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Antibacterial Characteristics of YTR 830, a Sulfone β-Lactamase Inhibitor, Compared with Those of Clavulanic Acid and Sulbactam

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The antibacterial activity, binding to penicillin-binding proteins, and morphological changes effected by YTR 830, a sulfone β-lactamase inhibitor, were studied in comparison with those of other β-lactamase inhibitors. YTR 830 had very poor antibacterial activity, bound to PBP 2 of gram-negative organisms, and at the MIC caused rapid lysis of spheroplasts formed.

New beta-lactams are more stable and resistant to hydrolysis by β-lactamases, and they are also inhibitors of the enzymes. Beta-lactam compounds, such as clavulanic acid and sulbactam, exhibit poor or weak intrinsic antibacterial activity but are good inactivators of β-lactamases, and thus they are used strictly as inhibitors in potentiating the activity of an otherwise β-lactamase-labile beta-lactam. YTR 830 is a new derivative of penicillanic acid sulfone, which has been shown to be an active β-lactamase inhibitor (1, 7; F. Moosdeen, S. Yamabe, and J. Williams, 1st Eur. Congr. Clin. Microbiol., Bologna, Italy, 1983, abstr. no. 423). In this study, we compared the activities of YTR 830, clavulanic acid, and sulbactam against a series of gram-negative bacterial strains. The interaction of the inhibitors with the penicillin-binding proteins (PBPs) of these organisms was studied, along with morphological responses caused by the inhibitors at their MICs.

The bacterial strains selected for study were strains that had no detectable β-lactamases and were susceptible to ampicillin (MIC, 1 to 4 μg/ml). The following organisms were used: *Escherichia coli* J62-1, *Klebsiella aerogenes* K2, *Citrobacter freundii* 5, *Enterobacter cloacae* 92, and *Proteus mirabilis* 254. The MICs of the compounds were determined by using the agar plate dilution method. Final concentrations of the compounds, ranging from 1 to 256 μg/ml, were added to Diagnostic Sensitivity Test Agar (Oxoid Ltd.). The inoculum used was approximately 10^5 CFU. The MIC was taken as the lowest concentration of compound at which no growth was visible.

TABLE 1. Competition of β-lactamase inhibitors for PBPs of gram-negative bacteria

Organism and agent	Concn (μg/ml) required to give 90% binding of [^{14}C]benzylpenicillin (estimated by visual inspection)								MIC (μg/ml)
	PBP 1	PBP 1a	PBP 1b	PBP 2	PBP 3	PBP 4	PBP 5	PBP 6	
E. coli J62-1									
Clavulanic acid		128	256	8	256	128	256	256	32
Sulbactam		32	1,024	128	512	>1,024	>1,024	1,024	32
YTR 830		64	1,024	64	>1,024	256	>1,024	1,024	512
P. mirabilis 254									
Clavulanic acid		64	>256	32	>256	64	64	256	64
Sulbactam		32	512	512	>1,024	1,024	512	>1,024	128
YTR 830		32	>1,024	128	1,024	256	512	>1,024	512
K. aerogenes K2									
Clavulanic acid	>256			16	256	>256	>256	>256	32
Sulbactam	1,024			512	>1,024	>1,024	>1,024	>1,024	64
YTR 830	512			>1,024	>1,024	>1,024	>1,024	>1,024	512
C. freundii 5									
Clavulanic acid	>256			4	>256	128	128	>256	32
Sulbactam	1,024			128	256	1,024	512	>1,024	32
YTR 830	512			32	1,024	1,024	512	>1,024	512
E. cloacae 92									
Clavulanic acid	256			4	>256	128	>256	256	32
Sulbactam	>1,024			128	512	1,024	>1,024	1,024	32
YTR 830	>1,024			64	>1,024	>1,024	>1,024	>1,024	512

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Cell membrane was prepared by growing 3-liter cultures of the strains in Antibiotic Medium no. 3 (Oxoid) at 37°C on an orbital shaker until an optical density of 0.8 to 0.9 at 675 nm was reached. The cells were harvested by centrifugation, washed once with cold phosphate buffer, and suspended in 80 ml of cold 10 mM phosphate buffer (pH 7.0) containing 140 mM β-mercaptoethanol. The cells were sonicated, and unbroken bacteria were removed by centrifugation at 6,000 × *g* for 20 min at 4°C. Bacterial membranes contained in the supernatant were sedimented by centrifugation (10,000 × *g* for 30 min at 4°C). The membrane pellet was suspended in cold 10 mM phosphate buffer (pH 7.0) and washed once. The final pellet was then suspended in phosphate buffer, divided into aliquots, and stored at −70°C. Protein content was determined by the method of Lowry et al. (10) with bovine serum albumin as the standard. The membrane suspension was adjusted to give a protein concentration of 7 to 10 μg/ml before use.

The final concentrations of the compounds in the PBP assay ranged from 0.25 to 256 μg of clavulanic acid and 1 to 1,024 μg of sulbactam and of YTR 830 per ml. To each tube containing 10 μl of the dilutions of compounds (equilibrated to 30°C), 90 μl of membrane preparation (thawed and warmed to 30°C) was added at timed intervals. The mixture was vortexed briefly and incubated at 30°C for 10 min, after which 10 μl of [^{14}C]benzylpenicillin (50 μCi/ml; 54 μCi/mol) was added to each tube, mixed, and further incubated for 10 min. Nonradioactive benzylpenicillin (10 μl of 6 mg/ml), followed by 10 μl of 20% (wt/vol) aqueous Sarkosyl, was added to each tube to terminate the reaction. After 20 min of standing at room temperature (extraction) followed by centrifugation at 10,000 × *g* (30 min at 10°C), the supernatant (Sarkosyl-soluble extracts) was mixed with sample buffer and heated at 100°C for 3 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) with modifications as described by Spratt and Pardee (13). The running gel was composed of 10% (wt/vol) acrylamide with a ratio of acrylamide to *N*, *N*′-methylenebisacrylamide of 100 to 1. Electrophoresis was done at constant currents of 15 mA through the stacking gel and 25 mA through the running gel. The gels were stained with Coomassie brilliant blue R, followed by destaining in multiple changes of destaining solution. The gels were prepared for fluorography by the method of Bonner and Laskey (2), and the dried gels were exposed to prefogged Kodak X-Omat XRP film at −70°C for 4 to 6 weeks. PBP binding at the 90% level was estimated by visual examination of the developed X-ray films.

The strains used in studies of morphological effects were those used in the PBP assay. A 0.1-ml sample of broth cultures grown overnight in Antibiotic Medium no. 3 was used to inoculate 10 ml of fresh prewarmed (37°C) medium, and incubation continued. During the early logarithmic phase of growth, the β-lactamase inhibitors were added to give final concentrations at the MIC. At 0, 1, 2, 4, 6, and 8 h of incubation, 0.05 ml of the culture was removed. Ten-microliter volumes of the cultures were mixed with equal volumes of 1% formaldehyde on a glass slide and examined for morphological changes.

The affinities of the three compounds for PBPs measured by a competition assay for five gram-negative strains are shown in Table 1. PBP 2 was the prime target of clavulanic acid for all strains. The primary affinities of YTR 830 and sulbactam differed in different species. In *K. aerogenes* and *P. mirabilis*, the prime target of YTR 830 was PBP 1; in *E. coli*, *C. freundii*, and *E. cloacae*, the binding was mainly to PBP 2. Sulbactam bound primarily to PBP 1 in *E. coli*, *P. mirabilis*, and *K. aerogenes* and to PBP 2 in *C. freundii* and *E. cloacae*.

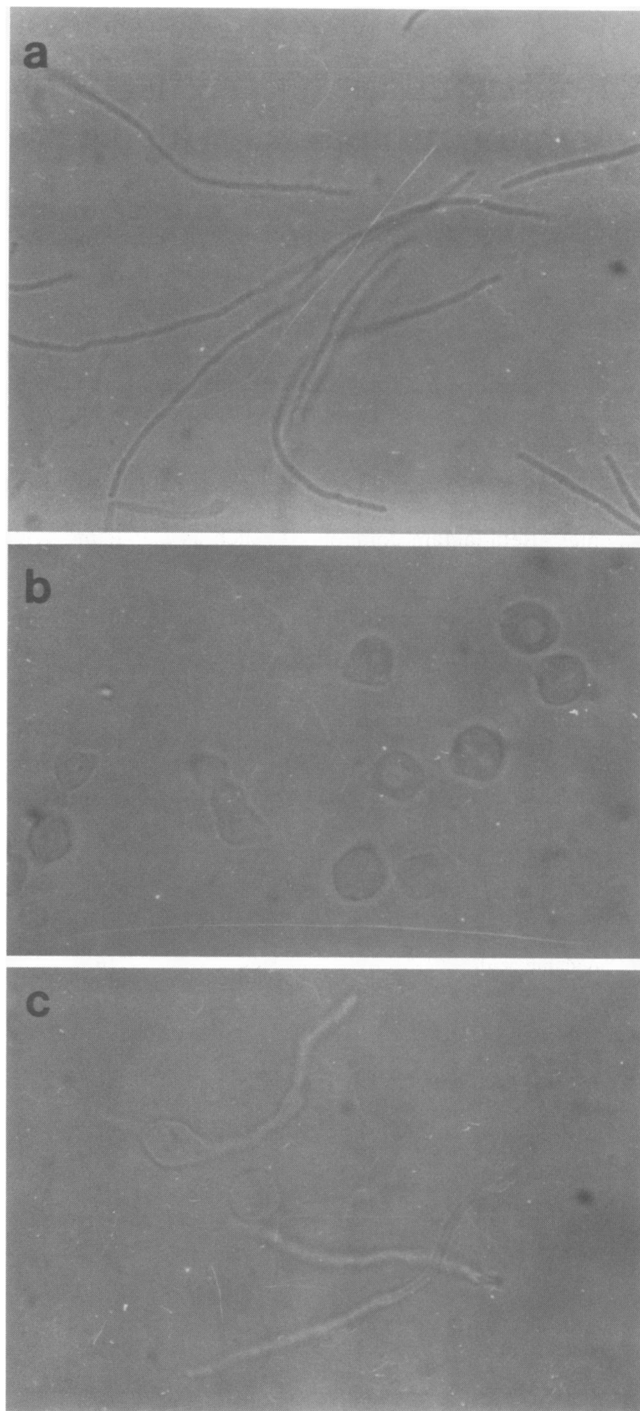

FIG. 1. Morphological changes effected by β-lactamase inhibitors at MIC. (a) Elongation of *E. coli* by sulbactam at 4 h. (b) Spheroplasts of *P. mirabilis* by YTR 830 at 2 h. (c) Outpouch formation on filament of *P. mirabilis* caused by sulbactam at 4 h.

At the MIC, clavulanic acid caused spheroplast formation in all strains during the first 2 h. Most of the spheroplasts lysed at 6 h. Sulbactam, however, caused filament formation. The cells elongated three to six times their original

length after contact (Fig. 1a). Lysis of *E. coli*, *K. aerogenes*, and *E. cloacae* occurred at about 6 h after contact. YTR 830 (at the MIC) had a more dramatic effect on the cells than the other two compounds. Spheroplasting occurred rapidly during the first 2 h of contact with the compound. A proportion of *Klebsiella* cells were lysed as early as 2 h after contact, and most of the cells were lysed by 4 h. The morphological effects on *P. mirabilis* were different from those on other species tested. Although the general effect by YTR 830 and clavulanic acid was spheroplasting, the cytoplasm contained materials of different densities (Fig. 1b). In addition to filamentous forms in *P. mirabilis*, sulbactam produced prominent bulges within the filaments (Fig. 1c).

YTR 830 had much lower intrinsic activity (MIC, ≥256 μg/ml) than clavulanic acid or sulbactam (mode MIC, 32 μg/ml) against strains of the family *Enterobacteriaceae* and the nonfermentative gram-negative organisms tested. The activity of these inhibitors was not related to the presence or absence of β-lactamases, since organisms producing β-lactamases had similar spectra of inhibition (data not shown). However, YTR 830, like clavulanic acid and sulbactam, is active against *Acinetobacter* species, which are known to be resistant to many beta-lactams (except imipenem) (MIC, >50 μg/ml) (5).

The affinity of clavulanic acid for PBP 2 of the organisms was comparable to the values obtained by Spratt (12) and Masson et al. (11) for PBP 2 of *E. coli* K-12. Formation of bulging round cells which accompanied this PBP 2 binding was common to all five strains. Affinity values of sulbactam for PBPs 1a and 2 of *E. coli* were comparable to those obtained by Masson and co-workers (11) and Curtis et al. (4). Binding to PBP 1 and 2 would result in round forms or spheroplasts (13). In this study, we observed filamentation. Our results of filamentation were in accord with the observation of Greenwood and Eley (6), when low concentrations (8 to 256 μg/ml) of sulbactam were used. This inconsistency between PBP affinity and morphological response could be due to unstable complexes between PBPs and the beta-lactam (8). Temocillin induces filamentation (14) despite its affinity for PBP la, as determined by the classical competitive assay. Modification of temperature and incubation time increased the affinity for PBP 3 about 300-fold. Binding of tigemonam to PBPs was also found to be temperature dependent (3). Direct labeling or modifications in the assay could therefore give a better indication of binding of beta-lactams to PBPs.

β-Lactamase inhibitors are mainly used in combination with a second beta-lactam primarily to inhibit the β-lactamase. They may nevertheless exert some intrinsic activity at sub-MICs reached in patients or in susceptibility tests.

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