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PC-904, a Novel Broad-Spectrum Semisynthetic Penicillin with Marked Antipseudomonal Activity: Microbiological Evaluation

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PC-904, sodium 6-{D(−)-α-(4-hydroxy-1,5-naphthyridine-3-carboxamido) phenylacetamido}-penicillanate, is a novel semisynthetic penicillin derivative that possesses a broad spectrum of in vitro and in vivo antibacterial activities. In low concentrations, PC-904 inhibits growth against large proportions of the gram-positive and gram-negative organisms susceptible to carbenicillin and gentamicin. In addition, PC-904 is several times more potent than carbenicillin against organisms such as *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Shigella, Salmonella, Neisseria gonorrhoeae,* and *Bacteroides fragilis.* Most striking are the inhibitory effects of PC-904 against *P. aeruginosa* and *K. pneumoniae.* Against these two clinical isolates, PC-904 is, respectively, 35 and 100 times more active than carbenicillin. The minimum inhibitory concentrations of PC-904 against *P. aeruginosa* are comparable to those of gentamicin. PC-904 acts bactericidally. The effect of inoculum size on the antibacterial activity is often small and generally comparable to carbenicillin. The rate of binding to serum protein is high (88 to 98%), but the effect of the addition of serum on the drug's activity is not marked, because such binding is reversible. It is confirmed that PC-904 has a very potent in vivo antibacterial activity against gram-negative and gram-positive organisms. Against systemic infections with *P. aeruginosa, K. pneumoniae,* and *E. coli* in mice, PC-904 is 7 to 10 times, over 8 times, and 2 to 15 times more active than carbenicillin, respectively.

For the last two decades, the incidence of *Pseudomonas aeruginosa* infection has progressively increased, along with infections from other gram-negative bacilli, and is one of the infections that are extremely refractory to chemotherapy (2, 8, 14, 19).

Carbenicillin was the first penicillin effective for treatment of *P. aeruginosa* infection (1, 6), and was followed by sulbenicillin (12, 13) and ticarcillin (18, 21), which is currently undergoing clinical trials. However, these penicillins are not potent against *P. aeruginosa*, so large amounts of these agents are required for effective treatment. Therefore, further research has led to the synthesis of other penicillins that have more potent antipseudomonal activities.

It has been reported elsewhere that acylation or other *N* substitution of the amino group in the side chain of a penicillin such as ampicillin has resulted in some penicillins that have improved antipseudomonal activity: sulfoaminobenzylpenicillin, BL-P 1462, guanylureido, and other acylureidobenzylpenicillins, BL-P 1654, BL-P 1597, BAY e6905, and others (4, 7, 15, 16).

In our laboratory, heteroaromatic carboxylic acids, such as pyridino, pyridazino, and naphthyridino carboxylic acids, were introduced to the α-amino group of ampicillin. Some of these substituted penicillins have been found to display excellent antipseudomonal activity. As a result, PC-904 has been confirmed as having marked antipseudomonal activity and a broader spectrum than carbenicillin. The present report is concerned with the in vitro and in vivo microbiological evaluation of PC-904.

MATERIALS AND METHODS

Antibiotics. PC-904, sodium 6-{D(−)-α-(4-hydroxy-1,5-naphthyridine-3-carboxamido) phenylacetamido}-penicillanate, shown in Fig. 1, was synthesized by members of the Research Department of the Pharmaceuticals Division of Sumitomo Chemical Co., Ltd. This new semisynthetic penicillin is a white crystalline substance that is soluble in water. All the other agents employed for comparison were commercial products.

Media. Media used in this experiment were as

follows. Nutrient agar, Tryptosoya broth (TSB), heart infusion broth, Tryptosoya agar, heart infusion agar (HIA), deoxycholate agar, Gifu anaerobic medium (GAM) agar, and Sabouraud agar were products of Nissui Pharmaceutical Co., Ltd., Japan. Mueller-Hinton agar and Dubos medium were products of Eiken Chemical Co., Ltd., Japan.

Test strains. Strains stocked in our laboratory were employed as standards. Clinical strains were isolated at the Osaka National Hospital in 1973 and kindly given to us by T. Kamiki.

In vitro antibacterial activity. Minimal inhibitory concentrations (MICs) of agents were determined by means of a standard twofold serial dilution method using agar or broth media. *Streptococcus* species were inoculated as 18-h broth cultures diluted 10^{-2}-fold onto plates of HIA with 10% defibrinated rabbit blood. *Proteus* spp. were inoculated onto deoxycholate agar at a 10^{-2}-fold dilution. *Staphylococcus aureus* FS-289 was tested with the tube dilution method and TSB. Anaerobic bacteria were subcultured in GAM semisolid agar, inoculated onto GAM agar, and cultured under anaerobic conditions for 48 h at 37 C (22). *Haemophilus influenzae* strains were inoculated as undiluted 18-h broth cultures onto chocolate agar and then incubated under 5% CO_2 for 72 h at 37 C. *Mycobacterium tuberculosis* subcultured in Dubos medium was inoculated into the broth and cultured for 14 days at 37 C. Fungi were subcultured on Sabouraud agar, inoculated onto this agar as appropriately diluted suspensions, and then cultured for 7 days at 30 C. For all other species, MIC values were determined after overnight incubation by inoculating a 10^{-2}-fold dilution of an 18-h culture in TSB onto HIA plates. (In our studies MIC means the lowest concentration required to inhibit macroscopic growth of bacteria.)

The effects of inoculum size and pH on antibacterial activity of PC-904 were determined by means of a twofold serial agar dilution method with HIA as the test medium. Inocula were prepared by making 10^{0}- to 10^{-5}-fold dilutions of overnight culture. Test media were adjusted to pH values between 5 to 9. The effect of culture media on activity was determined with nutrient agar, Tryptosoya agar, HIA, and Mueller-Hinton agar as media.

Effects of human serum on the antibacterial activity were examined with a twofold broth dilution method by inoculating at a cell concentration of 10^{4} to 10^{5} organisms/ml.

Bactericidal activity. The bactericidal activity of PC-904 was compared with that of carbenicillin by two methods. In one method, the minimal bactericidal concentration (MBC) was determined by a standard twofold serial dilution method with heart infusion broth as follows. MICs were determined macroscopically by final inoculation of a preculture diluted 10^{-4}-fold after incubation for 18 h at 37 C. Then a loop transfer was made from each tube of the MIC series to a tube of antibiotic-free broth. After further overnight incubation, the lowest concentration of antibiotic in the original MIC series from which no growth was obtained, the MBC, was determined. Another method consisted of counting the number of viable cells at the appropriate time intervals after addition of antibiotics.

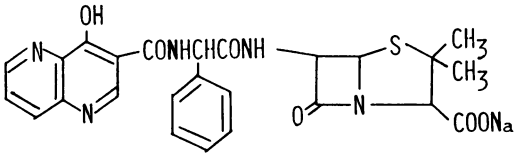

FIG. 1. *Structure of PC-904, sodium 6-{D(−)-α-(4-hydroxy-1,5-naphthyridine-3-carboxamido) phenylacetamido}-penicillanate.*

The influence of inoculum size on bactericidal activity of PC-904 and carbenicillin was tested with the above-mentioned loop transfer method.

Emergence of drug resistance. The rates of development of resistance to PC-904 and carbenicillin were determined by the successive-transfer method on antibiotic-containing medium every 24 h, according to the technique of Price et al. (16).

The degree of cross-resistance between PC-904 and carbenicillin was examined with the resistant strains obtained by the previously mentioned technique.

Stability to β-lactamase. Rates at which β-lactamase from *Escherichia coli* 33, *Klebsiella pneumoniae* 10, and *P. aeruginosa* IAM 1095 and 163 hydrolyze PC-904 and other β-lactam antibiotics were determined by adding antibiotics to each crude β-lactamase preparation and incubating the mixture at 37 C. The crude enzyme preparations derived from *E. coli* and *K. pneumoniae* were cell-free culture media of these organisms. In the instance of *P. aeruginosa* β-lactamase, the sonically treated extract of cells that had been cultured in the presence of penicillin G was employed as an enzyme source. The residual activity at various intervals of incubation was bioassayed with *Bacillis subtilis* PCI-219. The inactivation rate of each antibiotic was expressed as the relative amount of antibiotic hydrolyzed, compared to penicillin G, which was hydrolyzed at the rate of 100 nmol/h at 37 C.

Stability in acid. The stability of PC-904 at low pH (2.0) was determined by incubating the penicillin at a concentration of 200 μg/ml at 37 C in 0.1 M sodium citrate-hydrochloride buffer. Samples were removed periodically, neutralized with 1 M phosphate buffer (pH 7.2), and bioassayed for residual activity on agar plates seeded with *B. subtilis* PCI-219.

Serum protein binding. The binding rate of PC-904 to serum protein was determined by an ultrafiltration method. The antibiotics were dissolved in serum at a concentration of 50 μg/ml and incubated for 1 h at 37 C, and then ultrafiltrated with membrane ultrafilters (Centriflo, CF 50A, Amicon Co., Ltd.; cut off level molecular weight 50,000) at 2,000 rpm for 20 min at 4 C. After ultrafiltration, the filtrate was bioassayed for residual activity.

In vivo antibacterial activity. The in vivo antibacterial activity of PC-904 was determined in experimental gram-positive and gram-negative systemic infections in mice. Eight male mice of the ICR

strain weighing 18 to 22 g were used for each dose level. Mice were challenged via the intraperitoneal route with sufficient microorganisms to kill all nontreated mice within 48 h. Challenge doses of bacterial cells were about 10 to 100 times the number of bacterial cells per milliliter required to kill 50% of the infected but nontreated mice. All gram-negative organisms given were suspended in 5% mucin. No mucin was utilized to produce infection by *Streptococcus pyogenes*.

Antibiotics to be tested were administered by the subcutaneous route. For the majority of infections, treatment was at 1 and 4 h postinfection. However, with infections caused by *S. pyogenes*, mice were treated at 23, 27, 30, and 47 h postchallenge. In all cases, a dose-response system, in which drug concentrations were increased twofold, was used. The total number of surviving mice was recorded, usually 1 week after infection, and the amount of a single dose, in milligrams per kilogram, that gave protection to 50% of the infected mice (ED_{50}) was estimated by means of a log-probit plot (11).

RESULTS AND DISCUSSION

In vitro activity. The spectrum of antibacterial activity of PC-904 against gram-positive organims is shown in Table 1, as compared with carbenicillin and gentamicin. PC-904 was active against all gram-positive organisms susceptible to carbenicillin. In a concentration of 1.56 μg/ml PC-904 completely inhibited the growth of the carbenicillin-susceptible organisms. It was several times more active than carbenicillin and gentamicin against *Streptococcus* and *Micrococcus*. Moreover, PC-904 was highly active against anaerobic organisms such as *Clostridium* spp. resistant to gentamicin. PC-904 was also comparable to carbenicillin in that it had potent inhibitory effects against the other susceptible gram-positive organisms, including *S. aureus*, which does not produce penicillinase. It was moderately active against *S. faecalis* (*Enterococcus*), which constitutes an important cause of endocarditis and urinary tract infections because of its relative resistance to penicillins (9).

PC-904, like carbenicillin, was inactive against penicillinase-producing *S. aureus* and *M. tuberculosis*.

As shown in Table 2, PC-904 proved to be remarkably active against gram-negative organisms. Carbenicillin and gentamicin have been known to possess potent antibacterial activities against gram-negative organisms, including *P. aeruginosa*. These agents are also effective and recommended for infections caused by those organisms (5, 10). Also, in the present experiments, both agents were found to have potent inhibitory effects against gram-negative organisms. In contrast, PC-904 displayed a broader spectrum and more potent antibacterial activity than either gentamicin or carbenicillin, inhibiting all gram-negative organisms tested at a low concentration: less than 3.13 μg/ml.

TABLE 1. *Antibacterial spectra of PC-904, carbenicillin, and gentamicin on gram-positive organisms*[a]

Organism	MIC (μg/ml)		
	PC-904	Carbenicillin	Gentamicin
Staphylococcus aureus 209P JC-1	0.39	0.39	0.05
S. aureus Smith	0.78	0.78	0.1
S. aureus Terashima	0.39	0.78	0.05
S. aureus 1200A	1.56	1.56	0.05
S. aureus FS-289	>200	>200	>200
Streptococcus pyogenes Cook	0.1	0.39	6.25
Streptococcus group A type 1	0.1	1.56	25
S. mitis	0.2	1.56	6.25
S. faecalis ATCC 8043	12.5	50	25
S. pneumoniae Neufeld	0.05	0.39	12.5
Micrococcus luteus PCI-1001	0.0125	0.05	0.39
Peptostreptococcus anaerobius B-38	0.39	3.13	>100
Bacillus subtilis PCI-219	0.2	0.1	0.05
B. cereus var. *mycoides* ATCC 9634	0.78	100	0.78
Clostridium chauvoei Okinawa	1.56	1.56	>100
C. tetani T_3	1.56	1.56	>100
C. septicum	1.56	1.56	>100
Corynebacterium xerosis NCTC 9755	1.56	3.13	0.05
Propionibacterium acnes P-11	0.39	0.78	12.5
Mycobacterium tuberculosis H37 Rv	>200	200	6.25

[a] These tests were carried out by means of a standard twofold serial dilution method using agar (based upon the standard method of the Japan Society of Chemotherapy) or broth media.

Whereas the MICs of carbenicillin against *P. aeruginosa* ranged from 12.5 to over 200 μg/ml, those of PC-904 were between 0.78 and 3.13 μg/ml and comparable to the MICs of gentamicin. PC-904 displayed potent antibacterial activity against *K. pneumoniae*, which is resistant to carbenicillin, and also against *Proteus mirabilis*, which is moderately resistant to gentamicin. Moreover, PC-904 was several times more active than carbenicillin against *E. coli*, *P. vulgaris*, *Shigella*, *Salmonella*, and *Neisseria gonorrhoeae*. Its superb action on *Bacteroides fragilis*, which is highly resistant to gentamicin, suggests the effectiveness of PC-904 for infection due to this anaerobic organism, which has recently been marked (2, 23). Compared to carbenicillin and gentamicin, PC-904 displayed

TABLE 2. *Antibacterial spectra of PC-904, carbenicillin, and gentamicin on gram-negative organisms*[a]

Organism	MIC (μg/ml)		
	PC-904	Carbenicillin	Gentamicin
Neisseria gonorrhoeae A	0.1	0.39	3.13
N. gonorrhoeae Morikawa	0.39	0.78	6.25
N. gonorrhoeae Sugiyama	0.2	0.78	12.5
Escherichia coli NIHJ	0.39	6.25	0.39
E. coli O111	0.1	0.78	0.78
E. coli K-12	0.78	3.13	0.39
Klebsiella pneumoniae PCI-602	3.13	>200	0.39
K. pneumoniae GN-45	1.56	6.25	0.39
Proteus mirabilis GN-2425	0.78	1.56	25
P. vulgaris HX-19	0.006	1.56	0.39
Shigella flexneri 2-2a	0.1	0.78	1.56
S. flexneri V-5	0.2	1.56	0.78
Salmonella typhi 901	0.39	1.56	0.39
S. typhi 58	1.56	3.13	0.39
S. paratyphi 1015	0.1	0.78	0.1
S. schottmuelleri 8006	0.1	0.78	0.39
Pseudomonas aeruginosa IAM 1095	3.13	>200	6.25
P. aeruginosa IFO 12045	0.78	50	0.78
P. aeruginosa IFO 3445	0.39	12.5	0.78
P. aeruginosa IFO 3451	0.78	25	1.56
P. aeruginosa NCTC 10490	0.39	0.78	6.25
P. aeruginosa 104	0.78	25	0.78
P. aeruginosa T	3.13	100	1.56
Bacteroides fragilis Ju-9-1	0.1	12.5	>100

[a] Tests were carried out as described in the footnote to Table 1.

either comparable or more potent inhibitory effects on the remaining gram-negative organisms, yet it was as inactive as the comparable agents against fungi such as *Trichophyton, Aspergillus,* and *Candida*.

Finally, PC-904 was shown to possess a broader spectrum than carbenicillin or gentamicin. It was superior to carbenicillin against all gram-negative organisms tested, including *P. aeruginosa, K. pneumoniae,* and *Proteus* spp. (indole-positive and -negative) and superior to gentamicin against *P. mirabilis, N. gonorrhoeae,* and anaerobic organisms. Moreover, this agent exhibited more potent antibacterial activities than either of the other agents against gram-positive organisms such as *Streptococcus* and *Micrococcus*.

When the antibacterial activity of PC-904 was compared with ampicillin, the former was generally found to have a comparable or diminished activity against gram-positive organisms, but was more potent against gram-negative organisms, particularly *P. aeruginosa* and *K. pneumoniae*.

On the other hand, compared with cefazolin, PC-904 was found to display several times more potent antimicrobial activity than this cephalosporin against all of the gram-negative and gram-positive organisms except for *Staphylococcus* and *Clostridium* spp.

The susceptibility pattern of PC-904 against bacteria isolated from a patient at the Osaka National Hospital in 1973 is illustrated in Fig. 2 and 3.

In the case of *P. aeruginosa,* carbenicillin inhibited growth at a concentration of 50 to 200 μg/ml. However, PC-904, as well as gentamicin, inhibited this species at a low concentration: 0.78 to 6.25 μg/ml. The MIC_{50} (the concentration required to inhibit the growth of 50% of the total number of clinically isolated strains) of PC-904 was 1.4 μg/ml; MIC_{50} values of carbenicillin and gentamicin were 49 and 1.5 μg/ml, respectively. Therefore, PC-904 was approximately 35 times more active than carbenicillin, and comparable to gentamicin in effectiveness.

Against *H. influenzae,* PC-904 was active at a concentration of less than 0.39 μg/ml, which was comparable to carbenicillin and ampicillin. Cefazolin and gentamicin were less active than these penicillins, by 77 and 5 times, respectively.

It was confirmed that PC-904 possesses marked antibacterial activity against species of *Enterobacteriaceae* (Fig. 3).

About 60% of *E. coli* isolates were susceptible to PC-904 at a concentration of less than 6.25 μg/ml, and it was three to four times more effective than carbenicillin and ampicillin. Against the remaining *E. coli* strains that are highly resistant to these penicillins, however, PC-904 was only moderately active, whereas gentamicin and cephalothin were very active. This phenomenon is considered to be related to penicillinase production by resistant strains. PC-904 was fairly active against *K. pneumoniae* strains, most of which were not susceptible to ampicillin and carbenicillin. About 85% of strains were fairly resistant to both penicillins. In contrast, PC-904 was rather inactive against one-fourth of the strains tested. But it inhibited the remaining three-fourths in a concentration ranging from 0.78 to 12.5 μg/ml, comparable to cephalothin. PC-904 was more active than carbenicillin and ampicillin, but inferior to gentamicin.

Against *P. mirabilis* and indole-positive *Proteus* resistant to gentamicin, carbenicillin is known to be extremely active and clinically effective (6). PC-904 was twice as active as carbenicillin against indole-positive *Proteus;* this agent was almost as active as carbenicillin against *P. mirabilis*.

Recently, there have been many reports that *Enterobacter* and *Serratia* species have been

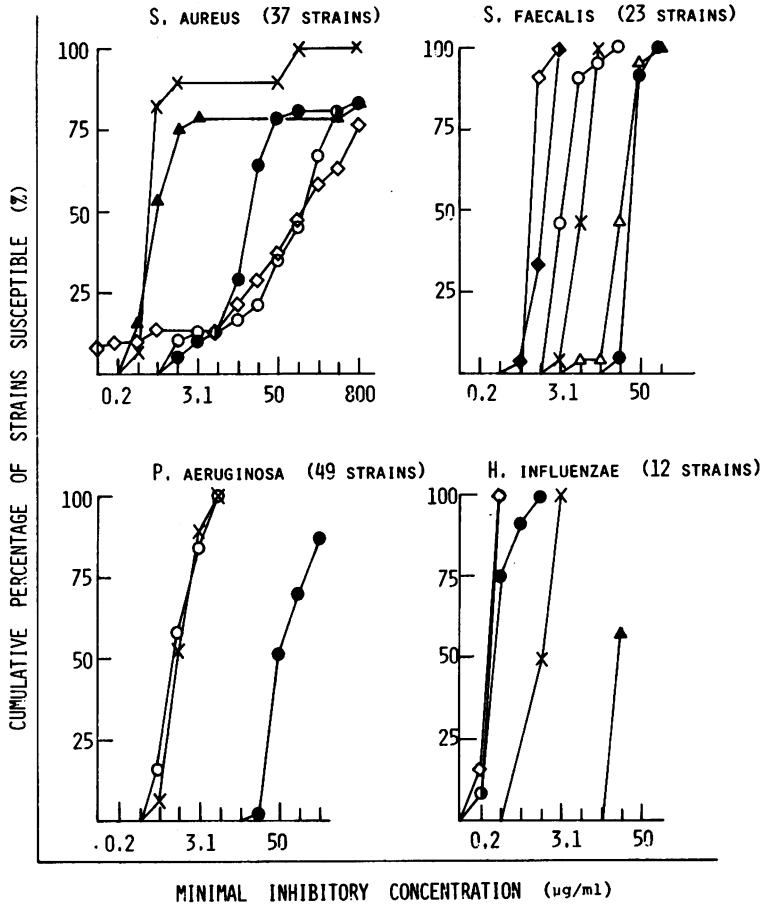

FIG. 2. *Susceptibility of several species of gram-positive and gram-negative clinical isolates to PC-904 (○), carbenicillin (●), ampicillin (◇), penicillin G (◆), cephalothin (△), cefazolin (▲), and gentamicin (X). MICs were determined by the agar dilution method. Inoculum size was 1 loopful of 10^{-2}-fold-diluted preculture. With S. aureus and H. influenzae, undiluted preculture was used.*

increasingly isolated together, as combined pathogenic agents (2, 3). These organisms were reported to be highly resistant to β-lactam antibiotics and other chemotherapeutics, except for the aminoglycosides. Against these organisms, PC-904 was fairly active, but inferior to gentamicin.

Finally, PC-904 displayed potent antibacterial activity against *Enterobacteriaceae*.

When *S. aureus* strains, 1 loopful of nondiluted overnight culture, were inoculated onto agar, almost all of the strains were not susceptible to PC-904, a pattern similar to that seen with ampicillin. In contrast, carbenicillin inhibited about 80% of strains in a concentration of less than 50 μg/ml. On the other hand, when a 10^{-2} dilution of preculture was inoculated, PC-904 and ampicillin were superior to carbenicillin, and the MIC_{50} values were 3.0, 2.0, and 6.6 μg/ml, respectively, for PC-904, ampicillin, and carbenicillin. As previously mentioned, the antibacterial activities of PC-904 and ampicillin tended to be highly influenced by inoculum size, possibly because of the production of penicillinase, but the activity of carbenicillin was less influenced. Gentamicin and cefazolin exhibited excellent activities against *S. aureus*, regardless of inoculum size.

Against *S. faecalis*, PC-904 was active and superior to carbenicillin, cephalothin, and gentamicin, but slightly inferior to ampicillin and penicillin G.

The influence of inoculum size on the antibacterial activity of PC-904 and carbenicillin was determined in agar dilution tests with several strains (Table 3). Changes in inoculum size had no significant effect on the activity of PC-904, as well as that of carbenicillin, against

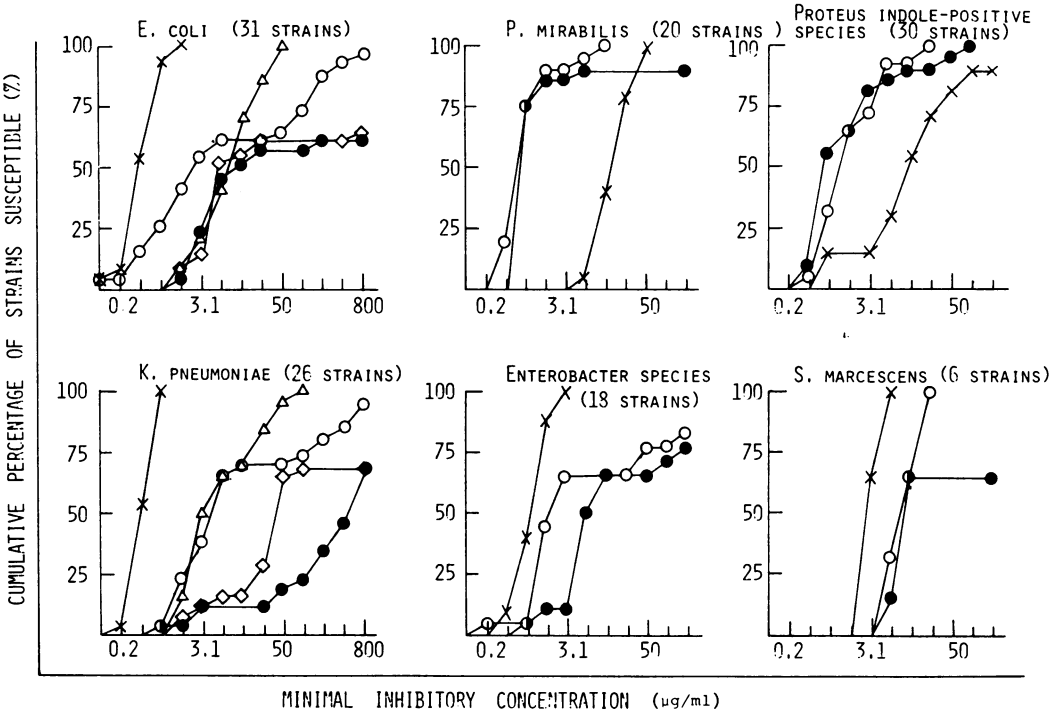

FIG. 3. *Susceptibility of various Enterobacteriaceae species to PC-904 (○), carbenicillin (●), ampicillin (◇), cephalothin (△), and gentamicin (X). MICs were determined by the agar dilution method. Inoculum size was 1 loopful of 10^{-2}-fold-diluted preculture.*

TABLE 3. *Effects of inoculum size on antibacterial activity of PC-904 and carbenicillin*

Test organism	No. of cells/ml in undiluted culture	Penicillin	MIC[a] (μg/ml) with dilution of culture				
			10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Staphylococcus aureus 209P	3×10^{8}	PC-904	0.78	0.78	0.2	0.1	0.05
		Carbenicillin	0.78	0.78	0.1	0.1	0.05
Streptococcus faecalis ATCC 8043	1×10^{8}	PC-904	12.5	6.25	6.25	6.25	3.13
		Carbenicillin	200	50	50	25	25
Escherichia coli NIHJ	1.4×10^{9}	PC-904	0.39	0.39	0.39	0.05	0.05
		Carbenicillin	100	50	3.13	1.56	1.56
Klebsiella pneumoniae PCI-602	1.4×10^{9}	PC-904	6.25	3.13	3.13	1.56	0.78
		Carbenicillin	400	400	200	100	50
Pseudomonas aeruginosa 104	7×10^{8}	PC-904	1.56	0.78	0.78	0.78	0.39
		Carbenicillin	200	50	25	25	25
P. aeruginosa NCTC 10490	2×10^{9}	PC-904	0.2	0.2	0.2	0.1	0.05
		Carbenicillin	1.56	1.56	1.56	0.78	0.39
Proteus mirabilis GN-2425	1.9×10^{9}	PC-904	0.78	0.78	0.39	0.39	0.2
		Carbenicillin	12.5	0.78	0.78	0.78	0.39
P. vulgaris HX-19	1.7×10^{9}	PC-904	1.56	0.39	<0.025	<0.025	<0.025
		Carbenicillin	1.56	0.78	0.78	0.78	0.39

[a] Determined by the twofold serial dilution method in HIA.

TABLE 4. *Effects of human serum on antibacterial activity of PC-904 and carbenicillin*[a]

Penicillin	Serum concn (%)	Test organism			
		S. aureus 209P	*P. aeruginosa* 104	*P. aeruginosa* NCTC 10490	*P. mirabilis* GN-2425
PC-904	0	0.39	0.78	0.39	0.78
	10	0.39	0.78	0.39	0.78
	25	0.78	1.56	0.78	1.56
	50	0.78	1.56	0.39	3.13
Carbenicillin	0	0.78	50	0.78	0.78
	10	0.39	25	0.78	0.78
	25	0.78	12.5	1.56	0.78
	50	0.78	6.25	0.39	3.13

[a] These tests were conducted by a twofold serial tube dilution method with TSB.

TABLE 5. *Bactericidal activity of PC-904 and carbenicillin*[a]

Organism	PC-904		Carbenicillin	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Staphylococcus aureus 209P	0.39	0.78	0.39	0.78
S. faecalis ATCC 8043	25	25	100	100
Escherichia coli NIHJ	<0.39	<0.39	6.25	6.25
Klebsiella pneumoniae PCI-602	6.25	6.25	>400	>400
K. pneumoniae GN-45	<0.78	<0.78	6.25	6.25
Proteus mirabilis GN-2425	3.13	3.13	3.13	3.13
P. vulgaris HX-19	0.1	0.1	1.56	1.56
Pseudomonas aeruginosa 104	1.56	1.56	100	100
P. aeruginosa NCTC 10490	0.78	>12.5	0.78	1.56

[a] Tests were carried out by the usual twofold serial dilution method with heart infusion broth. The overnight preculture was seeded into tubes with a final dilution of 10^{-4}-fold as an inoculum size. MIC was determined macroscopically after 24-h incubation at 37 C. Then, MBC was determined by a loop transfer method.

almost all of the tested strains. However, MICs of both penicillins tended to be higher as the inoculum size increased. In *P. vulgaris* HX-19, the MIC of PC-904 fell dramatically when the inoculum size was decreased. Although a previous study has shown that the MIC of PC-904, rather than of carbenicillin, against clinical isolates of *S. aureus* was greatly influenced by inoculum size, the MIC of both penicillins against *S. aureus* 209P (a strain that does not produce penicillinase) was similarly influenced by inoculum size. It seems likely that the effect of inoculum size on antibacterial activity may have been exerted via the combination of penicillinase-producing organisms and penicillinase-susceptible antibiotics.

Effects of different culture media and pH values on the MICs of PC-904 and carbenicillin were not great.

Effects of the addition of human serum on the antibacterial activity of PC-904 were compared with the effects on carbenicillin in a tube dilution test (Table 4). Human serum, at a final concentration of 50%, raised MIC values of PC-904 and carbenicillin, respectively, two to four times and one-fourth to four times more than in human-serum-free media.

Bactericidal activity. As indicated in Table 5, PC-904 and carbenicillin displayed bactericidal action against almost all organisms when inoculum size was about 10^4 to 10^5 cells/ml. The MBCs were almost equal to the MICs. Of the organisms tested, however, PC-904 was not bactericidal for only one strain, *P. aeruginosa* NCTC 10490. Another strain, *P. aeruginosa* 104, was killed by both penicillins. This fact could be explained almost completely by studying the effect of inoculum size on MBC. As seen in Table 6, the MBC of PC-904 against *P. aeruginosa* was greatly influenced by inoculum size, as was the MBC of carbenicillin. PC-904 was bactericidal only when inoculum size was less than 10^5 organisms/ml. This finding was also seen in species other than *P. aeruginosa*. PC-904 and carbenicillin were bactericidal to *E. coli* NIHJ when the inoculum size was about 10^7 or less organisms per ml. Against *S. faecalis* ATCC 8043, PC-904 displayed bactericidal

action when about 10^3 or less cells per ml were inoculated, as was found with ampicillin and penicillin G. Generally, penicillins did not exert bactericidal action when a heavy inoculum was used.

Bactericidal activity of PC-904 against *P. aeruginosa* was examined by counting viable cells (Fig. 4). As *P. aeruginosa* 104 was grown to a concentration of 7.5×10^4 cells/ml, one-fourth- to twofold the MICs of PC-904 and carbenicillin were added. The MICs of PC-904 and carbenicillin for this strain were 1.56 and 50 μg/ml, respectively. Two hours after addition, both penicillins reduced the number of viable cells to 1/30 at a concentration of one to two times the MIC and then displayed bactericidal action. As seen in Fig. 4, PC-904 displays 32 or more times as potent a bactericidal activity as carbenicillin on *P. aeruginosa* 104.

Drug resistance, susceptibility to β-lactamase, acid stability, and protein-binding rate. The rates at which PC-904 and carbenicillin develop resistance to *E. coli* NIHJ and *P. aeruginosa* 104 were studied by a tube transfer method (Fig. 5). Repeated exposure of both strains to PC-904 produced stepwise increases in resistance that closely paralleled those found with carbenicillin. Resistance developed in this manner appears to be fairly stable, as no decrease in final resistance levels occurred when strains were subjected to several transfers on penicillin-free medium. Cross-resistance between PC-904 and carbenicillin was found in both resistant strains, obtained as previously mentioned .

The inactivation rate of PC-904 by crude β-lactamase preparations was compared with those of other β-lactam antibiotics (Table 7). Ampicillin was degraded at the same rate as penicillin G by *E. coli* and *K. pneumoniae* β-lactamases, which predominantly hydrolyzed penicillins. In contrast, PC-904 and carbenicillin were moderately stable in the presence of these β-lactamases. Their inactivation rates were only half that of penicillin G. Moreover, PC-904 was as highly resistant to hydrolysis by inducible *P. aeruginosa* β-lactamase as carbenicillin and ampicillin, whereas cefazolin was highly susceptible to this enzyme.

Although PC-904 gave the same inactivation rates as carbenicillin, its MIC values were much lower than those of the latter, as shown in Table 7. These results may be explained by the fact that the antibacterial activity of PC-904 is determined not only by susceptibility to β-lactamase, but also by other intrinsic resistance factors, one of which is probably the differences in cell wall and membrane permeability of antibiotics (17, 20).

PC-904 was found to be fairly stable at low pH (Table 8). Its half-life at pH 2.0 was estimated to be about 4.7 h. The corresponding values for carbenicillin and ampicillin were 0.4 and 7.1 h, respectively. Although PC-904 is

TABLE 6. *Effects of inoculum size on MIC and MBC of penicillins against two strains of Pseudomonas aeruginosa and Escherichia coli NIHJ*[a]

Organism[b]	Final dilution (-fold)	PC-904		Carbenicillin	
		MIC	MBC	MIC	MBC
P. aeruginosa 104	10^{-1}	>200	>200	>200	>200
	10^{-2}	>200	>200	200	>200
	10^{-3}	3.13	>200	200	200
	10^{-4}	1.56	1.56	50	100
	10^{-5}	1.56	3.13	50	50
	10^{-6}	1.56	1.56	50	50
P. aeruginosa NCTC 10490	10^{-1}	>200	>200	>200	>200
	10^{-2}	>200	>200	6.25	>200
	10^{-4}	0.39	100	1.56	6.25
	10^{-6}	0.2	0.39	0.78	1.56
E. coli NIHJ	10^{-1}		100		200
	10^{-2}	12.5	25	50	100
	10^{-3}	6.25	6.25	12.5	25
	10^{-4}	0.2	0.2	6.25	12.5
	10^{-5}	0.2	0.2	3.13	3.13
	10^{-6}	0.1	0.1	3.13	3.13

[a] These tests were conducted by the usual twofold serial dilution method with heart infusion broth. MICs were determined with a variety of inoculum sizes, and then MBCs were determined by a loop transfer method.

[b] Initial cell counts were: *P. aeruginosa* 104, 7×10^8/ml; *P. aeruginosa* NCTC 10490, 2×10^9/ml; *E. coli* NIHJ, 1×10^9/ml.

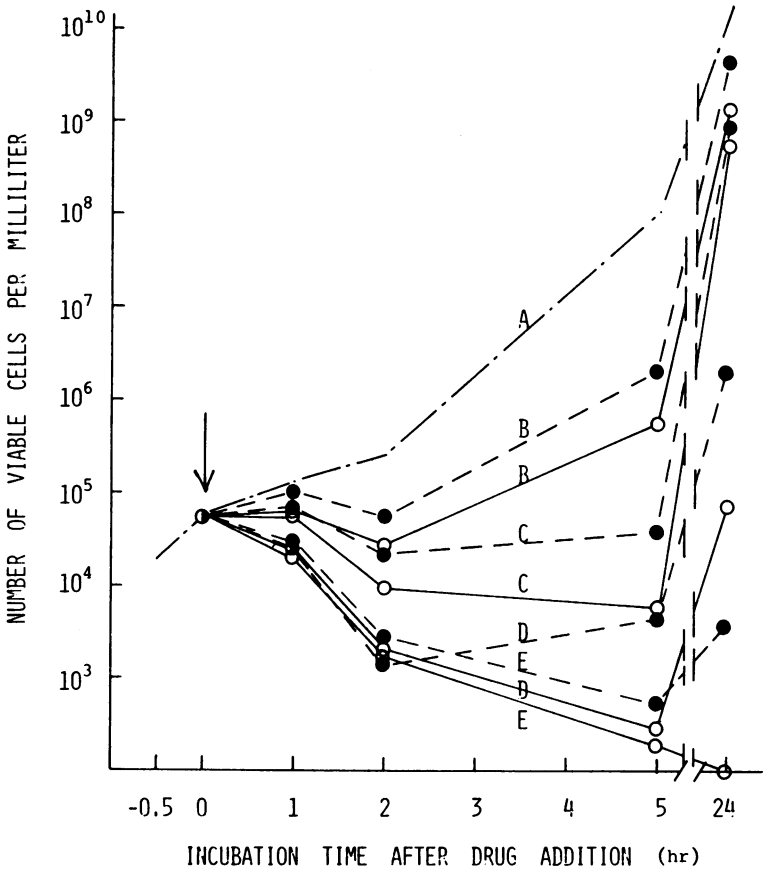

FIG. 4. *Bactericidal effects of PC-904 (○) and carbenicillin (●) against P. aeruginosa 104. The number of viable cells at the appropriate time intervals after addition of antibiotics (arrow) was counted on antibiotic-free agar. The MIC against P. aeruginosa 104 was 1.56 μg/ml for PC-904 and 50 μg/ml for carbenicillin. Final drug concentrations by addition were each one-fourth of MIC (B), one-half of MIC (C), MIC (D), and two times MIC (E) (micrograms per milliliter); (A) no drugs added.*

probably not degraded by highly acidic gastric juice, it was not well absorbed after oral administration to rats.

The binding rate of PC-904 to serum protein ranged from about 88 to 98%, regardless of the kind of serum used (Table 9). In the case of carbenicillin, this value varied from 30 to 45% (6). Although PC-904 has a high rate of binding to serum protein, this binding seemed to be rather reversible. With ultrafiltration method for estimation of protein binding, the nonfiltrable fraction had much less antibacterial activity, probably due to protein binding. When diluted with an excess of buffer, however, the antibacterial activity of this fraction was restored. Furthermore, about 100% of the activity was recovered in the protein-free fraction when the mixture of PC-904 and serum was applied onto Sephadex G-25 column chromatography and eluted with buffer. These results are consistent with results from a previous study indicating that the effect of the addition of human serum on antibacterial activity is not marked in the broth dilution system.

In vivo antibacterial activity. PC-904 proved to be an extremely effective chemotherapeutic agent in mice experimentally infected with a variety of gram-negative pathogens.

Chemotherapeutic effects of PC-904 on experimental infections in mice caused by three strains of *P. aeruginosa* are shown in Table 10. PC-904 displayed 7 to 10 times more potent activity than carbenicillin on infections caused by *P. aeruginosa* strain T, but was inferior to gentamicin, which is one of the most effective agents for *P. aeruginosa* infection (5). Against infections with *P. aeruginosa* 19 and the highly virulent strain NC-5, PC-904 was, respectively, four to seven times as active as carbenicillin. In vivo effectiveness of PC-904 was actually lower

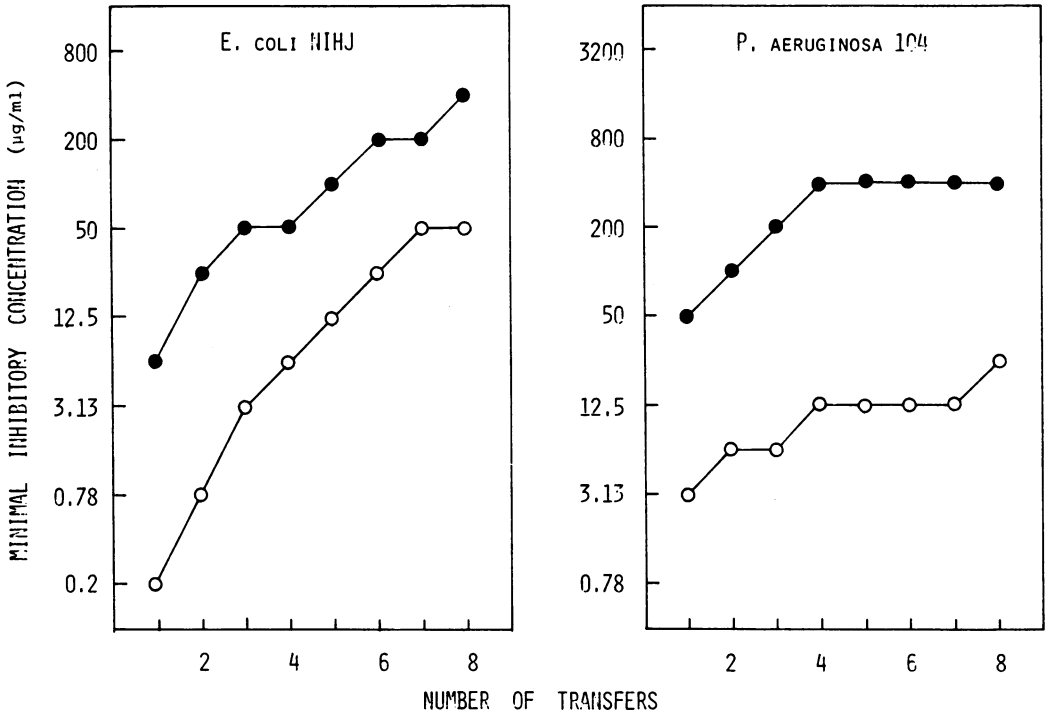

FIG. 5. *Emergence of drug resistance to PC-904 (○) and carbenicillin (●). The rates of development of resistance to PC-904 and carbenicillin were determined by the successive-transfer method on antibiotic-containing medium every 24 h.*

TABLE 7. *Relative inactivation rates of PC-904 and other antibiotics by β-lactamase*

Antibiotic	Enzyme source							
	E. coli 33		*K. pneumoniae* 10		*P. aeruginosa* IAM1095		*P. aeruginosa* 163	
	MIC (μg/ml)[a]	RIR (%)[b]	MIC (μg/ml)	RIR (%)	MIC (μg/ml)	RIR (%)	MIC (μg/ml)	RIR (%)
Penicillin G	1,600	100	>3,200	100	>3,200	100	>3,200	100
PC-904	100	68	800	57	3.13	0	3.13	0
Carbenicillin	>3,200	51	>3,200	58	50	0	200	0
Ampicillin	1,600	111	>3,200	108	1,600	0	1,600	0
Cefazolin	6.25	13	25	20	>3,200	1,130	>3,200	1,013

[a] MIC was determined by the usual twofold serial dilution method with HIA.

[b] RIR, Relative inactivation rate. The inactivation rate value convertedly assigned to penicillin G for each of the crude β-lactamase preparations was 100.

TABLE 8. *Stability in acid*[a]

Penicillin	Half-life (h)
PC-904	4.7
Carbenicillin	0.4
Ampicillin	7.1

[a] The stability at pH 2.0 was determined by incubating the compound at 37 C in 0.1 M sodium citrate-hydrochloride buffer. Samples were removed periodically, neutralized with phosphate buffer (pH 7.2), and bioassayed for residual activity.

TABLE 9. *Serum protein binding*

Serum type	Binding rate (%)[a]	
	PC-904	Carbenicillin
Bovine	98	45
Dog	88	34
Human	96	30

[a] The rate of binding to serum protein was estimated by an ultrafiltration method with Centriflo membrane ultrafilters (Amicon Co.).

than had been expected on the basis of the excellent MICs of PC-904, which were almost 30-fold lower than those of carbenicillin. A partial explanation is that the tissue distribution of PC-904 is slightly different from that of carbenicillin. Also, less of the former is absorbed into blood than the latter after parenteral administration.

Table 11 compares the therapeutic activity of PC-904, carbenicillin, ampicillin, cephalothin, and cefazolin in a series of infections induced by *S. pyogenes* and a variety of gram-negative organisms other than *P. aeruginosa*. PC-904 displayed marked protective effects in systemic infections of mice caused by *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *P. vulgaris*.

In mice infected with *E. coli* NIHJ, PC-904 displayed 15 times more potent protective effects than carbenicillin and several times more potent activity than cefazolin and was comparable to ampicillin. There appeared to be a fairly good correlation between MIC and ED_{50} values. The ED_{50} was highly influenced by challenge doses, as was also found in other experiments and with other penicillins. PC-904 and carbenicillin had relatively comparable levels of activity against other strains of *E. coli*.

Carbenicillin was found to be ineffective against experimental infections caused by *K. pneumoniae*, as is well known. In contrast, PC-904 displayed excellent protective activity, comparable to ampicillin and cephalothin, and was over eight times more active than carbenicillin on *K. pneumoniae* infection. Cefazolin was found to be superior to cephalothin and the other penicillins on this organism.

Also, on infections induced in mice by *P. mirabilis* (indole negative) and *P. vulgaris* (in-

TABLE 10. *Chemotherapeutic effects of PC-904 on experimental infections of mice caused by P. aeruginosa*[a]

P. aeruginosa challenge strain[b]	MIC (μg/ml)[c]			ED_{50} (mg/kg)[d]		
	PC-904	Carbenicillin	Gentamicin	PC-904	Carbenicillin	Gentamicin
T	3.13	100	1.56	17[e] 56[f] 25	175[e] 400[f]	7
NC-5	6.25	200		56	390	
19	3.13	100		80	300	

[a] Eight male mice of ICR strains weighing 18 to 22 g were used at each dose level.
[b] Organisms were diluted with 5% mucin solution, and mice were challenged intraperitoneally.
[c] MICs were determined by the usual twofold dilution method with HIA.
[d] Test compounds dissolved in distilled water were administered subcutaneously twice, 1 and 4 h postinfection. ED_{50} was calculated from survival rates 1 week after infection.
[e] Challenge dose was light.
[f] Challenge dose was heavy.

TABLE 11. *Chemotherapeutic effects of PC-904 on experimental infections in mice*[a]

Challenge organism	MIC (μg/ml)					ED_{50} (mg/kg)				
	PC-904	CAR[b]	AMP	CET	CEZ	PC-904	CAR	AMP	CET	CEZ
Streptococcus pyogenes A-1[c]	<0.2	1.56				50	100			
Escherichia coli NIHJ	0.78	6.25	0.78	0.78	3.13	13 1.4	>20 21	1.4		3.2
E. coli O111	0.1	0.78				30	22			
E. coli 8	1.56	12.5				1.8	4.4			
Klebsiella pneumoniae 18	25	>200	25	1.56	1.56	32 11	270		19 4	
						16	390	23		0.7
K. pneumoniae 26	25	>200	100	1.56	1.56	<12.5 2	165		2	
Proteus mirabilis GN 2425	0.78	1.56				10	34			
P. vulgaris 1	<0.05	0.1				32	50			

[a] See footnote *a* to Table 10.
[b] CAR, Carbenicillin; AMP, ampicillin; CET, cephalothin; CEZ, cefazolin.
[c] Mice challenged intraperitoneally with *S. pyogenes* without mucin were treated four times at 23, 27, 30, and 47 h postinfection.

dole positive), PC-904 was several times more active than carbenicillin in therapy.

Finally, as might be expected from its excellent in vitro activity, PC-904 displayed striking protective activity against systemic infections in mice challenged with a variety of gram-negative organisms.

Moreover, PC-904 had twice the efficacy of carbenicillin in systemic infections induced by a gram-positive pathogen, *S. pyogenes*.

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