A SULFONE β-LACTAM COMPOUND WHICH ACTS AS A β-LACTAMASE INHIBITOR

NALINEE ASWAPOKEE and HAROLD C. NEU*

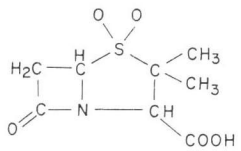
Departments of Medicine and Pharmacology, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032, U.S.A.

(Received for publication September 28, 1978)

CP-45,899 [3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3,2,0)heptane-2-carboxylic acid, 4,4-dioxide, [2S-(2α,5α)]] has low intrinsic activity against most Gram-positive cocci, Enterobacteriaceae and *Pseudomonas*. It inhibits *Neisseria* at concentrations of 0.1 ~ 6.2 μg/ml. The combination of CP-45,899 and ampicillin inhibited *Staphylococcus aureus* and Enterobacteriaceae resistant to ampicillin by virtue of β-lactamase activity. Combination of CP-45,899 and cephalothin was synergistic less often, and CP-45,899 did not act synergistically with carbenicillin or ticarcillin against *Pseudomonas* resistant to these agents. CP-45,899 acted synergistically with ampicillin against *Bacteroides*. Synergy of CP-45,899 and ampicillin was demonstrated at varying concentrations suggesting that it may significantly enlarge the antibacterial activity of ampicillin against resistant bacteria.

β-Lactamases have become increasingly important as mediators of resistance not only among Gram-negative bacteria[6,12] but in organisms such as *Neisseria gonorrhoea*[1] and *Haemophilus influenzae*[14]. Although structural modifications of the basic penicillin nucleus have yielded compounds resistant to hydrolysis by both Gram-positive and Gram-negative β-lactamases, these agents, such as oxacillin and cloxacillin, have not proved clinically useful in the treatment of most infections due to β-lactamase producing bacteria except for urinary tract infections[13]. Recently naturally occurring β-lactamase inhibitors have been found[2,5,11,15]. Clavulanic acid has been shown to be a potent inhibitor of selected β-lactamases[7,11]. CP-45,899 is a penicillanic acid sulfone (Fig. 1) which acts as an inhibitor of β-lactamases of many Gram-positive and Gram-negative aerobic and anaerobic species. We have investigated its interaction with other β-lactam antibiotics.

Fig. 1. Structure of CP-45,899: 3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3,2,0)heptane-2-carboxylic acid, 4,4-dioxide, [2S-(2α,5α)]



CP-45,899

Materials and Methods

CP-45,899 was provided as a dry crystalline powder by Pfizer Laboratories. Clavulanic acid, amoxicillin and ampicillin were provided by Beecham Laboratories. Piperacillin was a gift of Lederle. Cephalothin was a gift of Eli Lilly. Bacterial isolates were obtained from patients recently hospitalized at the Columbia-Presbyterian Medical Center, N.Y.C., as well as isolates from our collection of bacteria saved because of β-lactamases[6].

Susceptibility and Synergy Tests:

Minimal inhibitory concentrations (MICs) were determined by either broth or agar dilution using MUELLER-HINTON medium (BBL) incubated at 35°C for 18 hours. An inoculum of 10^5 colony forming

* Address Reprint Requests to: Department of Medicine, 630 West 168 Street, New York, N.Y. 10032, U.S.A.

units (CFUs) was used. The MIC was the lowest concentration at which there was no visible growth on agar or in the broth. Minimal bactericidal concentrations (MBCs) were determined by plating 0.1 ml from clear tubes to blood agar. Synergy studies were performed using 10^5 CFU added to MUELLER-HINTON agar containing equivalent amounts of CP-45,899 and the other antibiotic. Synergy was defined as a 4-fold reduction in the MIC or MBC of both agents. Partial synergy was defined as a 4-fold reduction in the MIC of one agent and a 2-fold or no reduction in the MIC of the other agent.

Presence of β-lactamase activity was determined by use of the chromogenic cephalosporin 87/312[10] or by use of an iodometric assay[8]. Organisms which gave negative tests for β-lactamase activity when assayed without exposure to β-lactam antibiotics were also tested after induction using either methicillin for *S. aureus* or cephalothin for the Enterobacteriaceae. Methicillin at a concentration of 0.5 μg/ml was added to a growing culture of *S. aureus*. After 3 hours a sample of culture was tested for β-lactamase activity. Cephalothin at a concentration of 25 μg/ml was added to growing cultures which were incubated for 3 hours and then the cells assayed for β-lactamase activity.

Time Growth Curves:

MUELLER-HINTON broth was incubated with an exponentially growing culture to yield approximately 10^6 CFU. Antibiotics were added at the MIC value or in combination at one-fourth the MIC of both agents. Cultures were incubated on a gyratory shaker at 35°C. Samples were removed, immediately diluted in broth and plated on MUELLER-HINTON agar which was incubated at 35°C for 18 hours to determine the surviving CFUs.

Results

CP-45,899 showed a low degree of intrinsic antibacterial activity when tested against clinically important Gram-positive cocci such as *S. aureus* or *S. epidermidis* with a range of MIC values of 25~400 μg/ml and a mode MIC of 100 μg/ml, Table 1. This was unrelated to the presence or absence of β-lactamase since strains with a penicillin G MIC value of 0.1 μg/ml and strains with penicillin G MIC values above 100 μg/ml both had CP-45,899 MIC values of 200 μg/ml. CP-45,899 poorly inhibited *Haemophilus influenzae* strains, but it was active against *Neisseria gonorrhoeae* and *N. meningitidis* isolates at concentrations of 0.1~6.2 μg/ml. The majority of the Enterobacteriaceae had CP-45,899 MIC values above 50 μg/ml and *Pseudomonas aeruginosa* were uniformly resistant to 400 μg/ml.

Table 1. *In vitro* activity of CP-45,899

Organism	No. Tested	μg/ml	
		Range	Mode
Staphylococcus aureus	21	25~>400	100
Staphylococcus epidermidis	14	50~>400	200
Haemophilus influenzae	15	25~200	100
Haemophilus parainfluenzae	3	25~100	
Neisseria gonorrhoeae	9	0.1~3.2	0.6
Neisseria meningitidis	2	0.31	—
Escherichia coli	32	12.5~50	25
Klebsiella pneumoniae	27	25~400	50
Enterobacter cloacae	28	25~>400	100
Enterobacter aerogenes	5	25~100	25
Serratia marcescens	10	100~200	200
Proteus mirabilis	5	25~>400	100
Proteus morganii	14	50~100	50
Proteus rettgeri	12	25~400	100
Proteus vulgaris	6	25~100	50
Salmonella species	12	50~>400	100
Shigella species	12	50~>400	>400
Bacteroides	30	25~100	25
Pseudomonas aeruginosa	51	>400	>400

In contrast, when CP-45,899 was combined with ampicillin (Table 2), there was a marked lowering of the inhibitory concentrations of both agents even though the organism was resistant to both agents. This was found for *S. aureus* and *S. epidermidis* and for the Enterobacteriaceae. When CP-45,899 was combined with cephalothin (Table 3), synergy was less frequently demon-

Table 2. Synergy of ampicillin and CP-45,899

Organism	MIC in μg/ml		
	Ampicillin	CP-45,899	Ampicillin /CP-45,899
S. aureus	100	100	1.6
S. epidermidis	25	>200	1.6
N. gonorrhoeae	0.1	0.8	0.05
H. influenzae	0.2	0.8	0.025
E. coli	>400	25	6.2
K. pneumoniae	50	50	3.1
E. cloacae	>400	100	12.5
P. morganii	200	50	3.1
P. rettgeri	>400	100	6.2
B. fragilis	100	25	3.1
S. sonnei	100	>400	6.2
S. typhimurium	400	50	6.2

Table 3. Synergy of cephalothin and CP-45,899

Organism	MIC in μg/ml		
	Cephalothin	CP-45,899	Cephalothin/ CP-45,899
S. aureus	0.4	200	0.05
E. coli	50	50	12.5
K.pneumoniae	400	100	50
S.marcescens	>400	100	50
E. cloacae	>400	100	50
E. aerogenes	6.3	25	0.8
P. morganii	>400	100	12.5
P. mirabilis	>400	50	50
P. rettgeri	50	50	12.5
B. fragilis	400	25	3.1
B. melaninogenicus	50	25	0.4
S. sonnei	50	50	6.2
S. typhi	100	50	12.5

Table 4. Combination of CP-45,899 and carbenicillin or piperacillin tested against *Pseudomonas aeruginosa*

	CP-45,899	Carbenicillin	CP-45,899+ carbenicillin	Piperacillin	CP-45,899+ piperacillin
MIC μg/ml	>200	50	50	3.1	3.1
	>200	200	25	25	3.1
	>200	400	>200	12.5	12.5
	>200	25	25	1.6	1.6
	>200	>400	100	200	50
	>200	200	25	25	3.1

strated. Combination of CP-45,899 with either carbenicillin or with piperacillin did not result in synergy against isolates that were resistant to carbenicillin, sensitive to piperacillin nor with isolates resistant to both agents, Table 4.

The overall synergistic activity of CP-45,899 combined with ampicillin or combined with cephalothin when tested against 283 isolates is given in Table 5. The percent of strains of a species for which synergy could be demonstrated varied from 0 to 100% for both isolates which produced a β-lactamase and for those in which β-lactamase could not be detected by use of the chromogenic cephalosporin even after induction with substrates. Complete synergy, 4-fold reduction of the MIC values of both ampicillin and CP-45,899, was found for 66% of β-lactamase isolates and partial synergy for 25%. Complete synergy of ampicillin and CP-45,899 was found for 54% of the β-lactamase lacking isolates and partial synergy was found for 18%. Against most *Klebsiella pneumoniae* and *Bacteroides* isolates the combination of CP-45,899 and ampicillin was synergistic even though β-lactamase activity was not demonstrated by use of the chromogenic cephalosporin assay nor by the iodometric method. In contrast, with the Gram-positive staphylococci, synergy of CP-45,899 and ampicillin was demonstrated mainly in the β-lactamase producing isolates.

Combination of CP-45,899 and cephalothin showed complete synergy against 36% of β-lactamase positive isolates and against 44% of β-lactamase negative isolates. Partial synergy of CP-45,899 and

Table 5. Synergy of CP-45,899 and other β-lactam antibiotics against β-lactamase positive and negative species

Organism	No. tested	Presence of β-lactamase	Drug tested	No. complete synergy	No. partial synergy
S. aureus	10	+	Ampicillin	8	2
	11	−	Ampicillin	2	3
S. epidermidis	6	+	Ampicillin	6	0
	8	−	Ampicillin	0	5
H. influenzae	2	+	Ampicillin	2	0
	15	−	Ampicillin	3	2
N. gonorrhoeae	2	+	Ampicillin	2	0
	9	−	Ampicillin	1	3
E. coli	13	+	Ampicillin	7	3
			Cephalothin	2	6
	19	−	Ampicillin	1	6
			Cephalothin	0	1
Klebsiella	8	+	Ampicillin	1	4
			Cephalothin	3	5
	19	−	Ampicillin	16	1
			Cephalothin	1	10
Enterobacter	14	+	Ampicillin	8	3
			Cephalothin	4	3
	9	−	Ampicillin	5	3
			Cephalothin	3	3
Salmonella	7	+	Ampicillin	4	1
			Cephalothin	1	1
	5	−	Ampicillin	3	1
			Cephalothin	0	1
Shigella	6	+	Ampicillin	5	0
			Cephalothin	6	0
	6	−	Ampicillin	6	0
			Cephalothin	4	2
Serratia	9	+	Ampicillin	0	9
			Cephalothin	0	1
	1	−	Ampicillin	1	0
			Cephalothin	0	1
Proteus, indole-positive	20	+	Ampicillin	18	2
			Cephalothin	11	8
	12	−	Ampicillin	11	1
			Cephalothin	9	2
Pseudomonas	15	+	Carbenicillin	8	2
			Piperacillin	2	4
	21	−	Carbenicillin	5	6
			Piperacillin	5	3
Bacteroides	3	+	Ampicillin	3	0
			Cephalothin	3	0
	27	−	Ampicillin	27	0
			Cephalothin	27	0

cephalothin was found against 29% of the β-lactamase positive isolates and 20% of the β-lactamase negative isolates. The combination of cephalothin and CP-45,899 was synergistic against most *Bacteroides* but not against *Klebsiella*.

The combination of ampicillin and CP-45,899 was synergistic at ratios of ampicillin to CP-45,899 of 1: 1, 5: 1 and 10: 1, although complete synergy was shown for only 72% of isolates at an ampicillin to CP-45,899 ratio of 10: 1, Table 6.

The combination of ampicillin and CP-45,899 was synergistic as far as bactericidal values are concerned for both *S. aureus* and for Enterobacteriaceae, Table 7. Interestingly, the MBC values were identical or only 2-fold greater

Fig. 2. The killing curves of CP-45,899 and ampicillin against *E. coli* and *K. pneumoniae*

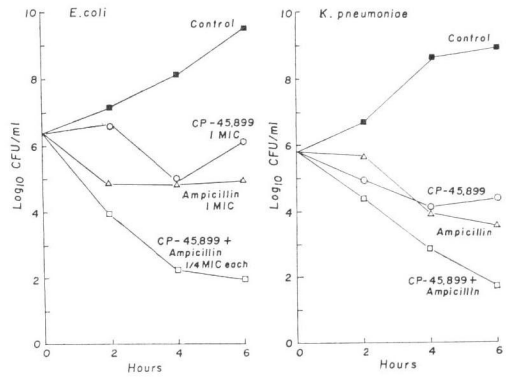


Table 6. Synergistic effect of CP-45,899 and ampicillin with different ratios against various organisms

Bacteria	No. Strains	Ampicillin CP-45,899 1: 1 Type of synergy	Ampicillin CP-45,899 5: 1 Type of synergy			Ampicillin CP-45,899 10: 1 Type of synergy		
		C*	C	P	I	C	P	I
S. aureus	5	5	4	1	0	4	0	1
E. cloacae	7	7	5	1	1	3	3	1
K. pneumoniae	8	8	9	0	0	7	0	1
E. coli	6	6	6	0	0	6	0	0
P. aeruginosa	5	5	3	0	2	3	0	2
Serratia	1	1	1	0	0	0	0	1

* C=Complete 4-fold reduction in MIC of both agents;
P=Partial reduction in MIC of one agent;
I=Indifference.

Table 7. Effect of CP-45,899 on MIC and MBC values in tube dilutions

Organism	MIC μg/ml			MBC μg/ml		
	Ampicillin	CP-45,899	Ampicillin/ CP-45,899 1: 1	Ampicillin	CP-45,899	Ampicillin/ CP-45,899 1: 1
S. aureus 3993	0.8	200	0.2	100	>400	6.2
S. aureus 4114	50	>400	6.2	>400	>400	6.2
S. aureus 4275	6.2	200	3.1	>400	>400	3.1
E. coli 3885	200	25	6.2	200	25	6.2
E. coli 4111	400	50	12.5	400	50	12.5
Klebsiella 2920	25	50	3.1	50	50	6.2
Klebsiella 3990	12.5	25	1.6	12.5	25	12.5
Salmonella	>400	100	25	>400	200	50
Salmonella	200	50	6.2	200	50	6.2
Shigella 2951	100	50	12.5	100	50	25
Shigella 3704	>400	200	25	>400	200	50

than the MIC values in most cases. Killing curves (Fig. 2) also showed this effect.

Discussion

There has been an increased search for β-lactamase resistant compounds in the past few years, but only recently have β-lactamase inhibitors been discovered which show significant promise[2,11,16]. Although antistaphylococcal penicillin such as methicillin or cloxacillin are effective inhibitors of certain types of β-lactamases, their low intrinsic activity against Gram-negative bacteria and high protein binding has interfered with their use clinically[3,4,9]. CP-45,899 has been shown in this study to act synergistically with ampicillin and with cephalothin to inhibit *S. aureus* and Enterobacteriaceae. This inhibition can be shown for both strains that contain β-lactamases and for strains in which β-lactamases cannot be demonstrated by conventional techniques even utilizing induction with β-lactam antibiotics.

The fact that the combination of CP-45,899 and ampicillin is synergistic in terms of both MIC and MBC values would seem to indicate that the inhibition is irreversible in intact cells. It is not clear whether the lack of synergy of CP-45,899 and β-lactams against organisms such as *P. aeruginosa* and many *Serratia* is due to failure of CP-45,899 to enter the microorganism or to an inability to effectively bind to a receptor site on the β-lactamase. Also, the lack of synergy of CP-45,899 and ampicillin against some *Escherichia coli* containing the plasmid-mediated β-lactamase is probably due to poor entry of CP-45,899 into these strains since CP-45,899 is an excellent inhibitor of purified β-lactamases of this type (FU and NEU, in preparation).

It is clear that CP-45,899 can markedly broaden the spectrum of ampicillin to include *Klebsiella, Bacteroides* and many of the *E. coli*, *Salmonella* and *Shigella* which in recent years have become increasingly resistant to ampicillin. Further studies of the ability of this compound to enhance the activity of β-lactamase labile β-lactams in animal protection experiments are indicated.

References

1) ASHFORD, W. A.; R. G. GOLASH & V. G. HEMMING: Penicillinase producing *Neisseria gonorrhoeae*. Lancet 1976-2: 657~658, 1976
2) BROWN, A. G.; D. BUTTERWORTH, M. COLE, G. HANSCOMB, J. D. HOOD, C. READING & G. N. ROLINSON: Naturally occurring β-lactamase inhibitors with antibacterial activity. J. Antibiotics 29: 668~669, 1976
3) COLE, M.; S. ELSON & P. D. FULLBROOK: Inhibition of the β-lactamases of *Escherichia coli* and *Klebsiella aerogenes* by semisynthetic penicillins. Biochem. J. 127: 295~308, 1972
4) HAMILTON-MILLER, J. M. T.: The demonstration and significance of synergism between β-lactam antibiotics. J. Med. Microbiol. 4: 227~237, 1971
5) HATA, T.; S. ŌMURA, Y. IWAI, H. OHNO, H. TAKESHIMA & N. YAMAGUCHI: Studies on penicillinase inhibitors produced by microorganisms. J. Antibiotics 25: 473~474, 1972
6) NEU, H. C.: The role of beta-lactamases in the resistance of Gram-negative bacteria to penicillin and cephalosporin derivatives. Infect. Dis. Rev. 11: 133~149, 1974
7) NEU, H. C. & K. P. FU: Clavulanic acid—a novel inhibitor of β-lactamases. Antimicr. Agents & Chemoth. 14: 1978 (in press)
8) NOVICK, R. B.: Microiodometric assay of penicillinase. Biochem. J. 82: 236~240, 1962
9) O'CALLAGHAN, C. H. & A. MORRIS: Inhibition of β-lactamases by β-lactam antibiotics. Antimicr. Agents & Chemoth. 2: 442~448, 1972
10) O'CALLAGHAN, C. H.; A. MORRIS, S. M. KIRBY & A. H. SHINGLER: Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicr. Agents & Chemoth. 1: 283~288, 1972
11) READING, C. & M. COLE: Clavulanic acid; a beta-lactamase-inhibiting beta-lactam from *Streptomyces clavuligerus*. Antimicr. Agents & Chemoth. 11: 852~857, 1977
12) RICHMOND, M. H. & R. B. SYKES: The beta-lactamases of gram-negative bacteria and their possible physiological role. Adv. Microbiol. Physiol 9: 31~88, 1973
13) SABATH, L. D.; H. A. ELDER, C. E. MCCALL & M. FINLAND: Synergistic combinations of penicillins in the treatment of bacteriuria. N. Engl. J. Med. 277: 232~238, 1967

14) THOMAS, W. J.; J. W. MCREYNOLDS, C. R. MOCK & D. W. BAILEY: Ampicillin-resistant *Haemophilus influenzae* meningitis. Lancet 1974–1: 313, 1974

15) UMEZAWA, H.; S. MITSUHASHI, M. HAMADA, S. IYOBE, S. TAKAHASHI, R. UTAHARA, Y. OSATO, S. YAMAZAKI, H. OGAWARA & K. MAEDA: Two β-lactamase inhibitors produced by a streptomyces. J. Antibiotics 26: 51～54, 1973

16) WISE, R.; J. M. ANDREWS & K. A. BEDFORD: *In vitro* study of clavulanic acid in combination with penicillin, amoxicillin and carbenicillin. Antimicr. Agents & Chemoth. 13: 389～393, 1978