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Influence of β-Lactamase Inhibitors on the Potency of Their Companion Drug with Organisms Possessing Class I Enzymes

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The ability of β-lactamase inhibitors to induce class I β-lactamases in certain organisms in vitro suggests a potential for antagonism in vivo. Therefore, a study was designed to assess the ability of sulbactam and clavulanate to induce β-lactamases in two strains each of *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, and *Pseudomonas aeruginosa* both in vitro and in vivo. Induction in vitro was observed only with *S. marcescens* and *P. aeruginosa* and generally only when inhibitor concentrations greater than 2 μg/ml were examined. A mouse model of lethal infection, designed to detect in vivo antagonism arising from β-lactamase induction, was used to determine what effect sulbactam and clavulanate would have on the 50% protective doses (PD_{50}s) of cefoperazone and ticarcillin. Antagonism (a significant increase in the PD_{50}) was observed in only 4 of 32 tests. Three of these involved antagonism of cefoperazone by clavulanate, and one involved antagonism of cefoperazone by sulbactam. In 6 of 32 tests, enhancement of efficacy (a significant decrease in PD_{50}) was observed. In four of these, sulbactam enhanced cefoperazone; in one, sulbactam enhanced ticarcillin; and in one, clavulanate enhanced ticarcillin. Four of the six cases of enhancement occurred when the β-lactamase inhibitor was given at the time of challenge. None of these positive or negative in vivo effects were predicted by in vitro tests. These data suggest that β-lactamase inhibitors can influence the in vivo potency of their companion drug in both a beneficial and detrimental fashion against organisms with class I β-lactamases and that these effects cannot be predicted from in vitro assays.

Previous studies have shown that β-lactamase inhibitors are in vitro inducers of chromosomal, class I β-lactamases in certain strains of the family *Enterobacteriaceae* and *Pseudomonas aeruginosa* (4, 11, 12, 16, 18–20). Quantitatively, clavulanate induces higher levels of β-lactamase than does sulbactam, although induction with these compounds is usually manifest only at high, clinically unachievable concentrations (6, 12, 18–20). The in vivo effects of these compounds against organisms possessing class I enzymes are unknown. However, the in vitro data suggest that it is possible for these β-lactamase inhibitors to antagonize the activity of their companion drug in vivo by inducing class I β-lactamases. Clinically, this could result in treatment failure. Since an animal model of lethal infection which can detect in vivo antagonism via induction of class I β-lactamases has been developed previously (7), a study was designed to (i) use this model to determine the influence of sulbactam and clavulanate on the efficacies of cefoperazone and ticarcillin and (ii) determine whether in vitro tests for induction predicted the results observed in vivo.

MATERIALS AND METHODS

Bacterial strains. Two strains each of *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, and *P. aeruginosa* were used in this study. These strains were clinical isolates that were susceptible to cefoperazone and ticarcillin. They were also found to be virulent in a mouse model of lethal infection, possessed inducible, chromosomal, class I β-lactamases, and lacked any plasmid-encoded β-lactamases.

Antimicrobial agents. Cefoperazone and sulbactam diagnostic powders were generously provided by Roerig-Pfizer Co. Ticarcillin and potassium clavulanate diagnostic powders were generously provided by Beecham Laboratories.

Antimicrobial susceptibility tests. Organisms were tested for their susceptibilities to ticarcillin, cefoperazone, clavulanate, sulbactam, ticarcillin-clavulanate, ticarcillin-sulbactam, cefoperazone-clavulanate, and cefoperazone-sulbactam. MICs were determined by an agar dilution method described by the National Committee for Clinical Laboratory Standards (13) by using an inoculum of 10^4 CFUs per spot. Drug combinations were tested by using a fixed concentration of 2 μg/ml for each β-lactamase inhibitor.

In vitro induction. Induction assays were performed as described previously (16). Organisms were incubated for 2 h with each β-lactamase inhibitor and with drug alone at a concentration of one-quarter the MIC of each drug. Each β-lactamase inhibitor was also tested at 2 μg/ml. Inductions with drug–β-lactamase inhibitor combinations were performed at a concentration of one-quarter the MIC obtained with combinations of each drug plus 2 μg of each inhibitor per ml. Cells were also incubated with cefoxitin as a positive inducer control and without antibiotics as an uninduced negative control. After induction, protein synthesis was arrested by adding 1 mM 8-hydroxyquinoline. The cells were harvested by centrifugation, washed, and disrupted by sonication. Crude sonic extracts were dialyzed overnight against phosphate buffer at pH 7.0 at room temperature. β-Lactamase activity in the sonic extracts was then determined by UV spectrophotometry by using cephalothin as a substrate. Protein was measured by the method of Bradford (3). Results were converted to an induction ratio (nanomoles of cephalothin hydrolyzed per minute per milligram of protein for induced cells divided by that for uninduced cells).

Mouse infection. The mouse model of lethal infection was performed as described previously (7). Logarithmic-phase cells were washed and suspended in 4 to 12% (depending on the organism) hog gastric mucin. Male CF-1 mice (weight, 20

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TABLE 1. Doses for pretreatment regimen

Strain	Drug	Concn (mg/kg)		
		Range	Sulbactam[a]	Clavulanate[a]
P. aeruginosa				
29	Cefoperazone	25–200	150	12.5
	Ticarcillin	100–300	150	12.5
162	Cefoperazone	25–50	62.5	3.9
	Ticarcillin	1–125	62.5	3.9
E. cloacae				
20	Cefoperazone	0.1–1.0	25	1.56
	Ticarcillin	10–50	25	1.56
58	Cefoperazone	100–300	200	12.5
	Ticarcillin	50–400	200	12.5
C. freundii				
20	Cefoperazone	0.1–5.0	15	0.94
	Ticarcillin	1–30	15	0.94
26	Cefoperazone	1–20	50	3.13
	Ticarcillin	10–100	50	3.13
S. marcescens				
2	Cefoperazone	400–600	300	15.6
	Ticarcillin	50–150	300	15.6
5	Cefoperazone	1–30	20	1.25
	Ticarcillin	5–40	20	1.25

[a] Pretreatment regimen.

to 25 g) were then injected intraperitoneally with a lethal dose (one 100% minimum lethal dose) of each organism. Groups of 10 mice each were treated subcutaneously with various doses (at least five different dose groups) of cefoperazone or ticarcillin at 1 and 3.5 h postchallenge. Mortality was assessed at 48 h postchallenge. All untreated animals died within this time period. The dose of drug which protected 50% of the mice (PD_{50}) was determined by logarithmic probit plot, and the 95% confidence intervals were calculated (9). In general, two to three repeat experiments were required to produce relatively tight confidence intervals.

Influence of β-lactamase inhibitors on drug efficacy. The influence of each β-lactamase inhibitor on the efficacies of cefoperazone and ticarcillin was examined by using two treatment regimens. These have been described previously, and they are designed to assess the in vivo antagonistic potentials of several β-lactam compounds (7). The first regimen, the simultaneous regimen, consisted of treating infected animals at 1 and 3.5 h postchallenge with the drug and β-lactamase inhibitor as a combination. A 2:1 ratio was used for drug-sulbactam, and a 32:1 ratio was used for drug-clavulanate. This regimen simulated the combinations as they would actually be used clinically. The second regimen, the pretreatment regimen, consisted of a single dose of inhibitor at the time of challenge followed by treatment at 1 and 3.5 h postchallenge with each drug alone. Such a pretreatment regimen has been shown previously to enhance in vivo antagonism produced via the induction of β-lactamase (7). The dose of inhibitor used in the pretreatment regimen was equal to 1/2 (for sulbactam) or 1/32 (for clavulanate) of the single highest dose of drug (usually ticarcillin)

TABLE 2. Susceptibilities of challenge strains to study compounds

Strain	MIC (μg/ml)[a]							
	SB	CA	CFP	CFP-SB	CFP-CA	TIC	TIC-SB	TIC-CA
P. aeruginosa								
29	>128	128	4	4	4	32	32	32
162	>128	64	1	1	2	4	4	2
E. cloacae								
20	64	32	≤0.06	≤0.06	0.25	8	4	4
58	64	32	0.12	0.06	0.12	4	4	4
C. freundii								
20	32	16	0.12	≤0.06	0.25	1	1	2
26	32	32	0.12	≤0.06	0.12	2	4	2
S. marcescens								
2	128	64	1	1	2	8	4	8
5	128	64	0.5	1	2	8	8	8

[a] SB, sulbactam; CA, clavulanate; CFP, cefoperazone; TIC, ticarcillin.

over the dose range examined (Table 1). Mortality was assessed at 48 h postchallenge, and PD_{50}s and confidence intervals were calculated as described above. As stated above, two to three repeat experiments were generally required to produce relatively tight confidence intervals. For the purposes of this study, antagonism was defined as a significant increase (nonoverlapping 95% confidence intervals; $P < 0.05$) in the PD_{50} with drug plus inhibitor versus that with drug alone. Enhancement was defined as a significant decrease in the PD_{50} with drug plus inhibitor versus that of the drug alone. All control mice treated with inhibitor alone by either regimen generally died. When some minimal survival was noted, the drug combination part of the experiment showed neither antagonism nor enhancement, with one exception (see Results).

RESULTS

In vitro susceptibility. As shown in Table 2, all strains included in this study were resistant to sulbactam and clavulanate alone. They were susceptible to cefoperazone and ticarcillin both alone and in combination with either inhibitor. In general, the presence of inhibitors at a concentration of 2 μg/ml had no significant effect on the activity of either ticarcillin or cefoperazone. There were two exceptions: MICs of cefoperazone against *E. cloacae* 20 and *S. marcescens* 5 increased fourfold or more with the addition of clavulanate.

In vitro induction. Results of in vitro tests for induction of class I β-lactamase are shown in Table 3. No induction was observed in tests with *C. freundii* or *E. cloacae* for any drug, inhibitor, or drug-inhibitor combination. In contrast, induction was observed with strains of *S. marcescens* and *P. aeruginosa*. Clavulanate at 16 to 32 μg/ml was a strong inducer for these strains; however, no induction was observed at 2 μg/ml. In contrast, sulbactam was a weak inducer at 32 μg/ml for all of these strains except *P. aeruginosa* 162, which was strongly induced. At 2 μg/ml, little or no induction was seen in the tests with sulbactam. Induction was not observed with cefoperazone alone for these strains; however, low-level induction was observed in the tests with ticarcillin alone. Drug-inhibitor combinations demonstrated weak or no inducer activity for these strains, again with the

TABLE 3. Inducer potential of study drugs against challenge strains

Inducer	Challenge strain															
	C. freundii 20 (ND)[a]		*C. freundii* 26 (2)		*E. cloacae* 20 (2)		*E. cloacae* 58 (2)		*S. marcescens* 2 (17)		*S. marcescens* 5 (10)		*P. aeruginosa* 29 (1)		*P. aeruginosa* 162 (4)	
	Concn (μg/ml)[b]	Ratio[c]	Concn (μg/ml)	Ratio	Concn (μg/ml)	Ratio	Concn (μg/ml)	Ratio	Concn (μg/ml)	Ratio	Concn (μg/ml)	Ratio	Concn (μg/ml)	Ratio	Concn (μg/ml)	Ratio
Cefoxitin	10	49	10	66	10	133	10	182	10	170	10	271	10	52	10	35
Clavulanate	4	1	8	1	8	1	8	1	16	9	16	7	32	25	16	42
	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1
Sulbactam	8	1	8	1	16	1	16	1	32	4	32	2	32	3	32	27
	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	3
Cefoperazone	0.03	1	0.03	1	0.03	1	0.01	1	0.25	1	0.12	1	1	1	0.25	1
+ Clavulanate[d]	0.03	1	0.03	1	0.06	1	0.03	1	0.5	2	0.5	1	1	1	0.5	10
+ Sulbactam	0.01	1	0.01	1	0.01	1	0.01	1	0.25	1	0.25	2	1	2	0.25	1
Ticarcillin	0.25	1	0.5	1	2	1	1	1	2	2	2	2	8	2	1	8
+ Clavulanate	0.5	1	0.5	1	1	1	1	1	2	4	2	3	8	4	0.5	9
+ Sulbactam	0.25	1	1	1	1	1	1	1	1	1	2	2	8	4	1	7

[a] Numbers in parentheses indicate the β-lactamase activity of uninduced cells in nanomoles of cephalothin hydrolyzed per minute per milligram of protein. ND, not detectable (induction ratio of 1 was assigned for results that were not detectable).
[b] Concentration of inducer.
[c] Induction ratio = β-lactamase activity of induced cells/β-lactamase activity of uninduced cells.
[d] The concentration of clavulanate or sulbactam was 2 μg/ml.

exception of *P. aeruginosa* 162, which was induced with cefoperazone-clavulanate, ticarcillin-clavulanate, and ticarcillin-sulbactam.

Influence of inhibitors on drug efficacy in vivo. In vivo induction, as suggested by a significant increase in the PD_{50} of drug-inhibitor over that of drug alone (antagonism), was observed in only 4 of 32 tests (Table 4). In each case, cefoperazone was the drug involved and in three of the four cases clavulanate was the inhibitor involved. In no test was the efficacy of ticarcillin significantly diminished by either inhibitor (Table 5).

In 6 of the 32 tests, the inhibitor significantly enhanced the efficacy of its companion drug (Tables 4 and 5). Two of these involved ticarcillin and four involved cefoperazone. In all but one instance, sulbactam was the inhibitor involved in the observed enhancement of efficacy. Four of the six cases of enhancement occurred in the pretreatment regimen. In one case of enhancement, *E. cloacae* 20 infected mice treated with ticarcillin-sulbactam in the pretreatment regimen (Table 5), significant protection (4 of 10 mice survived) was observed in control mice treated with sulbactam alone. Thus, enhancement in this case may have been due to an additive effect.

DISCUSSION

With the recent introduction of β-lactamase inhibitors such as clavulanate and sulbactam and the marketing of these agents in combination with broad-spectrum β-lactam antibiotics such as ticarcillin and cefoperazone, use of these

TABLE 4. Influence of inhibitors on the efficacy of cefoperazone in the mouse model of lethal infection

Strain (CFU/mouse)	PD_{50} (mg/kg [95% confidence interval])				
	Drug alone	Simultaneous treatment with:		Pretreatment with:	
		Sulbactam	Clavulanate	Sulbactam	Clavulanate
P. aeruginosa					
29 (10^5)	56 (43–74)	124 (75–205)[a]	114 (23–567)	63 (17–226)	176 (96–323)[a]
162 (10^6)	32 (26–39)	26 (19–37)	35 (26–48)	21 (13–36)	36 (22–59)
E. cloacae					
20 (10^6)	0.3 (0.2–0.5)	0.3 (0.2–0.5)	0.5 (0.3–0.7)	0.3 (0.2–0.5)	0.6 (0.5–0.9)
58 (10^7)	92 (78–109)	126 (97–165)	137 (110–171)	13 (7–22)[b]	226 (195–262)[a]
C. freundii					
20 (10^6)	1.3 (0.7–2.2)	0.4 (0.2–0.6)[b]	1.4 (0.6–3.4)	2.8 (0.9–8.9)	0.7 (0.3–1.4)
26 (10^7)	2.4 (0.8–7.1)	2.9 (0.4–23)	1.8 (0.2–16)	0.7 (0.2–2.0)	1.6 (0.7–3.7)
S. marcescens					
5 (10^6)	2.7 (1.7–4.3)	6.1 (4.1–8.9)	6.5 (4.4–9.7)[a]	5.5 (3.2–9.5)	2.0 (0.7–6.2)
2 (10^7)	569 (501–646)	266 (162–437)[b]	653 (478–892)	121 (88–168)[b]	395 (249–626)

[a] Significantly higher than that with drug alone.
[b] Significantly lower than that with drug alone.

TABLE 5. Influence of inhibitors on the efficacy of ticarcillin in the mouse model of lethal infection

Strain (CFU/mouse)	PD_{50} (mg/kg [95% confidence interval])				
	Drug alone	Simultaneous treatment with:		Pretreatment with:	
		Sulbactam	Clavulanate	Sulbactam	Clavulanate
P. aeruginosa					
29 (10^5)	119 (96–147)	100 (65–153)	103 (70–151)	94 (55–163)	111 (52–237)
162 (10^6)	20 (14–29)	15 (3–72)	5 (2–14)	17 (3–111)	13 (6–25)
E. cloacae					
20 (10^6)	11 (9–15)	10 (4–23)	15 (10–22)	4 (2–8)[a]	3 (1–7)[a]
58 (10^7)	85 (38–187)	94 (21–423)	97 (72–130)	22 (10–48)	85 (55–132)
C. freundii					
20 (10^6)	1.4 (0.3–5.6)	1.7 (1.0–2.9)	3.1 (1.8–5.6)	1.4 (0.3–7.9)	2.6 (1.7–4.2)
26 (10^7)	12 (7.6–19)	7.8 (4.2–14)	18 (11–31)	11 (4.3–28)	14 (1.5–122)
S. marcescens					
5 (10^6)	20 (15–26)	14 (10–21)	16 (11–24)	20 (14–30)	17 (11–27)
2 (10^7)	76 (56–102)	68 (53–87)	56 (36–87)	78 (61–100)	75 (51–108)

[a] Significantly lower than that with drug alone.

agents in patients with serious infections either empirically or therapeutically for broad-spectrum coverage is likely to occur. These infections may involve organisms possessing inducible class I β-lactamase enzymes (5). Others have shown that clavulanate and sulbactam can cause the induction of class I β-lactamase (4, 11, 12, 16, 18–20). In vitro antagonism has been demonstrated for most combinations of broad-spectrum β-lactams (including ticarcillin and cefoperazone) and clavulanate for organisms possessing class I enzymes (4, 10, 19). Although Rolinson (15) suggests that the potential for antagonism is minimal with ticarcillin-clavulanate treatment of these organisms, to date the potential for in vivo antagonism with β-lactam–β-lactamase inhibitor combinations has not been assessed systematically.

In the current study, the in vitro inducer activity of clavulanate and sulbactam for organisms possessing class I enzymes was demonstrated, confirming results of previous studies (4, 11, 12, 16, 18–20). This induction was strain and concentration dependent and occurred to a greater extent with clavulanate. The latter has been reported previously (16, 18, 20). Induction was much greater at 16 to 32 μg of inhibitor per ml than it was at 2 μg/ml. Similarly, Tausk and Stratton (19) reported that the magnitude of induction with clavulanate is directly proportional to its concentration. This may explain the lack of antagonism that was observed by susceptibility testing in which the addition of inhibitor at 2 μg/ml to cefoperazone or ticarcillin did not significantly affect the MIC except in tests with one strain each of *E. cloacae* and *S. marcescens* (Table 2). The MIC of cefoperazone for these latter two strains was increased fourfold or more by the presence of clavulanate.

The effects of inhibitors on the potency of the companion β-lactam drug were assessed in a mouse model of lethal infection which was developed previously (7). In this model, antagonism, as evidenced by an increase in the PD_{50}, occurs with the induction of class I β-lactamase. Antagonism was observed only sporadically for the strains and drugs tested (Tables 4 and 5). For three of the four times this was observed, clavulanate was involved. Antagonism occurred only once with sulbactam and was not observed with either inhibitor combined with ticarcillin.

An increased MIC, in vitro induction, and in vivo antagonism with the inhibitors were all poor predictors of each other (Table 6). This was a surprising and not readily explainable finding. It suggests that in vitro tests bear little relationship to what occurs in vivo or that mechanisms other than enzyme induction may be involved in in vivo antagonism. Further research is required to clarify these inconsistencies.

Another surprising finding of this study was the apparent in vivo enhancement of efficacy observed in tests with certain organisms. This phenomenon occurred six times, five of which involved sulbactam (Tables 4 and 5). In four of these five instances, the companion β-lactam was cefoperazone. This enhancement was most often seen (four of six times) when the inhibitor was given at the time of challenge (pretreatment regimen). These data suggest that β-lactamase inhibitors may be optimally effective if they are given prior to the β-lactam companion drug in order to allow for maximal β-lactamase inhibition to occur in a time-dependent fashion. Alternatively, the inhibitors may cause a nonspecific effect (e.g., the leakage of β-lactamase from the periplasmic space into the extracellular milieu) that renders β-lactamase less effective in protecting the cell from β-lactam antibiotics. This mechanism has been described previously for amdinocillin potentiation of β-lactam activity (17).

In vivo enhancement of a β-lactam drug, with the addition of either sulbactam or clavulanate for organisms possessing class I enzymes, has not been described previously. However, in vitro activity has been shown to be enhanced for combinations of sulbactam or clavulanate with azlocillin, mezlocillin, piperacillin, and apalcillin against members of the family *Enterobacteriaceae* possessing class I β-lactamases without any plasmid-mediated enzymes (8). This occurred more frequently with sulbactam (four of four strains tested) than it did with clavulanate (one of four strains tested). In contrast, Bayer et al. (2) found no in vitro synergy between sulbactam or clavulanate plus ceftazidime for a constitutive class I β-lactamase-overproducing strain of *P. aeruginosa*. Indeed, clavulanate has been shown to have little effect on class I enzymes (14), whereas sulbactam has 100-fold better inhibitory activity (1). The latter may explain why, in the current study, in vivo enhancement was often observed with sulbactam but was rarely observed with clavulanate.

In conclusion, these data suggest that β-lactamase inhibitors can affect the potency of their companion β-lactam in both a beneficial and detrimental fashion for organisms

TABLE 6. Summary of in vitro and in vivo test results

Strain	Drug-inhibitor combination showing each effect[a]			
	Increased in vitro:		In vivo efficacy:	
	MIC	Induction	Enhanced	Antagonized
P. aeruginosa				
29	ND	CFP-SB, CFP-CA, TIC-SB	ND	CFP-CA, CFP-SB
162	ND	CFP-CA, TIC-CA	ND	ND
E. cloacae				
20	CFP-CA	ND	TIC-CA, TIC-SB	ND
58	ND	ND	CFP-SB	CFP-CA
C. freundii				
20	ND	ND	CFP-SB	ND
26	ND	ND	ND	ND
S. marcescens				
5	CFP-CA	CFP-SB, TIC-CA	ND	CFP-CA
2	ND	CFP-CA, TIC-CA	CFP-SB	ND

[a] CFP, cefoperazone; TIC, ticarcillin; SB, sulbactam; CA, clavulanate. The effect is over that of the drug alone. ND, not different.

possessing class I enzymes. An effect was more likely observed when cefoperazone was the companion drug (eight events) than when ticarcillin was (two events). Combinations which included clavulanate were more likely to be indifferent or antagonistic versus drug alone. In view of the potential for induction and little evidence for any beneficial effect over ticarcillin alone, a conservative approach may be to avoid ticarcillin-clavulanate therapy for infections caused by organisms possessing class I enzymes. In contrast, combinations which included sulbactam were more likely to be indifferent or more efficacious than drug alone. The clinical relevance of these data are uncertain. However, in view of sulbactam's poor inducer ability coupled with the enhancement of cefoperazone efficacy in vivo, it may be worthwhile to explore this therapeutic combination further and to determine what in vitro tests may be used to predict this beneficial effect accurately.

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