

CP-45,899, a Beta-Lactamase Inhibitor That Extends the Antibacterial Spectrum of Beta-Lactams: Initial Bacteriological Characterization

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CP-45,899 {3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3.2.0)heptane-2-carboxylic acid, 4,4-dioxide, [2S-(2 α ,5 α)]} is an irreversible inhibitor of several bacterial penicillinases and cephalosporinases. In the presence of low concentrations of CP-45,899, ampicillin and other β -lactams readily inhibit the growth of a variety of resistant bacteria that contain β -lactamases. CP-45,899 used alone displays only weak antibacterial activity, with the notable exception of its potent effects on susceptible and resistant strains of *Neisseria gonorrhoeae*. CP-45,899 appears to be somewhat less potent but markedly more stable (in aqueous solution) than the recently described β -lactamase inhibitor clavulanic acid. The spectrum extensions provided by the two compounds are similar. A 1:1 mixture of CP-45,899 and ampicillin displays marked antimicrobial activity in mice experimentally infected with ampicillin-resistant *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Proteus vulgaris*.

A primary objective of penicillin and cephalosporin research over the past two decades has been to improve their antibacterial spectrum by chemically modifying their structures to prevent degradation by the β -lactamases of resistant bacteria. An alternate approach to this problem is to design an agent whose sole purpose is to irreversibly inhibit these β -lactamase enzymes. Such an agent need not possess antibacterial activity of its own, but would be used in conjunction with existing β -lactam antibiotics to afford activity against otherwise resistant bacteria. One such agent, the fermentation product clavulanic acid, has recently been described by Brown et al. (2). We describe here a new semi-synthetic β -lactamase inhibitor, CP-45,899, which has features in common with clavulanic acid but which also has some potential practical advantages.

MATERIALS AND METHODS

Materials. CP-45,899 is a water-soluble, white crystalline solid, which was designed and synthesized by one of us (W.E.B.). Antibiotics, ampicillin, penicillin G, and carbenicillin, were obtained from Pfizer Inc. Cefazolin and cephaloridine were purchased from local suppliers. Clavulanic acid was a gift from Beecham Laboratories. The bacteria used were primarily clinical isolates that have been maintained in our laboratories for some years.

In vitro susceptibility studies were performed in

brain heart infusion broth as the basal medium. The broth was enriched with 5% (vol/vol) sheep blood for studies with *Streptococcus pyogenes* and 5% Fildes plus 2% IsoVitaléX for *Haemophilus influenzae*. Tests with *Neisseria gonorrhoeae* were performed on gonococcus agar base (BBL) supplemented with hemoglobin and IsoVitaléX. Studies with *Bacteroides fragilis* were carried out in prerduced brain heart infusion as described in the *Anaerobe Laboratory Manual* (3); incubation was in an 80% N₂-10% CO₂-10% H₂ gas mixture either in an anaerobic chamber or in GasPak jars equipped with gas-exchange capability.

Methods. Minimal inhibitory concentrations (MICs) of antibiotics in combination with CP-45,899 were determined using a 7-by-7-concentration grid protocol in broth culture, as described by Sabath (8), with an inoculum of $\approx 10^6$ colony-forming units per ml. Testing for synergy against *B. fragilis* was performed by an agar dilution method similar to the broth dilution method.

β -Lactamase studies. The hydrolysis of ampicillin and penicillin G was determined by the microiodometric method of Novick (5). Cephaloridine hydrolysis was measured by following the decrease in ultraviolet absorbance at 255 nm (6). Conditions for both assays were identical: 0.5 M potassium phosphate, pH 6.5, and 37°C. Reactions were initiated by the addition of the cell-free β -lactamase, except in the case of preincubation experiments in which the inhibitor and enzyme were incubated together in the assay mixture for 10 min before initiation of the reaction by addition of substrate. With the cell-free extracts of *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, the substrate was

ampicillin at 33 μ M (13 μ g/ml). The specific activities of the β -lactamase preparations were, respectively, 6,019, 88,970, 260, and 76 μ mol/h per mg of protein. Penicillin G (33 μ M) was the substrate used with the *Enterobacter cloacae* β -lactamase, which showed a specific activity of 10,080 μ mol/h per mg of protein. With *B. fragilis* the substrate was cephaloridine (66 μ M), and the specific activity of the β -lactamase was 795 μ mol/h per mg.

Cell-free extracts were prepared by sonic treatment (using three 30-s bursts at 4°C except for *S. aureus*, which was broken with a French press) of cultures grown in brain heart infusion on a rotary shaker incubator. For the *S. aureus*, *P. aeruginosa*, and *E. cloacae* strains, de novo synthesis of β -lactamase was induced by growing a log-phase culture in the presence of a sublethal concentration of penicillin G at 100, 1,000, and 300 μ g/ml, respectively, for 2.5 h. In the case of *B. fragilis*, the enzyme was induced in the presence of 10 μ g/ml over 36 h. Induced cell-free extracts were dialyzed overnight at 4°C against a 1,000-fold-greater volume of buffer (0.005 M sodium cacodylate, pH 6.5, and 0.001 M 2-mercaptoethanol). Protein was determined by the biuret method (4).

Stability studies. Studies of the stability of solutions of CP-45,899 and clavulanic acid at 37°C were carried out at concentrations of 100 and 1,000 μ g/ml, prepared in 0.1 M citrate-phosphate buffer at pH 2.6 and 7.0 and in 0.1 M phosphate buffer at pH 8.0, in fresh pooled human serum at pH 7.4, and in pooled human urine adjusted to pH 4.5 and 9.0. At various times, samples of the preparations were removed and bioassayed for residual activity using *Comomonas terrigena* ATCC 8461 as the assay organism.

Acute systemic infections. Acute systemic infections in mice were produced by intraperitoneal inoculation of from 1 to 10 100% lethal doses of bacterial cultures suspended in 5% hog gastric mucin. Mice were treated orally via gavage or subcutaneously, commencing 0.5 h after challenge with subsequent treatments at 4 and 24 h. The dosage range consisted of four different antibiotic concentrations in a twofold dilution series administered to 10 mice per dosage level. Percent survival was recorded after a 4-day observation period. After four or five experiments were completed, survival data were averaged, and a 50% protective dose expressed in milligrams per kilogram was calculated by the method of Batson (1). Each experiment included CP-45,899 and ampicillin as single agents and a fixed 1:1 combination of these agents.

RESULTS AND DISCUSSION

The comparative in vitro spectrum and potency of CP-45,899 (Fig. 1), ampicillin, and CP-45,899-ampicillin combinations are presented in Tables 1 and 2. From these data it is clear that CP-45,899 per se has very weak antibacterial activity compared with ampicillin, even against the ampicillin-susceptible organisms listed in Table 1. The high activity of CP-45,899 against *N. gonorrhoeae* is an interesting exception. CP-45,899 neither improved nor antagonized the activity of ampicillin against susceptible strains.

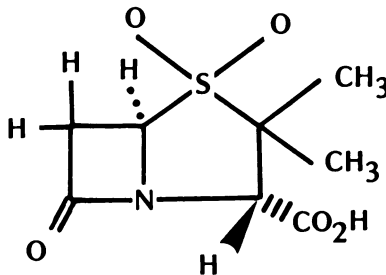


FIG. 1. CP-45,899 — {3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3.2.0)heptane-2-carboxylic acid, 4,4-dioxide, [2S-(2 α ,5 α)]}.

The results of the study with CP-45,899-ampicillin combinations against representative ampicillin-resistant bacteria are an interesting contrast. CP-45,899 reduced the amount of ampicillin required to inhibit growth to within a range encountered with susceptible organisms, and the combination displayed impressive synergistic activity against several microorganisms that elaborate penicillinases, namely, *S. aureus*, *H. influenzae*, *N. gonorrhoeae*, and *K. pneumoniae*. Against those organisms that primarily produce a cephalosporinase, the most notable activity of the CP-45,899-ampicillin combinations was demonstrated against resistant *B. fragilis*. Of all the resistant, gram-negative species listed in Table 2, only *E. coli* and *E. cloacae* remained relatively unsusceptible to CP-45,899-ampicillin combinations (9).

CP-45,899 also expanded the spectrum of β -lactam antibiotics other than ampicillin. As seen in Table 3, CP-45,899 improved the potency of penicillin G, carbenicillin, and cefazolin against a variety of resistant bacteria.

CP-45,899 and clavulanic acid were compared as to their respective abilities to inhibit cell-free β -lactamases, to render resistant organisms more susceptible to ampicillin, and to remain stable in solution over a range of pH conditions when stored at 37°C. The representative data shown in Table 4 demonstrate that CP-45,899 and clavulanic acid were very similar in their ability to inhibit the three types of penicillinases represented by *S. aureus*, *K. pneumoniae*, and the multiply drug-resistant *E. coli* 51A129. CP-45,899 was also very effective against the *B. fragilis* cephalosporinase, but relatively inactive against inducible cephalosporinases from *P. aeruginosa* or *E. cloacae*. Reading and Cole (7) have reported similar results for clavulanic acid.

When either CP-45,899 or clavulanic acid was allowed to react with β -lactamases before substrate addition, the subsequent addition of a large excess of substrate (500-fold in the case of *E. coli* 51A129) did not reverse the inhibition,

TABLE 1. *In vitro* activity of CP-45,899-ampicillin combination against ampicillin-susceptible organisms

Organism and strain no.	MIC ($\mu\text{g/ml}$)		
	CP-45,899 alone	Ampicillin alone	CP-45,899 + ampicillin ^a
<i>Streptococcus pyogenes</i> 0C203	50	0.025	0.025 + 0.025
<i>S. faecalis</i> 02A010	>50	1.56	1.56 + 1.56
<i>Staphylococcus aureus</i> 01A005	200	0.05	0.05 + 0.05
<i>Neisseria gonorrhoeae</i> F-18-CDC	0.15	0.07	0.07 + 0.07
<i>Haemophilus influenzae</i> 54A012	100	0.39	0.39 + 0.39
<i>Escherichia coli</i> 51A266	25	3.12	3.12 + 3.12
<i>Proteus mirabilis</i> 57C023	50	0.39	0.39 + 0.39

^a Figures presented were selected on the basis of minimal total drug concentration.

TABLE 2. *In vitro* activity of CP-45,899-ampicillin combination against ampicillin-resistant organisms

Organism and strain no.	MIC ($\mu\text{g/ml}$)		
	CP-45,899 alone	Ampicillin alone	CP-45,899 + ampicillin ^a
<i>Staphylococcus aureus</i> 01A400	200	200	3.12 + 1.56
01A137 ^b	>200	>200	6.25 + 6.25
<i>S. epidermidis</i> 01B116	>50	>25	3.12 + 1.56
01B087 ^b	>50	25	3.12 + 6.25
<i>Neisseria gonorrhoeae</i> CDC/res	1.2	>10	0.31 + 0.31
<i>Haemophilus influenzae</i> 54A042	100	200	1.56 + 1.56
<i>Bacteroides fragilis</i> 78C004 ^b	25	200	0.78 + 3.12
<i>Escherichia coli</i> 51A401	50	100	12.5 + 25
51A129 ^b	200	4,000	100 + 2,000
51A003 ^b	50	100	25 + 25
<i>Klebsiella pneumoniae</i> 53A079	50	50	6.25 + 6.25
<i>Enterobacter cloacae</i> 67B009	100	200	50 + 50
<i>Serratia marcescens</i> 63A095	100	200	12.5 + 12.5
<i>Proteus morganii</i> 57G001	200	50	3.12 + 3.12
<i>P. vulgaris</i> 57A067	100	>200	3.12 + 3.12
<i>Providencia alcalifaciens</i> 77B004	100	50	12.5 + 12.5
<i>Citrobacter diversus</i> 70C014	200	100	12.5 + 6.25
<i>Salmonella</i> sp. 58C004	100	400	12.5 + 6.25
<i>Pseudomonas aeruginosa</i> 52A104	>400	1,000	100 + 125

^a Figures presented were selected on the basis of minimal total drug concentration.

^b Strains which are also resistant to cefazolin (MIC > 50 $\mu\text{g/ml}$). *S. aureus* 01A137 and *S. epidermidis* 01B087 are resistant to methicillin. *E. coli* 51A003 contains a constitutive cephalosporinase-type β -lactamase.

indicating that both compounds acted as irreversible inhibitors of β -lactamases.

The data in Table 5 indicate that clavulanic acid-ampicillin combinations had somewhat

more potent antibacterial effects in vitro than CP-45,899-ampicillin combinations; against *E. coli*, the difference was quite significant. Since CP-45,899 appeared equally active as an inhibi-

TABLE 3. Susceptibility of ampicillin-resistant strains to selected β -lactams in combination with CP-45,899

Antibiotic	MIC ^a (μ g/ml) of resistant strain			
	<i>Staphylococcus aureus</i> 01A400	<i>Staphylococcus epidermidis</i> 01B116	<i>Haemophilus influenzae</i> 54A042	<i>Bacteroides fragilis</i> 78C004
CP-45,899	200	>50	100	25
Penicillin G	200	25	25	100
Penicillin G + CP-45,899	6.25 + 0.39	1.56 + 1.56	1.56 + 0.78	1.56 + 3.12
Carbenicillin	12.5	ND ^b	25	200
Carbenicillin + CP-45,899	6.25 + 0.39	ND	0.39 + 0.39	3.12 + 6.25
Cefazolin	0.39	ND	25	200
Cefazolin + CP-45,899	ND	ND	3.12 + 0.20	3.12 + 0.78

^a Figures presented were selected on the basis of minimal total drug concentration.

^b ND, Not done.

TABLE 4. Activity of CP-45,899 and clavulanic acid as inhibitors of cell-free β -lactamases

Source (and type of β -lactamase)	Inhibitor	% Inhibition of β -lactam hydrolysis at inhibitor concn			
		66 μ M	16.5 μ M	3.3 μ M	0.066 μ M
<i>Staphylococcus aureus</i> 01A400 (penicillinase)	CP-45,899	99	92	0(62) ^a	0(6)
	clavulanic	99	94	22(58)	0(0)
<i>Escherichia coli</i> 51A129 (penicillinase)	CP-45,899		100	100	6(92)
	clavulanic		100	100	14(71)
<i>Klebsiella pneumoniae</i> 53A079 (penicillinase)	CP-45,899		100	100	
	clavulanic		100	100	
<i>Pseudomonas aeruginosa</i> 52A104 (cephalosporinase)	CP-45,899	27	5		
<i>Enterobacter cloacae</i> 67B009 (cephalosporinase)	CP-45,899	6	0		
<i>Bacteroides fragilis</i> 78D022 ^b (cephalosporinase)	CP-45,899	100	94	81	

^a Value in parentheses is that percent inhibition when inhibitor is preincubated with the β -lactamase for 10 min at 37°C before substrate addition.

^b Substrate was not a penicillin, but cephaloridine at 66 μ M.

TABLE 5. Comparison of CP-45,899 and clavulanic acid as potentiators and ampicillin activity against ampicillin-resistant bacteria

Resistant strains	MIC (μ g/ml) ^a	
	CP-45,899 + ampicillin	Clavulanic acid + ampicillin
<i>Staphylococcus aureus</i> 01A400	3.12 + 1.56	0.39 + 0.78
<i>S. epidermidis</i> 01B116	3.12 + 1.56	0.39 + 1.56
<i>Haemophilus influenzae</i> 54A042	1.56 + 0.78	0.78 + 0.39
<i>Proteus vulgaris</i> 57A067	6.25 + 3.12	3.12 + 12.5
<i>Escherichia coli</i> 51A129	100 + 2,000	12.5 + 12.5
<i>Neisseria gonorrhoeae</i> CDC/Res	0.31 + 0.31	0.31 + 0.31

^a MICs of CP-45,899 and ampicillin alone against these strains are presented in Table 2. Clavulanic acid alone required ≥ 25 μ g/ml except for *S. epidermidis* which was inhibited by 6.25 μ g/ml and *N. gonorrhoeae* by 5.0 μ g/ml.

TABLE 6. Comparative stability of solutions of CP-45,899 and clavulanic acid at concentrations of 1,000 µg/ml and 100 µg/ml under variable conditions at 37°C

Test conditions	Estimated half-life (h) of:			
	CP-45,899 (µg/ml)		Clavulanic acid (µg/ml)	
	1,000	100	1,000	100
0.1 M citrate phosphate buffer at pH 2.6	>100	>100	0.7	0.8
0.1 M citrate phosphate buffer at pH 7.0	>100	>100	6.7	6.4
0.1 M phosphate buffer at pH 8.0	>100	>100	6.5	6.4
Human serum at pH 7.4	>100	>100	9.4	12.0
Human urine at pH 4.5	>100	82.1	17.3	9.2
Human urine at pH 9.0	>100	>100	1.6	1.3

TABLE 7. In vivo activity of CP-45,899 and ampicillin alone and combined in a fixed 1:1 ratio against systemic infections in mice produced by ampicillin-resistant bacteria

Infecting organism	Dosage route ^a	PD ₅₀ (mg/kg) ^b		
		CP-45,899	Ampicillin	Combination ^c
<i>Staphylococcus aureus</i>				
01A400 ^d	Oral	>200	>200	29.2 ± 3.12 ^e
01A137 ^f	Oral	>200	>200	38.0 ± 2.0
<i>Haemophilus influenzae</i>				
54A042	Oral	>200	>200	35.0 ± 9.1
<i>Klebsiella pneumoniae</i>				
53A009	Subcutaneous	>200	>200	34.0 ± 2.1
<i>Proteus vulgaris</i>				
57A067	Subcutaneous	>200	>200	12.5 ± 2.4

^a Drug was administered 0.5, 4, and 24 h postchallenge for all infections except those due to *H. influenzae*. Treatment against *H. influenzae* was administered three times on day of infection and twice on days 2, 3, and 4.

^b PD₅₀, 50% protective dose.

^c Value shown is the amount of each component in the combination.

^d Also resistant to erythromycin and tetracycline.

^e 95% confidence limits.

^f Also resistant to methicillin and cephalosporin.

tor of the cell-free *E. coli* β-lactamase, it was probable that differences in the susceptibility of *E. coli* cells to the two inhibitors were based on differences in permeability.

CP-45,899 is extremely stable during storage in solution at 37°C under all conditions listed in Table 6. The half-life was greater than 100 h under all conditions, with the exception of the 100-µg/ml solution in human urine at pH 4.5, which showed a half-life of 82 h. Clavulanic acid appeared much less stable under all the conditions tested.

The combination of CP-45,899 and ampicillin in a 1:1 weight ratio produced potent, synergistic antibiotic activity in mice challenged with ampicillin-resistant strains of *S. aureus*, *H. influenzae*, *K. pneumoniae*, and *Proteus vulgaris* (Table 7). The infections produced in these

model experiments were designed to provide severe tests of the effectiveness of CP-45,899, in that neither ampicillin nor CP-45,899, used as single agents, could affect them at doses equal to or less than 200 mg/kg. Potent antibacterial activity of CP-45,899-ampicillin combinations could be demonstrated against these infections after both oral and parenteral dosing.

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