THE ANTIBACTERIAL ACTIVITY OF TICARCILLIN/ CLAVULANIC ACID (BRL28500) AGAINST TICARCILLIN-RESISTANT BACTERIA

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(Received for publication January 17, 1986)

The efficacy of BRL28500, a formulation of ticarcillin (TIPC, 15 parts) and clavulanic acid (CVA, 1 part), against TIPC-resistant strains of *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* was studied both *in vitro* and *in vivo*. The MICs of BRL28500 against these β-lactamase producing strains were lower than those of TIPC or CVA alone against such strains. When BRL28500 was added during the logarithmic growth phase of bacteria at a concentration equivalent to the MIC, it demonstrated marked lytic activity. Cells treated with BRL28500 underwent morphological change, becoming filament-like, similar to those treated with TIPC alone. With CVA alone at concentrations above the MIC the cells assumed a stable round form.

In bacterial cultures of the β-lactamase-producing strains, TIPC was protected from hydrolysis by the presence of CVA.

The *in vivo* activity of BRL28500 against experimental infections in mice caused by β-lactamase-producing strains of bacteria was superior to that of TIPC alone. TIPC and CVA were found to be well distributed in peritoneal fluid following subcutaneous administration of BRL28500 into mice with peritoneal infections. The residual TIPC concentrations achieved were higher than when TIPC alone was administered.

These results suggest that BRL28500 will be effective in the treatment of human infections due to TIPC-resistant bacteria.

It is well known that ticarcillin (TIPC) has a very broad antibacterial spectrum[1,2]. However, TIPC is somewhat susceptible to hydrolysis by the β-lactamases produced by certain Gram-negative bacteria, resulting in loss of antibacterial activity[3]. Since clavulanic acid (CVA) irreversibly inhibits the β-lactamases belonging to Richmond types II, III, IV and V[4], a combined formulation of amoxicillin (2 parts) and CVA (1 part) (Augmentin) has been developed for oral use[5,6] and shown to be extremely effective against many infections caused by β-lactamase-producing bacteria.

BRL28500 is a new formulation of TIPC (15 parts) with CVA (1 part), developed for parenteral use[7~9] which has already shown some promise in the clinic[10]. In this report, we report the results of both *in vivo* and *in vitro* studies of the activity of BRL28500 against β-lactamase-producing strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*.

Materials and Methods

Antibiotics and Test Strains

Ticarcillin sodium salt (TIPC), BRL28500 (a formulation of 15 parts TIPC and 1 part CVA) and clavulanic acid potassium salt (CVA) were provided by Beecham Yakuhin K.K., Tokyo, Japan. The bacterial strains used were clinically isolated in Japan.

Table 1. Antibacterial activity of BRL28500, TIPC and CVA against organisms used.

Organism	β-Lactamase activity* (μmol/minute/mg protein)	Antibiotic	MIC** (μg/ml)
Escherichia coli 33	0.206	BRL28500	50
		TIPC	>800
		CVA	50
E. coli 113	0.218	BRL28500	100
		TIPC	>800
		CVA	50
Klebsiella pneumoniae 102	0.00998	BRL28500	12.5
		TIPC	800
		CVA	50
K. pneumoniae 200	0.00692	BRL28500	50
		TIPC	>800
		CVA	50
Staphylococcus aureus L-5	0.660	BRL28500	25
		TIPC	200
		CVA	50

* TIPC was used as substrate.

** Inoculum size: One loopful of 10^8 cells per ml.

Determination of MICs

MICs were determined by the standard serial 2-fold dilution method, using heart infusion agar (Nissui Pharmaceutical Co., Ltd., Tokyo). An overnight culture (about 10^8 cells per ml) in Trypticase soy broth (Nissui) was used for inocula. After incubation at 37°C for 18 hours, the MICs were defined as the minimum drug concentration which completely inhibited the growth of bacteria.

Preparation of Crude β-Lactamases

An overnight culture was inoculated into Trypticase soy broth to a final concentration of 5%. The cultures were incubated for 4 hours at 37°C with shaking, harvested by centrifugation, washed twice with 0.1 M phosphate buffer (pH 7.0) then resuspended in the same buffer. The bacteria were disrupted by sonicator (Heat Systems-Ultrasonic, Inc., Plainview, N.Y.) in an ice bath at 20 kHz for 10 minutes. Crude β-lactamase was prepared by ultracentrifugation (100,000 × *g*) for 40 minutes at 4°C.

Assay of β-Lactamase

β-Lactamase activity was determined by a modification of the NOVICK micro-iodometric method[11] using TIPC as substrate. Inhibition of β-lactamase hydrolysis of TIPC was measured by incubation in 0.05 M phosphate buffer (pH 7.0) containing both TIPC and CVA and the 50% inhibition (I_{50}) value was obtained. The kinetic parameters were estimated by the least-squares method according to LINEWEAVER-BURK plots. Protein content was determined by the method of LOWRY *et al.*[12] using bovine serum albumin as the standard.

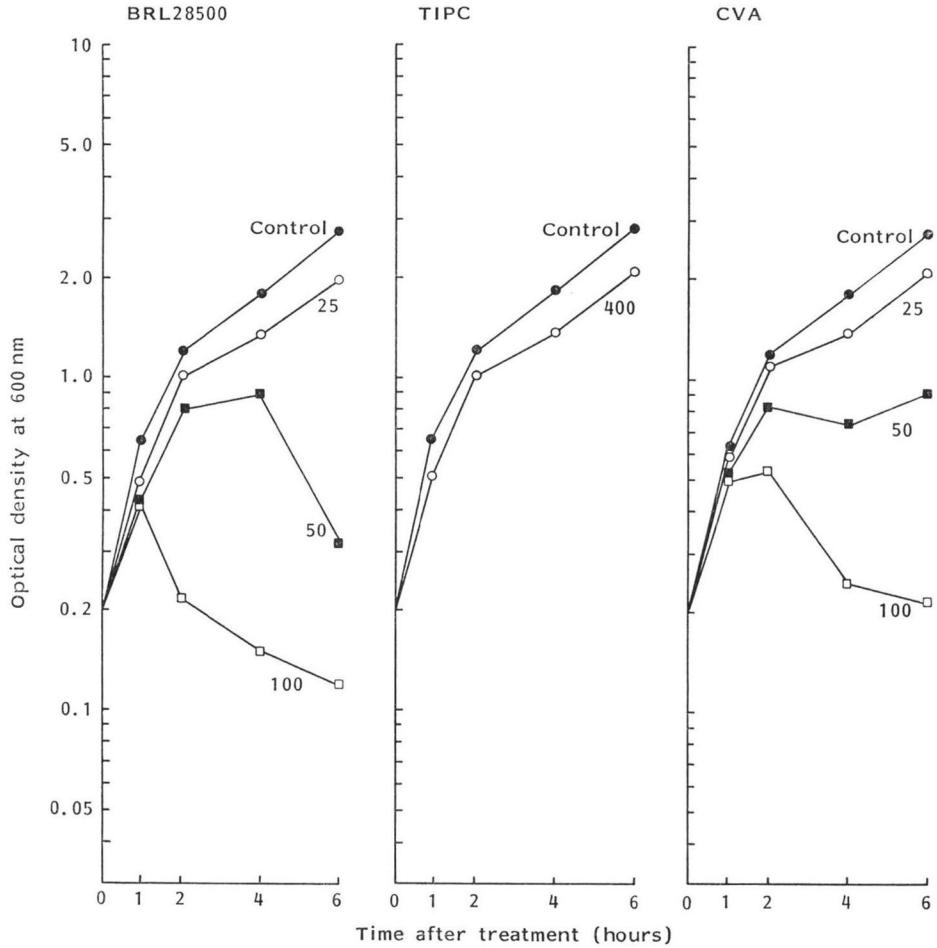
Lytic Activity and Residual Antibiotic Concentration

Change of optical density (OD) at 600 nm was measured in heart infusion broth at the logarithmic growth phase using a Shimadzu 189 spectrophotometer. At each sampling time, an equal volume of EtOH was added to the bacterial solution and after centrifugation, the concentration of antibiotics in the supernatant were determined by bioassay as described.

Observation of Morphological Changes

A small amount of bacterial cells at the logarithmic growth phase was dropped onto a heart infusion agar film containing antibiotic on a glass slide. A cover glass was placed on the agar film and sealed with paraffin. Morphological changes of the cells at 37°C were observed by differential interference microscopy.

Fig. 1. Lytic effect of BRL28500, TIPC and CVA on the growth of *Escherichia coli* 33.
MIC: BRL28500 50 μg/ml, TIPC >800 μg/ml, CVA 50 μg/ml.



Intraperitoneal Infections and Penetration of Antibiotics

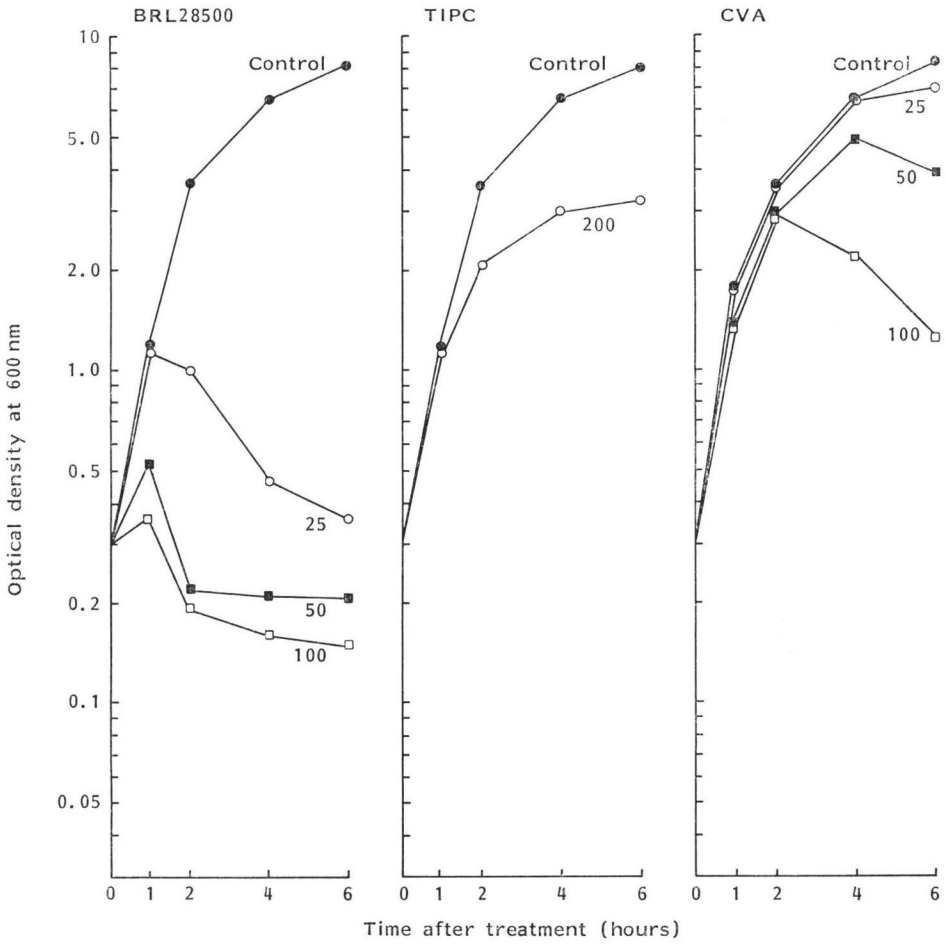
*dd*Y Male mice, weighing 18~20 g were inoculated by the intraperitoneal route with 0.5 ml of bacterial suspension in swine gastric mucin (Wako Co., Ltd., Tokyo) solution (pH 7.0). The inoculum was adjusted to 10× to 50× lethal doses by diluting the overnight culture of the test organism. Two hours after inoculation, mice were given subcutaneously 0.2 ml of antibiotic dissolved in saline. A group of ten mice was used for each dosage. The number of animals surviving after 7 days was recorded and the dose of antibiotic required to protect 50% of the infected animals (ED_{50}) was calculated using the method of LITCHFIELD-WILCOXON[13].

The concentrations of TIPC and CVA in peritoneal fluid of mice infected were also determined in the same manner as previously described[14]. At a predetermined time, the body fluids were taken from the peritoneal cavity and mixed with an equal volume of MeOH for sterilization. The supernatant was used for determination of the antibiotics by bioassay.

Bioassay

The concentration of TIPC was measured by paper-disc assay using *Pseudomonas aeruginosa* NCTC 10490 as the test organism. To determine CVA, a β-lactamase inhibition assay using heart infusion agar plates containing 60 μg/ml of benzylpenicillin and 3% of an overnight culture of *K. pneumoniae* NCTC 29665 producing penicillinase, was used.

Fig. 2. Lytic effect of BRL28500, TIPC and CVA on the growth of *Klebsiella pneumoniae* 200.
MIC: BRL28500 50 μg/ml, TIPC >800 μg/ml, CVA 50 μg/ml.



Results

Antibacterial Activity

Table 1 shows the β-lactamase activities against TIPC of the test organisms used. The table also shows the MICs of BRL28500 and its separate components, TIPC and CVA, against the organisms.

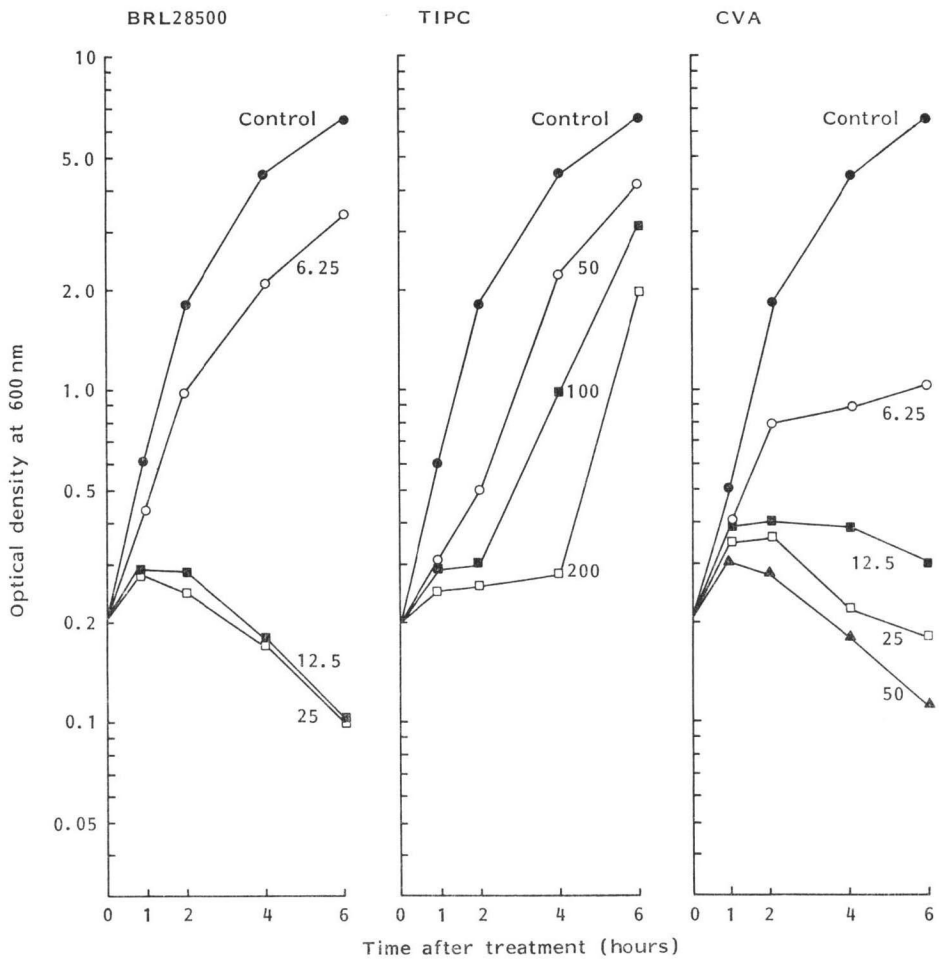
The β-lactamase activity of *E. coli* 113 was similar to that of *E. coli* 33, similarly the activities of *K. pneumoniae* 102 and *K. pneumoniae* 200 were similar. The production of β-lactamase was highest for the *S. aureus* strain followed by *E. coli* and *K. pneumoniae*.

MICs of TIPC alone against the above five strains ranged from 200 μg/ml to over than 800 μg/ml, and the strains could thus be regarded as TIPC-resistant. However, there was no apparent correlation between the β-lactamase activity and MICs of TIPC among the test organisms. In each case BRL28500 was substantially more effective than TIPC against β-lactamase-producing strains of *E. coli*, *K. pneumoniae* and *S. aureus*. Little or no advantage of BRL28500 over CVA was noted.

Lytic Activity

The time courses of changes in the optical density of culture solutions of *E. coli* 33 treated with

Fig. 3. Lytic effect of BRL28500, TIPC and CVA on the growth of *Staphylococcus aureus* L-5. MIC: BRL28500 25 μg/ml, TIPC 200 μg/ml, CVA 50 μg/ml.



various concentrations of BRL28500, TIPC and CVA are shown in Fig. 1.

At 400 μg/ml of TIPC the growth of cells was similar to that of the drug-free control. Addition of CVA at 50 μg/ml (MIC) to the cells showed inhibition of growth. BRL28500 at 50 μg/ml (MIC, equivalent to 47 μg/ml TIPC and 3 μg/ml CVA) showed strong lytic activity, particularly when compared with TIPC alone. This effect was further enhanced when BRL28500 was used at 100 μg/ml.

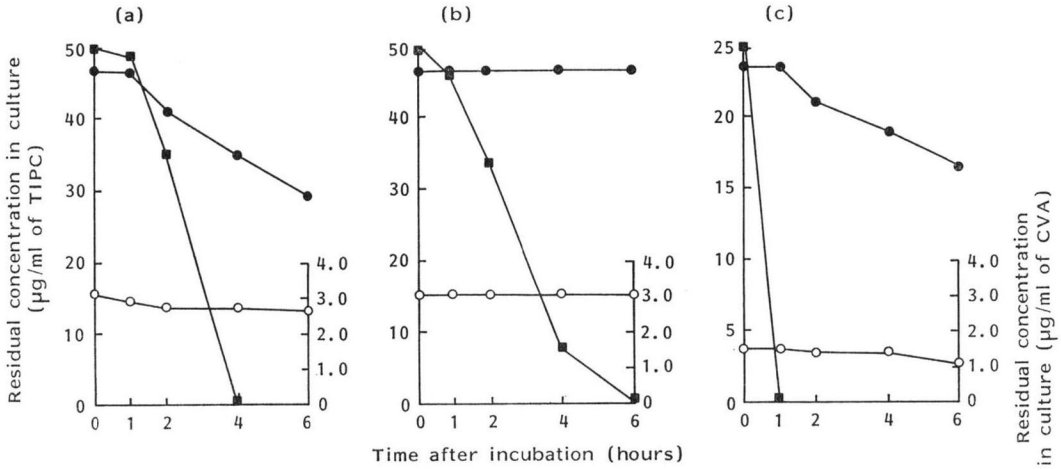
Fig. 2 shows the lytic activity of BRL28500, TIPC and CVA against *K. pneumoniae* 200 when the cells were treated in the same manner as above. TIPC at a concentration of 200 μg/ml did not suppress growth. Cells treated with 50 μg/ml (MIC) of CVA lyzed 4 hours after addition. In contrast, marked lytic activity of BRL28500 was observed at a concentration of 25 μg/ml (1/2 MIC, equivalent to 23.5 μg/ml TIPC and 1.5 μg/ml CVA) at 1 hour after addition.

Fig. 3 shows the lytic effect of BRL28500 on the *S. aureus* L-5 strain. Growth was suppressed in the cells treated with 200 μg/ml (MIC) of TIPC for 4 hours but the cells grew again at the normal growth rate 6 hours after treatment. CVA at 25 μg/ml (1/2 MIC) showed lytic action and at 12.5 μg/ml bacteriostatic action. On the other hand, BRL28500 at 12.5 μg/ml (1/2 MIC, equivalent to

Fig. 4. Residual activity of BRL28500 and TIPC in the growth cultures.

The antibiotic was added to cultures of *Escherichia coli* 33 (a) and *Klebsiella pneumoniae* 200 (b) at the concentration of 50 μg/ml and to cultures of *Staphylococcus aureus* L-5 at 25 μg/ml (c).

● TIPC after addition of BRL28500, ○ CVA after addition of BRL28500, ■ TIPC after addition of TIPC.



11.75 μg/ml TIPC and 0.75 μg/ml CVA) showed marked lytic action throughout the time of observation. The BRL28500 clearly showed stronger lytic activity against the test strains of *E. coli*, *K. pneumoniae* and *S. aureus* than either CVA or TIPC alone.

Residual Activity in Liquid Cultures

The residual concentrations in bacterial cultures treated with BRL28500, TIPC and CVA were measured simultaneously with the lytic activities.

TIPC at an initial concentrations of 50 μg/ml in the culture of *E. coli* 33 was decomposed completely 4 hours after addition, as shown in Fig. 4(a) while CVA was stable under similar conditions. In contrast, when BRL 28500 was tested about 62% of the TIPC was protected from hydrolysis for 6 hours.

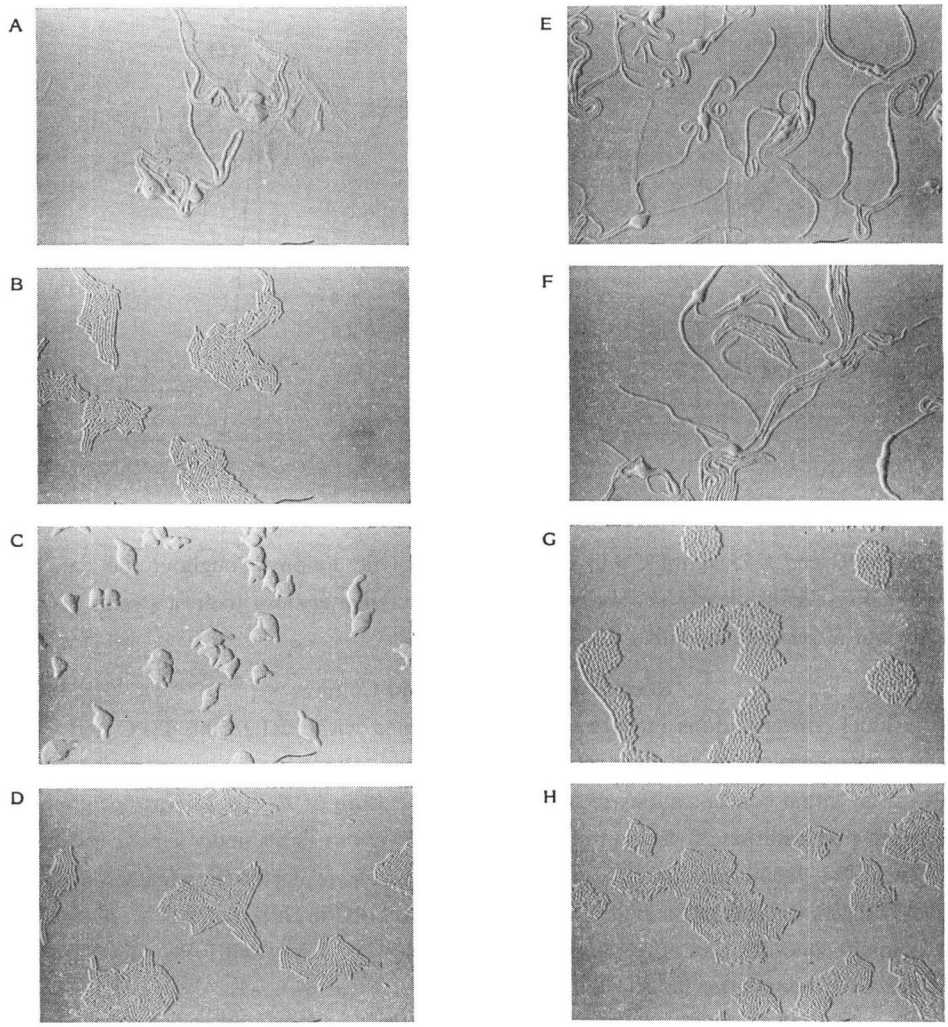
Fig. 4(b) shows the residual activity of BRL28500 and TIPC in the culture of *K. pneumoniae* 200. TIPC at an initial concentration of 50 μg/ml decomposed slowly. CVA and TIPC added as BRL28500 were not affected 6 hours after inoculation.

Fig. 4(c) shows the β-lactamase activity of *S. aureus* L-5, which hydrolyzed 25 μg/ml of TIPC almost completely within 1 hour. In contrast, TIPC in BRL28500 was hydrolyzed only weakly. CVA was stable in the culture of *S. aureus* L-5.

Morphological Changes

Fig. 5 shows a micrograph of *E. coli* 33 exposed to various concentrations of BRL28500, TIPC and CVA for 3 hours on an agar film. No morphological change was observed in the bacterial cells exposed to 400 μg/ml of TIPC (Fig. 5B), when compared to the control (Fig. 5D). The cells exposed to more than 25 μg/ml (1/2 MIC) of CVA assumed a round formation attended by "rabbit ears" (Fig. 5C). In contrast, the cells exposed to BRL28500 at the MIC (50 μg/ml, equivalent to 47 μg/ml TIPC and 3 μg/ml CVA) became filamentous, and several spherical and lyzed cells were observed (Fig. 5A). When the cells were exposed at half the MIC (25 μg/ml) rod shaped cells appeared but filamentous cells did not.

Fig. 5. Micrographs of *E. coli* 33 or *K. pneumoniae* 200 exposed to BRL28500, TIPC and CVA for 3 hours. *Escherichia coli* 33: A; BRL28500 50 μg/ml, B; TIPC 400 μg/ml, C; CVA 25 μg/ml, D; control. *Klebsiella pneumoniae* 200: E; BRL28500 12.5 μg/ml, F; TIPC 100 μg/ml, G; CVA 6.25 μg/ml, H; control.



Morphological changes in cells of *K. pneumoniae* 200 treated with the test compounds were similar to those in *E. coli* 33. The morphology of untreated cells is shown in Fig. 5H. When the cells were exposed to 100 μg/ml of TIPC, filamentous and spherical cells were formed gradually as shown in Fig. 5F. At less than 100 μg/ml of TIPC, filamentous forms were more dominant. Cells exposed to the MIC (25 μg/ml) or 1/2 MIC of CVA became spindle-shaped, similar to the changes observed in *E. coli* exposed to CVA alone. At 6.25 μg/ml (1/4 MIC), the cells assumed a round shape (Fig. 5G). When treated with concentrations of BRL28500 ranging from the MIC (50 μg/ml) to 1/16 MIC (3.13 μg/ml) filamentous cells were dominant and spherical cells appeared only occasionally (Fig. 5E).

Efficacy against Experimental Infections

Table 2 shows the therapeutic effects of BRL28500, TIPC and CVA against *E. coli*, *K. pneumoniae*

Table 2. Protective effect of BRL28500, TIPC and CVA against experimental infection in mice.

Organism	Challenge dose (cells/mouse)	Antibiotic	ED_{50} (mg/mouse)
Escherichia coli 33	1.4×10^5	BRL28500	8.0 (5.0~12.8)*
	($11\times LD_{50}$)	TIPC	>16
		CVA	>16
Klebsiella pneumoniae 102	1.8×10^8	BRL28500	2.0 (1.2~3.3)
	($9\times LD_{50}$)	TIPC	>16
		CVA	>16
Staphylococcus aureus L-5	3.9×10^7	BRL28500	10.0 (6.2~16.2)
	($39\times LD_{50}$)	TIPC	>16
		CVA	>16

Mice were treated with antibiotic subcutaneously 2 hours after intraperitoneal infection by bacteria.
* 95% confidence limit.

and *S. aureus* infections in mice.

All untreated mice died within 24 hours of infection. Beneficial therapeutic effects of BRL28500 were observed with 3 test strains at doses of 2~10 mg/mouse, against which TIPC or CVA alone was not effective under the experimental conditions. In particular, the greatest chemotherapeutic effect of BRL28500 was seen against *K. pneumoniae* 102. The *in vivo* efficacy against *K. pneumoniae* 200 could not be demonstrated because this strain is non-virulent in mice.

The Distribution of the Test Compounds in Peritoneal Fluids of Mice Infected with Test Strains

As shown in Fig. 6(a) the maximum peritoneal level for both BRL28500 and TIPC in mice infected with *E. coli* 113 occurred 15 minutes after dosing and decreased gradually thereafter. The peak level of TIPC achieved in the peritoneal cavity of mice treated with BRL28500 was higher than in mice treated with TIPC alone. Furthermore, the TIPC levels following dosing of BRL28500 were sustained longer than with TIPC alone. The elimination profile of CVA from the mice treated with BRL28500 was similar to the TIPC profile.

In the case of mice treated with *K. pneumoniae* 102 (Fig. 6b), the maximum peak level of TIPC achieved in the peritoneal fluids of mice receiving BRL28500 occurred at 60 minutes after dosing but in the mice dosed with TIPC alone the peak level was detected at 30 minutes.

Thus the maximum peritoneal half-life of TIPC dosed as BRL28500 was higher and longer than when TIPC was administered alone. The maximum concentration of CVA dosed as BRL28500 was achieved after 60 minutes and coincided with the peak level of TIPC from BRL28500.

When the mice infected with *S. aureus* L-5 were treated with BRL28500 (Fig. 6c), peak levels of TIPC and CVA were observed after 30 minutes. Both compounds were still detectable 3 hours after dosing. In contrast in mice treated with TIPC alone the level fell rapidly from the peak reaching the minimum in 1 hour.

β-Lactamase Inhibitory Activity of Clavulanic Acid

The I_{50} values for CVA against β-lactamases produced by the 5 strains used in this experiment are shown in Table 3. The β-lactamase from *S. aureus* L-5 was the most strongly inhibited. The I_{50} values of CVA against β-lactamases from *E. coli* were similar to that against β-lactamases from *K. pneumoniae*.

Fig. 6. Concentration of TIPC and CVA in peritoneal washings after treatment with BRL28500 or TIPC to mice infected intraperitoneally with β-lactamase-producing bacteria.

Mice were given subcutaneously 4 mg/mouse of antibiotic.

(a) *Escherichia coli* 113, (b) *Klebsiella pneumoniae* 102, (c) *Staphylococcus aureus* L-5.

● TIPC after treatment with BRL28500, ○ CVA after treatment with BRL28500, ■ TIPC after treatment with TIPC.

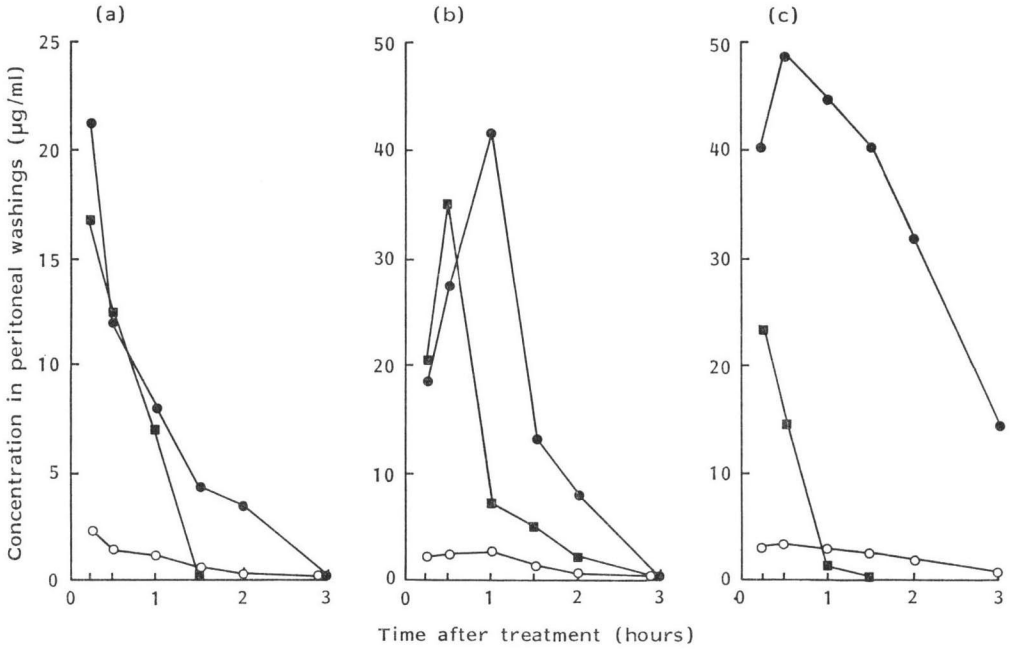


Table 3. Inhibitory effect of CVA on the β-lactamase hydrolysis of TIPC.

β-Lactamase source	β-Lactamase type (RICHMOND)	I_{50} (μg/ml)
Escherichia coli 33	III	0.220
E. coli 113	III	0.195
Klebsiella pneumoniae 102	IV	0.185
K. pneumoniae 200	IV	0.175
Staphylococcus aureus L-5	Other PCase	0.095

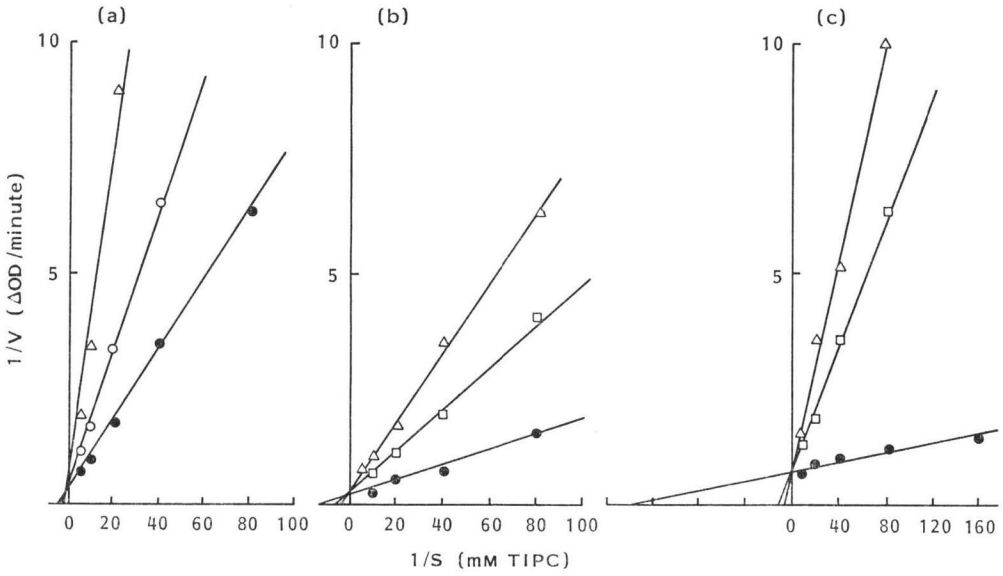
The TIPC concentration was 0.2 mM as substrate. TIPC and CVA were incubated simultaneously for 30 minutes at 30°C.

Kinetic studies of β-lactamase inhibition by CVA are shown in Fig. 7. The affinity of CVA to *S. aureus* L-5 β-lactamase was found to be higher than that to the other β-lactamases and the inhibitory action of CVA was found to be irreversible.

Discussion

It has been previously reported that the binding of TIPC to penicillin binding protein 3 (PBP 3) in *E. coli* causes the formation of filamentous cells[15]. On the other hand CVA causes the formation of round cells, due to specific binding to PBP 2[16]. Generally speaking, a combination of β-lactam antibiotics having affinity for different PBPs exerts a synergistic effect. The lytic effect of BRL28500 was more pronounced than that of TIPC or CVA alone, indicating synergism of these β-lactams. The morphological changes of *E. coli* 33 and *K. pneumoniae* 200 cells treated with BRL28500 at the

Fig. 7. Kinetic plot of the hydrolysis of TIPC by *Staphylococcus aureus* L-5 (a), *Escherichia coli* 33 (b) and *Klebsiella pneumoniae* 200 (c) β-lactamases.
● TIPC alone, ○ TIPC plus 2 μM of CVA, □ TIPC plus 5 μM of CVA, △ TIPC plus 10 μM of CVA.



MIC level were similar to those observed by treatment with TIPC alone, *i.e.* they became filamentous and, in part, spherical. Formation of round cells, typically induced by CVA, was observed only to a small extent in cells treated with BRL28500. Thus, synergistic morphological change was not markedly evidenced in BRL28500. A more detailed morphological study using electron microscopy may be needed to explain the enhanced antibacterial activity of BRL28500.

It has been reported that CVA irreversibly inhibits plasmid-mediated β-lactamase produced by Enterobacteriaceae, *Haemophilus influenzae*, *S. aureus*, *Neisseria gonorrheae*, *Branhamella catarrhalis*, and chromosomally-mediated β-lactamases produced by *Proteus vulgaris*, *K. pneumoniae* and *Bacteroides fragilis*[6,17~20]. However, inhibition of the cephalosporinases produced by Gram-negative bacilli is rather weak[4]. Consistent with these reports, *Ki* and I_{50} values of CVA against the *S. aureus* L-5 β-lactamase, which is plasmid-mediated, were lowest among the 5 strains used in this experiment. I_{50} values of CVA against type III β-lactamase of *E. coli* 33 and *E. coli* 113, and type IV β-lactamase of *K. pneumoniae* 102 and *K. pneumoniae* 200 were moderate and similar to each other, indicative of their similar enzymatic nature. The β-lactamase inhibiting activity of CVA in BRL28500 was well demonstrated by MIC value, lytic activity, ED_{50} value and residual drug levels as compared with TIPC alone. It is noted that the addition of CVA to TIPC in a ratio of 1: 15 was sufficient to suppress the β-lactamase activity of the tested strains.

Since BRL28500 is a combinatin of TIPC and CVA, it is important that the components show similar pharmacokinetic behavior *in vivo* in order to obtain maximum synergy. It has been reported earlier that TIPC and CVA are well distributed throughout the human body with similar pharmacokinetic parameters following administration of BRL28500[21]. Furthermore, the present work shows that, after administration to infected mice, TIPC and CVA are similarly distributed. The higher concentration and longer duration of TIPC in the peritoneal cavity of the infected mouse following administration of BRL28500 than those of achieved after administration of TIPC alone indicate that CVA of BRL28500 actually protected TIPC from hydrolytic inactivation by the β-lactamases of the tested strains. These observations explain the greater efficacy of BRL28500 for mice infected with TIPC-resistant strains when compared to that of TIPC or CVA alone.

Acknowledgments

The authors wish to thank Dr. S. INOUYE for his advice and assistance in the final preparation of the manuscript.

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