THE COMPARATIVE *IN VITRO* ACTIVITY AND β-LACTAMASE STABILITY OF A NEW UREIDO PENICILLIN

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The *in vitro* activity of Bay K-4999, 6[D-2-(3-furfurylidenamino-2-oxo-imidazolidine-1-carboxamido)-2-(4-hydroxy-phenyl) acetamido] penicillinate, a new ureido penicillin was evaluated against 555 clinical isolates and compared to selected β-lactams. Bay K-4999 inhibited most streptococci at concentrations less than 1 μg/ml, but was not active against β-lactamase producing *Staphylococcus aureus*. The activity of Bay K-4999 against streptococci including *Streptococcus faecalis* was similar to the activity of ampicillin. β-Lactamase-producing *Haemophilus influenzae* and *Neisseria gonorrhoeae* were inhibited by Bay K-4999; albeit at levels higher than needed for non-β-lactamase producing isolates. The activity of Bay K-4999 against the members of the Enterobacteriaceae varied from family to family and seemed to correlate with the presence of β-lactamases. Bay K-4999 was more active than ampicillin, piperacillin or mezlocillin against *Escherichia coli* lacking β-lactamases. It was more active against *Klebsiella* than piperacillin or mezlocillin, but cefazolin-resistant strains were not inhibited. It had activity against *Pseudomonas aeruginosa* comparable to piperacillin but was less active against *Bacteroides fragilis*. Bay K-4999 was hydrolyzed by β-lactamases of *S. aureus*, and by both plasmid and chromosomally-mediated β-lactamases of Enterobacteriaceae at rates comparable to ampicillin. It acted synergistically with gentamicin to inhibit resistant *Pseudomonas* isolates.

Although many new penicillins have been developed in this decade, there is still a need for compounds with a greater spectrum than were provided by penicillin G, ampicillin and carbenicillin[1,13]. The activity of these agents against *Klebsiella* is essentially nil. There are still significant numbers of *Pseudomonas aeruginosa, Enterobacter cloacae* and *Acinetobacter calcoaceticus*, as well as some of the indole-positive *Proteus*, resistant to carbenicillin and ticarcillin[5,6]. Moreover, infections due to these organisms most often occur in individuals with precarious cardiovascular and hematologic status. Thus the sodium content of carbenicillin and the binding of the agent to the adenosine diphosphate of platelets can mitigate against its use in the concentrations needed for successful therapy of life-threatening infections in patients with compromised host defenses[3,7].

These factors prompted us to evaluate the *in vitro* activity of a ureido penicillin, Bay K-4999, 6[D-2-(3-furfurylidenamino-2-oxo-imidazolidine-1-carboxamido)-2-(4-hydroxy-phenyl) acetamido] penicillinate, and compare its properties to other recently developed compounds[1,2,5,6]. We further wished to determine whether Bay K-4999 offers any advantage over the ureidopenicillins available for clinical use in Germany; namely, azlocillin[1,5] and mezlocillin[2,5] in terms of β-lactamase stability, or synergy when they are combined with aminoglycosides.

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Materials and Methods

Bay K-4999, 6[D-2-(3-furfurylidenamino-2-oxo-imidazolidine-1-carboxamido)-2-(4-hydroxy-phenyl) acetamido] penicillinate, was a gift of Delbay Pharmaceuticals. Mezlocillin and azlocillin were obtained from Delbay, piperacillin from Lederle Laboratories, carbenicillin and ticarcillin from Beecham Laboratories. Cefoxitin was from Merck Sharp and Dohme, cefazolin and cefamandole from Lilly Research and gentamicin from Schering Corporation. Fresh antibiotic dilutions were prepared daily in sterile distilled water or broth.

Bacteria:

Bacterial strains tested were recent clinical isolates from patients hospitalized at the Columbia-Presbyterian Medical Center, New York City, or selected β-lactamase-producing isolates which had been retained frozen from previous studies with other compounds[5,6,8,9].

Susceptibility Testing:

Antimicrobial activity was measured by agar or broth dilution methods as noted in the test, using MUELLER-HINTON broth or agar (BBL). An inoculum of 10^5 colony forming units (CFU) was used unless otherwise specified. Serial two-fold dilutions of antibiotic were prepared, and an overnight culture of bacteria was applied with an inoculating device. Organisms were incubated at 35°C for 18 hours. The minimal inhibitory concentration (MIC) of an antibiotic was defined as the lowest concentration that inhibited development of visible growth on agar or turbidity in a tube. Minimal bactericidal concentration (MBC) was determined by plating 0.01 ml from clear tubes of broth onto blood agar. The MBC was the concentration at which less than five colonies grew. The effect of growth medium on activity of Bay K-4999 was determined using brain-heart infusion, nutrient broth, and MUELLER-HINTON broth (BBL), and the effect of inoculum size was determined using 10^3, 10^5 and 10^7 CFU. Activity against anaerobic bacteria was determined on MUELLER-HINTON agar supplemented with sheep blood and vitamin K. Plates were incubated at 35°C for 48 hours in Gas Pak jars (BBL). *Haemophilus* were tested using chocolate agar, as were the *Neisseria*, which were incubated in the presence of 5% CO_2.

β-Lactamase Assays:

The presence of β-lactamase of a particular isolate was determined by use of a chromogenic cephalosporin as described before[1]. Staphylococci were induced by 0.5 μg/ml of methicillin, *Pseudomonas* by 1,000 μg/ml of penicillin G, and the other organisms by 12.5 μg/ml of cephalothin.

The enzymes were classified according to the schema of RICHMOND and SYKES[12]. The β-lactamase assay procedures used were either the microiodometric or spectrophotometric. The presence of a β-lactamase was detected in clinical isolates by the chromogenic technique[11].

β-Lactamase assays were performed using purified or partially purified enzymes that had been prepared by sonic disruption of bacteria, followed by centrifugation to remove debris and chromatography on Sephadex G50.

Synergy Study:

Synergy was measured by the agar dilution with a checkerboard technique as described previously[4]. Synergy was considered to be present when there was a four-fold decrease in the MICs of both agents.

Results

Bay K-4999 inhibited the majority of non-β-lactamase producing *Staphylococcus aureus* and *S. epidermidis* at MICs of 0.8 μg/ml, but the MICs against the β-lactamase isolates were above 400 μg/ml, Table 1. The majority of streptococci, *Streptococcus pyogenes, S. agalactiae, S. viridans*, and *S. pneumoniae*, were inhibited by concentrations of 0.2 μg/ml or less. Of the *S. faecalis* tested, 95% were inhibited by 3.1 μg/ml. The majority of *Haemophilus* species and *H. influenzae* and *H. parainfluenzae* were inhibited by 0.2 μg/ml as were *Neisseria*, including *N. gonorrhoeae* and *N.*

Table 1. Overall inhibitory activity of Bay K-4999 against Gram-positive and negative aerobic and anaerobic bacteria

Organism	No. tested	Minimum inhibitory concentration (μg/ml) Mode	Range
Staphylococcus aureus	16	12.5	0.8~400
Staphylococcus epidermidis	15	12.5	0.8~100
Streptococcus pyogenes	12	0.1	<0.05~0.8
S. agalactiae	12	0.2	<0.05~0.8
S. viridans	5	0.2	0.2~0.8
S. pneumoniae	5	0.2	<0.05~0.4
S. faecalis	19	1.6	0.2~6.2
Haemophilus influenzae	15	0.1	<0.1~6.2
H. parainfluenzae	14	0.2	0.1~6.2
Neisseria gonorrhoeae	19	0.2	0.1~3.1
N. meningitidis	5	0.1	0.1~0.4
Listeria monocytogenes	10	0.8	0.1~3.1
Bacillus subtilis	2		0.1
Escherichia coli	46	0.4	<0.1~>400
Klebsiella pneumoniae	32	3.1	0.4~>400
Enterobacter aerogenes	18	0.8	0.2~25
E. cloacae	18	0.4	0.4~>400
E. agglomerans	3		6.3~25
E. hafniae	2		0.4~0.8
Serratia marcescens	32	100	12.5~>400
Citrobacter diversus	10	0.8	0.1~3.1

Organism	No. tested	Minimum inhibitory concentration (μg/ml) Mode	Range
C. freundii	22	0.8	0.2~200
Proteus mirabilis	32	0.4	0.2~12.5
P. morganii	15	0.2	0.2~50
P. vulgaris	4	0.4	0.2~400
P. rettgeri	11	0.8	0.4~>400
Providencia stuarti	16	25	0.2~>400
Salmonella species	15	0.2	0.2~400
S. typhi	5	0.2	0.2~1.6
Shigella sonnei	16	0.2	0.1~>100
S. flexneri	5	0.2	0.2~12.5
Clostridium perfringens	2		0.2
Fusobacterium varium	2		1.6
Bifidobacterium	1		1.6
Peptostreptococcus	2		0.8
Bacteroides fragilis	15	25	12.5~400
Bacteroides species	12	25	12.5~100
Acinetobacter	15	6.3	0.8~50
Pseudomonas aeruginosa	64	6.3	0.8~400
Pseudomonas other	10	50	6.3~>400
Total	555		

meningitidis. β-Lactamase producing isolates of *H. influenzae*, *H. parainfluenzae* and *N. gonorrhoeae* were inhibited by 3.1~6.2 μg/ml. *Listeria monocytogenes* were inhibited by concentrations of 0.1~3.1 μg/ml.

The activity of Bay K-4999 against the members of the Enterobacteriaceae varied from family to family. Non-β-lactamase producing *Escherichia coli* were inhibited by 0.8 μg/ml or less. The activity of Bay K-4999 against *Salmonella* and *Shigella* isolates was similar to its activity against *E. coli* and correlated with the presence of β-lactamases. Bay K-4999 inhibited 53% of *K. pneumoniae*, 94% of *Enterobacter aerogenes*, and 75% of *E. cloacae* at 6.3 μg/ml. *Citrobacter diversus* were all inhibited by 3.1 μg/ml. Although 75% of *C. freundii* were inhibited by 6.3 μg/ml, as was observed with *E. cloacae*, a small number of isolates had MICs of Bay K-4999 greater than 400 μg/ml. Most, greater than 80%, of *P. mirabilis* and *P. morganii* were inhibited by less than 1 μg/ml, while some hospital isolates of *P. vulgaris*, *P. rettgeri* and *Providencia stuartii* were resistant to 400 μg/ml. The susceptibility of anaerobic species ranged from 0.2 μg/ml to greater than 400 μg/ml against some *Bacteroides fragilis*. The *P. aeruginosa* were hospital isolates and so of the more resistant types, but 55% were inhibited by 12.5 μg/ml and 89% by 100 μg/ml.

No significant effect of the type of medium used to determine susceptibility was detected for five isolates each of *E. coli*, *K. pneumoniae*, *E. cloacae*, *E. aerogenes*, *P. morganii* and *P. aeruginosa*, when tests were performed simultaneously in MUELLER-HINTON, brain-heart infusion, trypticase soy or

Table 2. Comparison of MICs and MBCs of Bay K-4999

Organism	No. tested	No. of isolates with identical MIC & MBC	No. of isolates with MBC greater than MIC (fold)			
			2	4	8	≥16
Escherichia coli	10	3	4	1	2	
Enterobacter	10		7	1	2	
Klebsiella	10					
Proteus, indole-positive	5	1	2		1	
Pseudomonas	10	4	3	2		1
Neisseria gonorrhoeae	3	2			1	
Haemophilus influenzae	3	1	1		1	

nutrient broth. Furthermore, there was no significant difference in MICs whether determined in either MUELLER-HINTON broth or agar. The MIC, MBC values agreed within one tube dilution.

Comparison of MICs and MBCs is given in Table 2. The MBCs were identical or only two-fold greater for 55% of the 53 isolates tested. Isolates of *P. morganii* and *P. aeruginosa* with inducible β-lactamases had MBCs eight-fold or greater than the MICs of Bay K-4999. Isolates of *H. influenzae*, *N. gonorrhoeae* and *E. coli* with MBCs of eight-fold greater than the MIC contained plasmid-mediated β-lactamases whose substrate profile was that of the TEM or RICHMOND type III[12].

The inoculum size effect for a number of different isolates is shown in Table 3. At 10^3 and 10^5 CFU the MICs were virtually identical; whereas, at 10^7 CFU most of the bacteria were resistant—MICs were 200 μg/ml or greater. Of 45 isolates so tested, ten of ten *E. coli*, eight of ten *Klebsiella*, nine of ten *Enterobacter*, five of five indole-positive *Proteus* and ten of ten *P. aeruginosa* isolates were resistant with MICs of 200 μg/ml at an inoculum of 10^7 CFU; whereas, their MICs were 12.5 μg/ml at 10^5 CFU. There was no clear correlation of the presence of β-lactamases and this inoculum effect since bacteria which lacked β-lactamases detectable by the iodometric or chromogenic cephalosporin technique also showed the inoculum effect.

Table 3. Comparison of MIC values at different colony forming units inocula

Organism	MIC values (μg/ml) at CFU of		
	Agar	Broth	
	10^5	10^5	10^7
Escherichia coli	0.4	0.4	200
E. coli	0.4	0.4	>200
Enterobacter cloacae	0.4	0.4	6.3
E. cloacae	6.3	3.1	>200
Klebsiella pneumoniae	1.6	1.6	12.5
K. pneumoniae	6.6	6.3	>200
Proteus morganii	0.2	1.6	>200
P. morganii	3.1	6.3	>200
Pseudomonas aeruginosa	3.1	6.3	>200
P. aeruginosa	12.5	12.5	>200

Overall activity of Bay K-4999 against *S. aureus, S. epidermidis, E. coli, Shigella sonnei* and *S. typhimurium* correlated with the presence of β-lactamases or the presence of β-lactamases which could be induced, Table 4. This was not true for *Klebsiella*, the indole-positive *Proteus, P. morganii, P. rettgeri* and *P. vulgaris,* nor for *Pseudomonas.*

Bay K-4999 was less stable to hydrolysis by β-lactamases whether of a plasmid or chromosomal mediated type, Table 5, than was carbenicillin. Plasmid-mediated enzymes primarily active against penicillins were more active in hydrolyzing Bay K-4999 than were the chromosomally mediated, inducible enzymes which have cephalosporins as their primary substrates.

Table 4. Correlation of β-lactamase activity with susceptibility to Bay K-4999

Organism	β-Lactamase	No.	Sensitive	Resistant
Staphylococcus aureus	Negative	3	3	0
	Positive	2	0	2
	Induced	6	0	6
S. epidermidis	Negative	5	3	2
	Positive	—		
	Induced	4	0	4
Escherichia coli	Negative	15	15	
	Positive	6	2	4
	Induced	—		
Klebsiella pneumoniae	Negative	7	7	0
	Positive	5	5	0
	Induced			
Enterobacter	Negative	2	2	
	Positive	2	2	
	Induced	3	3	
Proteus morganii	Negative			
	Positive	5	4	1
	Induced	9	9	
P. rettgeri	Negative	2	2	
	Positive	4		4
	Induced	1	1	
P. vulgaris	Negative	1	1	
	Positive	2		2
	Induced	1	1	
Pseudomonas aeruginosa	Negative	20	15	3
	Positive	2		2
	Induced	10	8	2

The comparative activity of Bay K-4999 and other β-lactam antibiotics is shown in Table 6. The cephalosporin which was used for comparison differs from family to family so as to reflect the agent which previous studies have shown to be the most active against the particular species[8,9]. Selection of 50% and 90% inhibition does not fully reflect the activity of Bay K-4999. For example, at 0.4 μg/ml 52% of *E. coli* were inhibited; whereas all of the other agents inhibited less than 5% of isolates at this concentration. Against *Klebsiella* the activity of Bay K-4999 was similar to that of mezlocillin and piperacillin, although Bay K-4999 was much more active at a concentration of 1.6 μg/ml, inhibiting 44% of isolates compared to 6% inhibited by mezlocillin and 13% by piperacillin. Table 7 shows the activity of Bay K-4999 against *K. pneumoniae* resistant to cefazolin, MIC

Table 5. Comparative β-lactamase stability of Bay K-4999

Source of enzyme	Enzyme type*	Relative activity**		
		Ampicillin	Carbenicillin	Bay K-4999
Pseudomonas	I	59	8	30
Providencia	I	16	0	0
P. morganii	I	4	4	3
Providencia	II	85	23	42
P. aeruginosa	III	87	9	73
E. coli	III	150	18	124
Klebsiella	IV	81	12	45
S. sonnei	V	102	24	58
S. aureus	—	119	2	31

* Classification based on RICHMOND system as determined by substrate and inhibitor specificity.

** Hydrolysis rate was relative to the rate of hydrolysis of penicillin G which was given a value of 100. Rates should not be compared between organisms since the specific activity of the enzymes differed.

Table 6. Comparative activity of Bay K-4999 with other β-lactam antibiotics

Organism (No. of strains)	MIC (μg/ml) for 50 and 90% of test strains					
	Bay K-4999	Carbenicillin	Ampicillin	Mezlocillin	Piperacillin	Cephalosporin
Escherichia coli (46)	0.4 >400	25 >400	12.5 >400	3.1 >400	1.6 >400	1.6[a] 25
Klebsiella pneumoniae (32)	3.1 >400	400 >400	400 >400	100 >400	25 >400	3.1[a] 50
Enterobacter (29)	0.8 25	6.3 100	100 400	6.3 50	3.1 50	1.6[b] 25
Serratia (30)	>400	>400	>400	>400	>400	>400[c]
Proteus mirabilis (32)	0.4 6.3	0.8 3.1	1.6 12.5	0.8 3.1	0.4 50	— —
Proteus, indole-positive (30)	0.8 >400	6.3 >400	>400	6.3 >400	6.3 >400	6.3[c] 12.5
Providencia (16)	25 >400	100 >400	>400	50 >400	100 >400	— —
Pseudomonas (62)	12.5 100	50 200	12.5[d] 100	25 400	6.3 200	25[e] 400
Salmonella (15)	0.2 50		0.8 >400	3.1 >400	0.8 200	—
Citrobacter (32)	0.8 50	100 >400	50 >400	6.3 200	3.1 200	—
Shigella (16)	0.2 1.6	— —	1.6 100	0.8 12.5	0.4 6.3	— —
Acinetobacter (15)	6.3 25	25 >400	— —	50 200	12.5 100	— —
Bacteroides fragilis (16)	25 100	50 200	— —	— —	12.5 50	12.5 50
Streptococcus faecalis (19)	1.6 3.1	25 50	0.8 1.6	0.8 1.6	3.1 6.3	— —

[a] Cefazolin, [b] Cefamandole, [c] Cefoxitin, [d] Azlocillin, [e] Ticarcillin

Table 7. Comparative activity of Bay K-4999 and cefazolin against *Klebsiella*

Klebsiella strain No.	MIC (μg/ml)		*Klebsiella* strain No.	MIC (μg/ml)	
	Cefazolin	Bay K-4999		Cefazolin	Bay K-4999
4077	50	1.6	4247	50	>400
4030	50	>400	4246	25	>400
4028	50	400	4244	25	200
4021	25	3.1	4243	>400	6.3
3973	100	100	3528	50	>400
3713	25	0.4	3600	25	>400
3636	25	0.4	3529	50	>400

>25 μg/ml. The majority of the isolates are also resistant to Bay K-4999. Bay K-4999 was eight to 16-fold more active than the other penicillins against the *Enterobacter* tested which included *E. cloacae, E. aerogenes* and the less common species. Bay K-4999 had virtually identical activity with piperacillin and mezlocillin against *P. mirabilis*, but it was two to four-fold more active than mezlocillin or piperacillin against indole-positive *Proteus*. Bay K-4999 and carbenicillin had almost identical activity against *Providencia* with 44% of the isolates inhibited by 1.6 μg/ml compared to 16% inhibited

Table 8. Activity of Bay K-4999 against carbenicillin-resistant organisms

	Carbenicillin	Bay K-4999	Mezlocillin	Piperacillin
Escherichia coli 3695	>400	0.1	3.1	0.4
E. coli 3714	>400	100	200	100
Enterobacter cloacae 3922	>400	25	100	50
E. cloacae 4259	400	12.5	50	50
Klebsiella 4243	200	6.3	12.5	12.5
Klebsiella 4077	>400	1.6	6.3	6.3
Citrobacter 4296	>400	50	200	200
Pseudomonas 4241	400	25	100	12.5
Pseudomonas 4238	>400	25	50	25
Proteus mirabilis 3378	>400	>400	>400	>400
P. rettgeri 4105	400	50	100	50
Bacteroides fragilis 27	100	25	25	12.5
B. fragilis 89	100	50	25	25
Acinetobacter 4272	>400	63	25	12.5

by mezlocillin and 19% inhibited by piperacillin at this concentration. The highly resistant *Providencia* were resistant to all the agents tested. The activity of Bay K-4999 against a select group of *Serratia* resistant to carbenicillin and to aminoglycosides was not better than that of mezlocillin or piperacillin. The activity of Bay K-4999 and piperacillin against *Pseudomonas* was similar with 44% inhibited by Bay K-4999 and 52% inhibited by piperacillin at 6.3 μg/ml and 93% inhibited by Bay K-4999 and 81% inhibited by piperacillin at 50 μg/ml. Bay K-4999 was two to eight-fold more active than other penicillins against *Salmonella, Shigella, Citrobacter* and *Acinetobacter*. Piperacillin and cefoxitin were the most active agents against *B. fragilis*. Bay K-4999 was two-fold more active against some strains. Bay K-4999, mezlocillin and ampicillin had similar activity against *S. faecalis* —16-fold greater than that of carbenicillin.

Table 8 shows that Bay K-4999 and piperacillin had similar MICs against Enterobacteriaceae and *P. aeruginosa* which were resistant to carbenicillin.

The synergistic activity of Bay K-4999 and gentamicin was tested against *P. aeruginosa* isolates. Complete synergy, four-fold reduction in MICs of both agents, was achieved for 69% and partial synergy for 18% of the isolates tested. No antagonism was seen. Synergy was achieved against β-lactamase-producing isolates and isolates which contained gentamicin-inactivating adenylating and acetylating enzymes, Table 9.

Table 9. Synergy of Bay K-4999 and gentamicin against *Pseudomonas aeruginosa*

Strain	MIC μg/ml		
	Bay K-4999	Gentamicin	Bay K-4999+Gentamicin
4060	25	6.3	6.3+0.31
3899	100*	3.1	12.5+0.62
4217	12.5	1.6	1.6+0.08
4283	100*	50**	6.3+0.31
4306	200*	100**	25+1.25
4235	50*	100**	12.5+12.5
4240	200*	50**	6.3+0.31

* Contains β-lactamase isolates
** Contains aminoglycoside inactivating enzyme

Discussion

Bay K-4999 has been shown to have inhibitory activity equivalent to ampicillin against most Gram-positive coccal species, including *S. faecalis*. It is several fold more active than the newest penicillin derivatives, mezlocillin and even piperacillin, against *E. coli, Salmonella, Shigella,* and

Citrobacter which lack β-lactamases. Bay K-4999 inhibited many *Klebsiella* at concentrations comparable to those of the other cephalosporins, but it did not inhibit cefazolin-resistant *Klebsiella* as do cefoxitin and cefamandole[8,9]. Bay K-4999 inhibited a number of *Enterobacter* resistant to carbenicillin. However, its activity against *Enterobacter* was similar to that of mezlocillin and piperacillin, and it offered no increased activity against *Serratia*. Bay K-4999 had activity against *P. aeruginosa* similar to that of piperacillin—the heretofore most active β-lactam compound—but Bay K-4999 was less active than piperacillin against certain *B. fragilis* isolates.

There was a discrepancy between MICs and MBCs for certain species. This discrepancy often correlated with the presence of β-lactamases whether plasmid-mediated or chromosomally inducible enzymes. Similar to other agents such as carbenicillin, the ureidopenicillins, azlocillin and mezlocillin, and the piperazine penicillin, piperacillin, Bay K-4999 did not inhibit many members of the Enterobacteriaceae or *Pseudomonas* at inocula of 10^7 CFU.

It is clear that the increased activity of Bay K-4999 was not related to β-lactamase stability since it was hydrolyzed by most of the different types of β-lactamases. It was hydrolyzed at a slower rate than ampicillin but to a much greater extent than was carbenicillin.

Bay K-4999 showed synergistic activity against *Pseudomonas* when combined with gentamicin. Significantly more isolates were inhibited by this combination than we had reported for the combination of azlocillin, mezlocillin and piperacillin and gentamicin[5,10].

Overall, this agent has a comparable *in vitro* activity to that of piperacillin. It is questionable whether the minor *in vitro* differences between these penicillins would be of clinical importance in the type of patient who develops such infections. Furthermore, since all agents are ampicillin derivatives, it is possible that they will produce similar untoward effects. Further animal and clinical studies are needed to delineate if significant differences in therapeutic potential exists between mezlocillin, piperacillin and Bay K-4999.

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