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# Clavulanic Acid, a Novel Inhibitor of β-Lactamases

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Clavulanic acid, Z-(2R,5R)-3-(β-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo-[3,2,0]heptane-2-carboxylic acid, has been shown to be an effective inhibitor of the β-lactamases of the Richmond types II, III, IV, and V. Inhibition is a time-dependent reaction and is irreversible. Clavulanic acid had poor antibacterial activity against *Staphylococcus aureus, Enterobacteriaceae,* and *Pseudomonas aeruginosa,* with minimal inhibitory levels greater than 25 μg/ml. It did inhibit the majority of *Neisseria gonorrhoeae* at 0.1 μg/ml and *Haemophilus influenzae* at 6.3 μg/ml. Clavulanic acid acted synergistically with penicillins and cephalosporins to inhibit β-lactamase-producing *S. aureus* and *Enterobacteriaceae.* Clavulanic acid combined with ampicillin inhibited β-lactamase-producing *N. gonorrhoeae, H. influenzae, Escherichia coli, Salmonella typhi,* and *Shigella sonnei.*

It is well recognized that the β-lactamases of both gram-positive and gram-negative bacteria contribute significantly to the resistance of bacteria to β-lactam antibiotics (8, 10). Structural modifications of the basic penicillin nucleus have produced penicillin compounds which are resistant to hydrolysis by the β-lactamases of *Staphylococcus aureus* (1). Many of these compounds—cloxacillin, oxacillin, etc.—also will inhibit the hydrolytic activity of β-lactamases produced by some gram-negative species (3, 4, 10). Although it has been possible to demonstrate synergism between β-lactam antibiotics in vitro, clinical application of the concept has been restricted to urinary infections in which the high concentrations of penicillins excreted in the urine are adequate for synergism (3, 14). Recently, naturally occurring β-lactamase inhibitors have been reported by several groups (6, 12, 17). One of these agents, clavulanic acid, has weak antibacterial activity but is a potent inhibitor of β-lactamases (1, 6, 12). We have studied the activity of this compound against clinical isolates and investigated its inhibition of gram-negative β-lactamases.

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## MATERIALS AND METHODS

Clavulanic acid was provided as a dry crystalline powder by Beecham Laboratories. Ampicillin and amoxicillin were also provided by Beecham Laboratories. Cephalothin, cephaloridine, and cefamandole were gifts from Eli Lilly Laboratories. Bacterial isolates were from our collection of organisms containing various β-lactamases.

**Preparation of β-lactamases.** The β-lactamases consisted of either ultrasonic extracts of bacterial cells or enzymes purified to homogeneity as previously published (9). Fresh overnight cultures of bacteria were inoculated into brain heart infusion broth and grown for 2 h at 35°C in a rotary shaker. *Klebsiella pneumoniae* and *Proteus mirabilis* were induced with cephalothin at 25 μg/ml. *Citrobacter freundii* and *Serratia marcescens* were induced with cephaloridine at 50 μg/ml, and *Pseudomonas aeruginosa* was induced with penicillin G at 500 μg/ml. Inducers were added, and incubation was continued for an additional 3 h. The bacterial cells were collected by centrifugation, resuspended, and washed with potassium phosphate buffer (0.05 M, pH 7.0) at 4°C. The bacteria were recentrifuged and subsequently resuspended in the same buffer, 10-fold concentrated. The bacteria were disrupted by sonic treatment for 3 min in an ice bath. Cellular debris was removed by centrifugation for 2 h at 40,000 × *g* at 4°C. The supernatant material was dialyzed for 24 h against phosphate buffer at 4°C. The dialyzed material was stored frozen. Periplasmic β-lactamases were obtained by the osmotic shock technique and purified by diethylaminoethyl-cellulose column chromatography (9). β-Lactamases were characterized by substrate against penicillin G, ampicillin, cephaloridine, and cephalothin and by inhibition by cloxacillin and *p*-chloromercuribenzoate (13).

**β-Lactamase assays and inhibition.** β-Lactamase activity was determined either by a spectrophotometric method, using the change in optical density at 255 nm with cephalosporins as substrate, or by a modification of the Novick microiodometric method (9). Inhibition of the hydrolysis of cephaloridine and ampicillin was measured by the same method by incubating β-lactamase with clavulanic acid before addition of the substrate. The chromogenic cephalospo-

rin, 87/312, was used to screen organisms for β-lactamase activity (11).

**Susceptibility and synergy tests.** Minimal inhibitory concentrations (MICs) were determined by either agar or broth dilution, using Mueller-Hinton medium (Baltimore Biological Laboratory) incubated at 35°C for 18 h. An inoculum of $10^5$ colony-forming units was used. Synergy studies and killing curves were peformed, using $10^5$ colony-forming units in Mueller-Hinton broth containing various concentrations of antibiotics in a checkerboard fashion. Samples (0.01 ml each) were plated to agar for bactericidal levels. Synergy was defined as a fourfold reduction in the minimal inhibitory or minimal bactericidal levels of both agents. Partial synergy was defined as a fourfold decrease in the MIC of one compound and either a twofold or no decrease in the MIC of the other compound.

## RESULTS

Clavulanic acid showed a low degree of antibacterial activity when tested against *S. aureus* (mean MIC was 25 μg/ml), most of the *Enterobacteriaceae* (mean MIC was 25 μg/ml), *Pseudomonas aeruginosa* (mean MIC was >200 μg/ml), *Bacteroides fragilis* (mean MIC was >50 μg/ml), and *Haemophilus influenzae* (mean MIC was 6.3 μg/ml). It did, at 0.1 μg/ml, inhibit 75% of the *Neisseria gonorrhoeae* tested. When clavulanic acid was combined with ampicillin (Table 1), it did result in a synergistic action of the two agents against selected organisms. Figure 1 illustrates the killing curve which resulted when clavulanic acid and ampicillin were combined against a Richmond type III β-lactamase-producing *E. coli*. There was a two-log decrease in colony-forming units after 6 h, during which time the organism increased by three logs in the presence of either agent alone. The overall results of the combination of clavulanic acid when tested against 163 organisms are shown in Table 2. It is apparent that the percentage of strains of a species for which synergy could be demonstrated varied from 100 to 14% for the β-lactamase-producing *Enterobacteriaceae* and from 100 to 0% for bacteria in which β-lactamase activity could not be detected when assayed by using noninduced bacteria and utilizing both the chromogenic assay and the iodometric assay.

TABLE 1. *Combination of clavulanic acid and ampicillin tested against β-lactamase-producing organisms*[a]

| Organism | MBC (μg/ml) of:[b] | | | |
|---|---|---|---|---|
| | Ampicillin | Clavulanic acid | Ampicillin and clavulanic acid[c] | |
| | | | 1[c] | 2[d] |
| *S. aureus* | 63 | 25 | 0.1 | 0.4 |
| *E. coli* | 400 | 25 | 6.3 | 0.8 |
| *Klebsiella* | 50 | 25 | 6.3 | 0.8 |
| *Enterobacter* | 400 | 12.5 | 3.1 | 0.8 |
| *S. typhi* | 400 | 25 | 25 | 0.8 |
| *S. sonnei* | 400 | 25 | 6.3 | 0.8 |
| *P. aerugi-nosa* | 400 | 25 | 100 | 3.1 |
| *P. morganii* | 25 | 50 | 3.1 | 3.1 |

[a] Twofold dilutions of antibiotic were made in Mueller-Hinton broth in a checkerboard fashion. $10^5$ colony-forming units of each organism were used. Clear tubes were plated (0.01 ml) to agar after overnight incubation.

[b] MBC, Minimal bactericidal concentration.

[c] Clavulanic acid was at 0.8 μg/ml.

[d] Clavulanic acid was at 1.6 μg/ml.

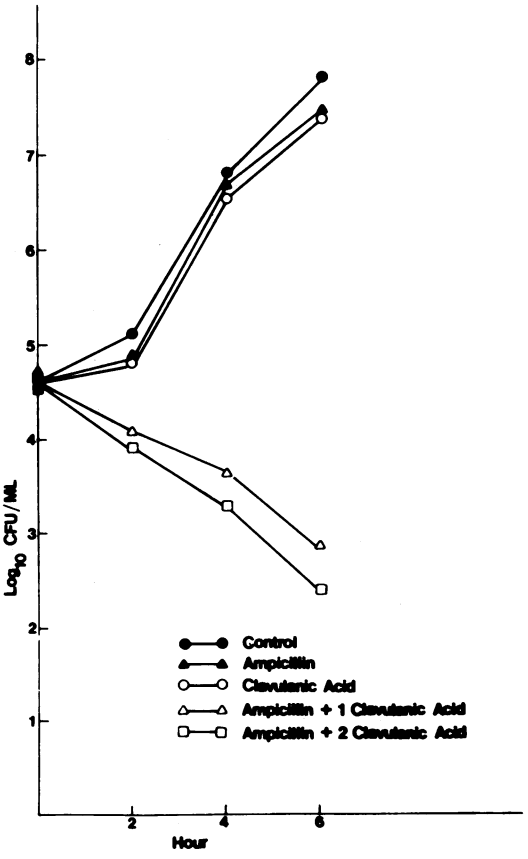


FIG. 1. *Bactericidal activity of ampicillin combined with clavulanic acid. An E. coli isolate containing a Richmond type III, plasmid-mediated, constitutive β-lactamase for which the MIC of ampicillin was >1,000 μg/ml and that of clavulanic acid was 25 μg/ml was grown overnight in Mueller-Hinton broth. It was diluted in fresh broth to yield 6 × $10^4$ colony-forming units. Symbols: ●, E. coli isolate alone; ▲, isolate in the presence of 6.3 μg of ampicillin per ml; △, isolate in the presence of 6.3 μg of ampicillin per ml and 0.8 μg of clavulanic acid per ml; □, isolate in the presence of 6.3 μg of ampicillin per ml and 1.6 μg of clavulanic acid per ml.*

Overall, however, synergy was more likely to be seen against β-lactamase-producing isolates (56%) than against bacteria which lacked β-lactamases (31%). The combination of clavulanic acid and ampicillin was synergistic over a concentration range of one part clavulanic acid and ten parts ampicillin to equal parts of the drugs.

Synergy of clavulanic acid was not limited to its combination with ampicillin. The combination of clavulanic acid with amoxicillin and with three cephalosporins resulted in synergy (Table 3) against some bacteria, but not against other bacteria. No synergy was found for *P. aeruginosa* which contained type I β-lactamase.

TABLE 2. *Synergy of clavulanic acid and ampicillin when tested against β-lactamase-positive and -negative species*

| Species | Presence of β-lactamase | No. tested | Synergy (%) |
|---|---|---|---|
| *S. aureus* | + | 4 | 100 |
| | − | 12 | 100 |
| *E. coli* | + | 5 | 100 |
| | − | 10 | 0 |
| *K. pneumoniae* | + | 5 | 100 |
| | − | 10 | 100 |
| *Enterobacter* sp. | + | 14 | 21 |
| | − | 2 | 0 |
| *P. mirabilis* | + | 1 | 100 |
| | − | 3 | 33 |
| *Proteus*, indole positive | + | 2 | 100 |
| | − | 10 | 0 |
| *Salmonella* sp. | + | 4 | 50 |
| | − | 12 | 8 |
| *Shigella sonnei* | + | 4 | 66 |
| | − | 12 | 10 |
| *P. aeruginosa* | + | 14 | 14 |
| | − | 2 | 0 |
| *N. gonorrhoeae* | + | 2 | 100 |
| | − | 12 | 0 |
| *H. influenzae* | + | 2 | 100 |
| | − | 12 | 0 |
| *B. fragilis* | + | 3 | 100 |
| | − | 6 | 100 |

To determine which β-lactamases were inhibited by clavulanic acid, its inhibitory activity was tested against isolated β-lactamases. Table 4, which compares the reaction velocities in the presence of clavulanic acid, demonstrates that clavulanic acid was a poor inhibitor of Richmond type I β-lactamases, whether the enzymes were induced or constitutive. It inhibited the hydrolysis of ampicillin by type II enzymes and the hydrolysis of ampicillin or cephaloridine by type III, IV, and V enzymes.

The inhibition of β-lactamase activity by clavulanic acid is a time-dependent reaction (Table 5). When enzyme and clavulanic acid were added simultaneously, there was minimal inhibition of the hydrolytic activity against either of the penicillin substrates. This preincubation requirement is seen with all of the types of β-lactamases for which clavulanic acid is an inhibitor. However, it is possible to overcome the requirement for preincubation of clavulanic acid and enzyme if the concentration of enzyme is low and the concentration of clavulanic acid is higher than that required for inhibition (Table 5). For example, a fourfold increase in the concentration of clavulanic acid resulted in a 50 to 60% increase in inhibition against type III and V enzymes, but had no effect against type I β-lactamases. It was possible to partially inhibit certain type I β-lactamases by preincubation, whereas others were not inhibited even after prolonged preincubation. Preincubation for 20 min produced an

TABLE 3. *Minimal bactericidal concentration of the combination of clavulanic acid and β-lactam antibiotics*

| Organism | Type β-lactamase | Cavulanic acid[a] | MBC (μg/ml) of:[a] | | | |
|---|---|---|---|---|---|---|
| | | | Amoxicillin | Cephaloridine | Cephalothin | Cefamandole |
| *E. coli* | III | − | 400 | 12.5 | 12.5 | 3.2 |
| | | + | 3.1 | 1.6 | 1.6 | 1.6 |
| *S. sonnei* | III | − | 400 | 12.5 | 12.5 | 12.5 |
| | | + | 3.1 | 6.3 | 1.6 | 1.6 |
| *P. mirabilis* | IV | − | 400 | 800 | 800 | —[b] |
| | | + | 3.1 | 6.3 | 25 | — |
| *E. aerogenes* | V | − | 50 | 400 | 400 | 400 |
| | | + | 0.4 | 400 | 400 | 400 |
| *P. aeruginosa* | I, | − | 400 | 400 | 400 | 400 |
| | | + | 400 | 400 | | |
| *P. aeruginosa* | I, III | − | 400 | 400 | 400 | 400 |
| | | + | 400 | 400 | 400 | 400 |

[a] The minimal bactericidal concentration (MBC) of clavulanic acid for all of the organisms was >25 μg/ml. The concentration of clavulanic acid in each test was 2.5 μg/ml.

[b] —, Not done.

inhibition of a *P. morganii* enzyme, but not of a *C. freundii* enzyme. The inhibition of type III β-lactamase activity by clavulanic acid was of an irreversible nature (Table 6). Clavulanic acid could not be dialyzed free from the enzymes once it had bound to the enzyme.

The kinetic data for the inhibition of Richmond type III β-lactamase is shown in Fig. 2. The reaction was run without preincubation to simulate the situation which might occur in vivo. Under these conditions, the reaction is competitive. The $K_m$ of ampicillin is 0.118 mM; the $K_m$ of ampicillin and clavulanic acid is 0.1818 mM, and the $K_i$ is 4.3 μM. The $K_m$ of cephaloridine is 0.5 mM; the $K_m$ of cephaloridine and clavulanic acid is 4 mM, and the $K_i$ is 0.17 μM. The $K_i$ of cloxacillin for this enzyme is 18.7 μM, indicating that clavulanic acid is a much more potent inhibitor. When clavulanic acid was preincubated with the enzyme for 10 min, the $K_i$ with ampicillin was $8.4 \times 10^{-3}$ μM.

TABLE 4. *Effect of clavulanic acid upon the inhibitory activity of β-lactamases of different species*[a]

| Organism | Type β-lactamase | Relative velocity (%) |
|---|---|---|
| *P. aeruginoa* | I (Induced) | 96 |
| *P. morganii* | I | 98 |
| *S. marcescens* | I (Induced) | 86 |
| *C. freundii* | I | 90 |
| *E. coli* | II | 30 |
| *P. mirabilis* | II | 20 |
| *E. coli* | III | 2 |
| *P. aeruginosa*[b] | III | 13 |
| *K. pneumoniae* | IV | 9 |
| *S. sonnei* | V | 6 |

[a] β-Lactamases were incubated with clavulanic acid and cephaloridine. Concentrations of clavulanic acid and of cephaloridine were 2.26 μM and 0.1 mM, respectively. Spectrophotometric assay was used.
[b] Purified enzymes.

## DISCUSSION

As β-lactamase-producing members of the *Enterobacteriaceae* and of *P. aeruginosa* have increased in clinical importance, the search for β-lactamase-resistant compounds has increased. β-Lactamase-resistant competitive inhibitors of the common β-lactamases are present in the form of antistaphylococcal penicillins of the isoxazoyl class, but these agents, probably by virtue of their bulky side chains, have not proved to be useful with intact bacteria and are even less useful clinically. Clavulanic acid has, as earlier studies (12, 18) and this one illustrate, excellent inhibitory activity against selected β-lactamases. This inhibitory activity covers β-lactamases of the Richmond classes II, III, IV, and V. The inhibition of β-lactamase hydrolytic activity is a time-dependent reaction and is irreversible. Some enzymes of the Richmond class I type, primarily cephalosporin-hydrolyzing β-lactamases, are inhibited by clavulanic acid at high concentrations after preincubation, but this inhibition does not result in synergy of clavulanic acid and other β-lactam compounds when tested with intact bacteria. The nature of the binding of clavulanic acid to β-lactamases of different classes may not be the same in all instances. The kinetic data suggest that if clavulanic acid and other penicillins are present simultaneously there is completion for receptor sites, but with previous exposure of enzyme to clavulanic acid it has bound irreversibly to the hydrolytic site.

Clavulanic acid acted synergistically to inhibit β-lactamase-producing *S. aureus* and *Enterobacteriaceae* when combined with ampicillin, amoxicillin, cephalothin, cephaloridine, and cefamandole. It also synergistically inhibited strains which failed to demonstrate β-lactamase activity as assayed by the cephalosporin chromogenic technique. The explanations for this phenomenon may be several. A number of these bacteria contain minute amounts of strategically placed β-lactamase (8) which protect penicillin

TABLE 5. *Effect of increasing clavulanic acid concentration and of preincubation upon inhibition of β-lactamase activity*

| Enzyme source (type) | Substrate[a] | % Hydrolysis of substrate | | | | | |
|---|---|---|---|---|---|---|---|
| | | Clavulanic acid concn (μg/ml) | | | | Preincubation time (min)[b] | |
| | | 0 | 0.5 | 1 | 2 | 10 | 20 |
| *E. coli* (III) | Ampicillin | 100 | 72 | 50 | 30 | 30 | 20 |
| *S. sonnei* (V) | Ampicillin | 100 | 94 | 61 | 35 | 35 | 20 |
| *P. morganii* (I) | Cephaloridine | 100 | 99 | 93 | 90 | 75 | 47 |
| *C. freundii* (I) | Cephaloridine | 100 | 96 | 95 | 95 | 95 | 95 |

[a] Concentrations of ampicillin and cephaloridine were 0.1 and 0.2 mM, respectively.
[b] Calvulanic acid was at 0.5 μg/ml.

TABLE 6. *Irreversible nature of clavulanic acid inhibition of β-lactamase*[a]

| Enzyme preparation | Substrate hydrolyzed (μmol/min) | Rate (%) |
|---|---|---|
| Enzyme[b] | 1.191 | 100 |
| Dialyzed enzyme[c] | 1.147 | 96 |
| Enzyme + clavulanic acid[b] | 0.063 | 5.3 |
| Enzyme + clavulanic acid[c] | 0.051 | 4.3 |

[a] Enzyme activity was determined with the starch-iodometric assay.

[b] β-Lactamase activity was assayed after 15 min of incubation.

[c] Assay was performed after 24 h of dialysis at 4°C.

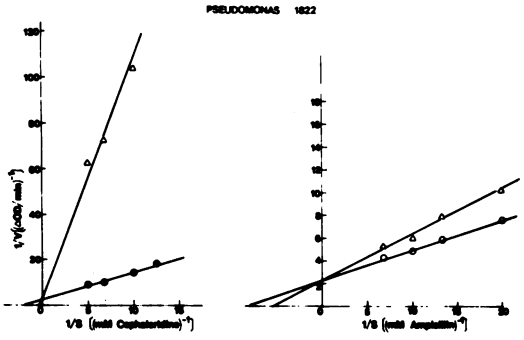


FIG. 2. *Plot of the hydrolysis of cephaloridine and ampicillin by a purified type III β-lactamase. The reaction was run without preincubation. Shown on the left are cephaloridine alone (●) and cephaloridine with 0.25 μg of clavulanic acid per ml (△); on the right are ampicillin alone (○) and in the presence of 0.5 μg of clavulanic acid per ml (△).*

binding proteins, and the clavulanic acid binds to this "cryptic" β-lactamase and inhibits hydrolysis of the entering β-lactam, which then can bind to a receptor protein (16). Secondly, many of these strains contain β-lactamases which are induced, and the clavulanic acid is able to bind to the enzyme as it is produced. Finally, some of the bacterial cell endopeptidases are now known to possess β-lactamase activity, and clavulanic acid may bind to these enzymes (7).

The fact that the combination of clavulanic acid and β-lactam compounds is synergistic in terms of both MICs and minimal bactericidal concentrations indicates that the inhibition also is irreversible in intact cells as well as with isolated, purified enzymes. Since clavulanic acid inhibits type III β-lactamases which are plasmid mediated, one might expect that it would inhibit all organisms with this enzyme. Unfortunately, some bacteria also possess low levels of the type I enzymes. Clavulanic acid is least useful against some of the organisms which are of greatest concern in the hospital, namely, *P. aeruginosa* and *S. marcescens*. These organisms contain cephalosporin-hydrolyzing enzymes which are inducible. Although clavulanic acid inhibits the plasmid-mediated β-lactamase of some *Pseudomonas* isolates, these organisms also contain a Richmond type Id β-lactamase, which is the inducible enzyme used by Sabath, Jago, and Abraham (15), and this enzyme is not inhibited by the concentration of clavulanic acid that normally could be achieved.

Clavulanic acid, however, will act synergistically with ampicillin or amoxicillin against the *E. coli*, *Shigella*, and *Salmonella* isolates, including *S. typhi*, which now often are resistant to ampicillin and amoxicillin. Furthermore, clavulanic acid and ampicillin act synergistically against most *Klebsiella* isolates which heretofore have not been inhibited by ampicillin.

These data suggest that clavulanic acid may be a most useful compound to reexpand the spectrum of aminopenicillins against the most important bacteria encountered in outpatient settings and in those hospital situations in which plasmid resistance-mediated β-lactamase production has become important. Clinical and animal studies are needed to demonstrate that these in vitro findings with isolated enzyme preparations and with intact bacteria have application to humans.

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