testing methods. Minimal inhibitory concentration (MIC) values were determined by tube dilution and agar dilution tests. In the tube dilution method, 0.5 ml of a 10⁻⁶ dilution of an overnight culture was used for the inoculum. Incubation was for 18 hr at 35 C. In the agar dilution method the antibiotic was incorporated into Trypticase Soy Agar (BBL). After the plates solidified, the surface was inoculated with an undiluted overnight culture; the end point was read as the lowest concentration that showed visible growth after overnight incubation at 35 C.

Assays for BRL2288 remaining in the supernatant of cultures were performed by using the agar diffusion method in plates (12 inch by 12 inch) with 5-mm wells in the agar. A 5-ml inoculum of an overnight culture of P. aeruginosa (ATCC23389) was added to the Trypticase Soy Agar before pouring the plate. Each plate contained a set of BRL2288 standards in concentrations of 5, 10, 20, and 40 μg/ml. The zone diameter was read with calipers, and a standard curve was constructed for the BRL2288 standard by plotting the logarithm of the drug concentration against zone size. The concentration of antibiotic in samples was calculated from the linear plot.

Test for synergy. A checkerboard arrangement of tests tubes was used to test the effect of BRL2288 and gentamicin. Tubes were arranged in five rows of 12 tubes each. A horizontal row contained the same concentration of BRL2288; a vertical row contained the same concentration of gentamicin. Twofold dilutions of both drugs were used. The initial row of each horizontal and vertical set contained the single drug; the
other rows were the mixture. Antibiotics were prepared fresh for each test. A $10^{-4}$ dilution of an overnight culture was added to each tube (final volume, 1.0 ml) and they were incubated for 18 hr at 35°C. The first clear tube was read as the MIC for the organism. Similar tests of synergy were performed for BRL2288 and cloxacinil.

Growth curves were obtained by growing cultures in 50-ml volumes in Erlenmeyer flasks and removing 1 ml at appropriate times to read the optical density at 600 nm in a Beckman DU spectrophotometer.

Purified Salmonella typhimurium and Escherichia coli penicillinases (6) were used to determine the $K_m$ against BRL2288. The penicillinase assay was a modification of the Novick microdilometric assay (7).

**RESULTS**

The MIC of BRL2288 for *Pseudomonas*, *Proteus*, *Enterobacter*, *Klebsiella*, *E. coli*, and *Serratia* are shown in Fig. 2. The majority of strains were inhibited by 100 μg or less of BRL2288 per ml except for strains of *Klebsiella pneumoniae*, 47% of which were not inhibited by 500 μg/ml. Of the *Pseudomonas* strains tested, 36% were inhibited by 25 μg and 89% by 100 μg of BRL2288 per ml. Eighty per cent of routine clinical isolates of *E. coli* were sensitive to 12.5 μg or less of the antibiotic per ml. Of the ampicillin-resistant *E. coli*, 28% were sensitive to 12.5 μg or less, 38% to 25 μg and 55% to 100 μg of BRL2288 per ml. The sensitivity of *Enterobacter* species to BRL2288 was surprising with 65% sensitive to 12.5 μg/ml and 91% sensitive to 100 μg/ml. *Proteus mirabilis* species fell into two groups, either very sensitive (i.e., less than 6.25 μg/ml) or resistant (i.e., greater than 100 μg/ml). All of the indole-positive *Proteus* strains tested (i.e., morganii, vulgaris, and rettgeri) were sensitive to less than 6.25 μg/ml. Rare *Serratia* strains were sensitive to BRL2288 but the majority of strains were extremely resistant.

The comparison of carbenicillin and BRL2288 (Fig. 3) demonstrates that BRL2288 is consistently one tube, twofold, more active than carbenicillin. This was also seen with *Enterobacter* species but was not seen for *Proteus* or *E. coli* species in which there was rarely even one tube difference in the MIC.

The activity of BRL2288 against most *P. aeruginosa* and some strains of *E. coli* and *P. mirabilis* was influenced by the size of the inoculum (Table 1). BRL2288 was two- to fourfold less active in tube dilutions at an inoculum of $10^{-3}$ as at an inoculum of $10^{-4}$. With the agar plate method, use of an undiluted overnight culture re-

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**FIG. 1. (A) Structure of BRL2288, 6-[(D)-α-carboxyl-3-thienylacetamido] penicillanic acid, (B) structure of carbenicillin, 6-[(D)-α-carboxyphenylacetamido] penicillanic acid.**

**FIG. 2. Susceptibility of gram-negative bacteria to BRL2288.**
sulted in the persistence of a small number of organisms at a drug concentration that was inhibitory at a 100-fold dilution of the overnight culture. This inoculum effect was seen with penicillinase and non-penicillinase strains.

The organisms that persisted were not more resistant. Colonies from the 400 µg/ml plate when subcultured in broth with a 10⁻² dilution were inhibited by 50 µg/ml of BRL2288. No significant difference in MIC of BRL2288 was observed when 10 strains of Pseudomonas were tested in broth containing 50% human serum as compared with parallel tests in broth without serum.

Resistance to BRL2288 of Enterobacteriaceae is due to destruction of the BRL2288 by β-lactamase. But this is not the mechanism in most Pseudomonas strains. Figure 4 demonstrates that there is no appreciable destruction of BRL2288 by a common Pseudomonas strain. An E. coli strain possessing an episomally mediated β-lactamase hydrolyzes all of the BRL2288 by 24 hr as does P. mirabilis (Fig. 5). An inoculum removed from the fully grown cells has the same MIC as the original organisms. Identical destruction and growth curves are obtained when carbenicillin is used instead of BRL2288. Pseudomonas strains which contain a penicillinase, the synthesis of which is

![FIG. 3. Comparison of MIC of BRL2288 and carbenicillin for 40 strains of Pseudomonas.](image)

**TABLE 1. Effect of inoculum size on MIC for Pseudomonas**

| Strain | Inoculum size—MIC (µg/ml)* | 10⁷ | 10⁵ | 10³ |
|--------|----------------------------|-----|-----|-----|
| 1      | 500                        | 125 | 62  |     |
| 2      | 1,000                      | 500 | 32  |     |
| 3      | 250                        | 125 | 125 |     |
| 4      | 1,000                      | 125 | 32  |     |
| 5      | 250                        | 50  | 25  |     |
| 6      | 500                        | 125 | 32  |     |
| 7      | 500                        | 50  | 25  |     |
| 8      | 250                        | 50  | 10  |     |

* MIC, minimal inhibitory concentration. Inoculum size refers to the number of colony-forming units in the 1-ml assay.
FIG. 5. Growth of Proteus mirabilis and Escherichia coli resistant to 125 μg of BRL2288 per ml. A 10⁻⁴ dilution of an overnight culture was added to broth containing 100 μg of BRL2288 per ml. Cultures were incubated at 37 °C with samples removed, at the times noted, to record optical density and membrane filtered (Millipore Corp.) to assay the hydrolysis of BRL2288. Assay of BRL2288 was performed by the agar plate method.

Episomally mediated, hydrolyze both BRL2288 and carbenicillin in a manner similar to the episome containing E. coli (3; H. C. Neu, in preparation).

Comparison of the hydrolysis of BRL2288, carbenicillin, and 6-D(-)α-sulfoaminobenzylacetamido penicillanic acid by purified penicillinase (6) is shown in Table 2. The Km would suggest that both carbenicillin and BRL2288 are hydrolyzed at equal rates. However a sulfamino penicillin derivative active against Pseudomonas (8) is hydrolyzed even more readily.

| Substrate | Km* (M) |
|-----------|---------|
| BRL2288, 6-D(-)α-carboxy-3-thi- | 1.12 × 10⁻⁵ |
| enylmethylacetamido penicil- | |
| lamic acid | |
| Carbenicillin, 6-D(-)α-carboxy- | 1.12 × 10⁻⁵ |
| phenylacetamido penicillanic | |
| acid | |
| BLP1462, 6-D(-)α-sulfaminophen- | 6 × 10⁻⁶ |
| ylacetamido penicillanic acid | |

*Km was determined by using a modification (6) of the Novick (7) starch-iodine penicillinase assay. A purified E. coli penicillinase (6) was used as the enzyme.

FIG. 6. Isobol plot of synergist action of BRL2288 and gentamicin.

FIG. 7. Susceptibility of gram-positive bacteria to BRL2288.
The synergism between BRL2288 and gentamicin was examined for strains with MIC for BRL-2288 from 15 to 250 µg/ml, and MIC for gentamicin from 0.5 to 2.5 µg/ml. Significant synergy was encountered in most strains examined (Fig. 6).

Combinations of BRL2288 and cloxacillin, a penicillinase-resistant penicillin, failed to show synergy against *Pseudomonas* or penicillinase-producing *E. coli* and *Klebsiella* strains.

The activity of BRL2288 against gram-positive cocci is shown in Fig. 7. The MIC of BRL2288 for *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and penicillin-sensitive *Staphylococcus aureus* strains is two- to eightfold greater than the MIC with ampicillin or benzylpenicillin G. Against penicillinase-producing *S. aureus*, BRL-2288 was fourfold as active as ampicillin or benzylpenicillin G. However, enterococci were quite resistant with MIC values as high as 100 µg/ml. BRL2288 was as active as ampicillin against *Haemophilus influenzae* strains, but less active against *Salmonella*.

**DISCUSSION**

Although BRL2288, 6-[D(-)-α-carboxy-3-thienylacetamido] penicillanic acid is slightly more active than carbenicillin, 6-[D(-)-α-carboxyphphenylacetamido penicillanic acid against *P. aeruginosa* and *Enterobacter* strains, it is very similar in most other aspects. Structurally the penicillins are quite similar except for the replacement of the R2-phenyl group by a thienyl moiety. It would appear that the anti-*Pseudomonas* activity is related to the carboxy group on the α-carbon in both instances. The lack of effect against *Klebsiella* and most *Serratia* strains is a major drawback, and the antibiotic is less active than carbenicillin against most gram-positive organisms. The increased activity of BRL2288 against *Pseudomonas* remains unexplained. It is not an effect of the assay system since there were no differences in the twofold increased activity of BRL2288 when the system used was broth, agar, or broth containing serum.

Both laboratory-induced and clinically isolated resistant strains of *Pseudomonas* do not inactivate carbenicillin, whereas resistance in the *Enterobacteriaceae* is due to β-lactamase production. *Pseudomonas* strains which hydrolyze carbenicillin or BRL2288 possess a β-lactamase identical to the *E. coli* and *Salmonella* penicillinases (H. Neu, in preparation). It is possible that the activity of BRL2288 and carbenicillin against *Proteus* strains is partially related to the apparent lower affinity of their β-lactamases for these antibiotics as compared with ampicillin. *Pseudomonas* strains which have a penicillinase which is episomal in origin and similar to the *E. coli* penicillinase hydrolyze BRL2288.

The synergy seen with combination of BRL-2288 and gentamicin is similar to that of carbenicillin (9). It is probably related to the fact that the gentamicin is active against the colonies that persist and eventually overgrow in the tube dilution assay. The absence of synergy with penicillinase-resistant penicillin is similar to that which we reported with carbenicillin (5). *Pseudomonas, Enterobacter*, and indole-positive *Proteus* strains resistant to carbenicillin will be resistant to BRL2288. *E. coli* and *P. mirabilis* strains which are resistant to ampicillin will, in the majority of instances, be resistant to carbenicillin and BRL2288.

It is possible that BRL2288 might offer some aid in the treatment of *Pseudomonas, Proteus*, and *Enterobacter* infections, since it may be possible to maintain bactericidal levels more readily than with carbenicillin. The synergy shown with gentamicin would be effective in overcoming problems of resistance development and superinfection with *Klebsiella* (4). However, Smith and Finland (9) showed that carbenicillin resistance increased despite use of carbenicillin and gentamicin. Animal experiments should be performed to determine whether the in vitro superiority of BRL2288 as compared with carbenicillin against *Pseudomonas* is seen in vivo.

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