6-ACETYLMETHYLENEPENICILLANIC ACID (Ro 15-1903), A POTENT β-LACTAMASE INHIBITOR

II. ANTIBACTERIAL PROPERTIES

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The β-lactamase inhibitor Ro 15-1903 showed low affinity for penicillin binding proteins (PBPs) of *Escherichia coli*. When used as a single compound, it displayed no substantial antibacterial activity but in combination with ampicillin, it was similar to clavulanic acid in conferring activity against ampicillin-resistant strains. Some synergy between Ro 15-1903 and piperacillin was found against high inocula of *Pseudomonas aeruginosa*. Ro 15-1903 markedly enhanced the activity of ceftriaxone against *Bacteroides fragilis*. In keeping with the *in vitro* findings, the combination Ro 15-1903 and ampicillin protected mice against systemic infections with β-lactamase-producing strains of *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus* sp. but not against those with *Enterobacter cloacae*, *Serratia marcescens*, and *E. coli* producing either chromosomally mediated β-lactamase of type I or plasmid-mediated β-lactamase of type TEM.

β-Lactamases are the most important mediators of resistance to β-lactam antibiotics in bacteria. Organisms producing β-lactamases are, therefore, often resistant to older penicillins and cephalosporins which generally are more susceptible to the action of β-lactamases than newer compounds. The antibacterial spectrum of those antibiotics can be extended to include bacteria producing β-lactamases by the addition of a suitable β-lactamase inhibitor. The usefulness of this therapeutic strategy has been substantiated in clinical trials[1]. Inhibitors of various types of β-lactamases are being found in an increasing number of structurally different classes of β-lactam derivatives[2~6]. Strong inhibitory properties were recently demonstrated among 6-acylmethylenepenicillanic acid derivatives, the most active compound being 6-acetylmethylenepenicillanic acid (Ro 15-1903)[7]. The antibacterial features of Ro 15-1903 both alone and in combination with other β-lactam antibiotics, are outlined in the following.

Materials and Methods

Antibiotics

Clavulanic acid was given to us by Beecham Laboratories. Sulbactam was prepared for this study in the Roche Laboratories, Basel. Ampicillin (Beecham Laboratories), cefazolin (Eli Lilly), and piperacillin (Lederle) were obtained as commercial products.

Organisms

R-Factor carrying strains of *Escherichia coli* and *Pseudomonas aeruginosa* were gratefully obtained from Prof. B. WIEDEMANN, University of Bonn. The other isolates were standard strains from our culture collection.

Penicillin Binding Protein (PBP) Assay

Membranes were prepared from *E. coli* W3110 and the PBP assay was performed according to the

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method of NOGUCHI *et al.*[8] except that preincubation with inhibitor (30°C, 10 minutes) was carried out before the addition of [^{14}C]benzylpenicillin potassium salt (53 Ci/mole, Radiochemical Centre) and that Enhance (New England Nuclear) was used for fluorography. PBP bands on the X-ray film (Eastman Kodak NS-2T) were quantified by densitometry using the Zeiss PMQII spectrophotometer assembly. The concentration at which [^{14}C]benzylpenicillin binding was inhibited by 50% was determined graphically and expressed as IC_{50}.

In Vitro Activity and Synergy Testing

Minimal inhibitory concentrations (MICs) for aerobically growing organisms were determined by the microdilution technique with Mueller-Hinton broth, (Difco), as the growth medium. The antibiotics were added in twofold serial dilutions to the wells of a plastic microtiter plate (MIC-2000 Inoculum Tray, Dynatech). Synergy was evaluated by determining the MICs of the combined compounds in a checkerboard fashion. The inoculum was 10^7 colony-forming units (CFUs) for *P. aeruginosa* and 10^5 CFUs for other organisms. The lowest concentrations of the single or combined compounds inhibiting visible growth after 20 hours of incubation at 37°C were taken as the MICs. Synergy was considered to be present when there was at least a fourfold decrease in the MICs of both antibiotics.

MICs against *Bacteroides fragilis* were determined by the agar dilution method using Wilkins-Chalgren agar[9]. The antibiotics were incorporated into the medium as single compounds or fixed 1:1 combinations in twofold serial dilutions. The surface of the agar was inoculated with the aid of a multipoint inoculator yielding approximately 10^4 CFUs per spot. After 20 hours of incubation at 37°C, the MICs were recorded as the lowest concentrations which prevented bacterial growth. Synergy was calculated as above.

In Vivo Activity

Acute systemic infections were induced in albino mice by intraperitoneal injection of the test organism. Some of the strains were injected as a suspension in 4% hog gastric mucin (American Laboratories, Inc.). Bacterial challenge doses ranged from 3 to 100 times the number of organisms required to kill 50% of the unmedicated control animals within 72 hours. Control and treatment groups consisted of five mice at each dose level. The antibiotics were given subcutaneously as single compounds or as fixed 1:1 combinations 1 hour and 3 hours after challenge except in the case of the pseudomonal infection which was treated with an additional dose 5 hours after challenge. The 50% effective dose (ED_{50}) was calculated by probit analysis from the survival rate on day 4 after infection.

Results

Affinity for PBPs

The IC_{50} values of Ro 15-1903 for the individual PBPs indicate that Ro 15-1903 exhibits highest affinity for PBP 1 (Table 1) which is considered to perform essential functions in cell elongation of *E. coli*[10]. In this respect, Ro 15-1903 resembles sulbactam rather than clavulanic acid. However, the affinity of Ro 15-1903 to PBP 1a is relatively weak, and no potent antibacterial properties of Ro 15-1903 on its own can be anticipated from these data.

In Vitro Activity

Antibacterial testing of Ro 15-1903 against various organisms, almost all of them producers of β-lactamases, revealed no activity by Ro 15-1903 alone. Strains of *Enterobacteriaceae, Staphylococcus aureus* (Table 2), *P. aeruginosa* (Table 3) and *B. fragilis* (Table 4) were not susceptible to concentrations up to 50 μg/ml. The addition of Ro 15-1903 to ampicillin, however, brought about inhibition of ampicillin-resistant strains. This synergy was most evident against *S. aureus*, isolates of *E. coli* producing plasmid-mediated OXA-2 and OXA-3 β-lactamases, *Klebsiella pneumoniae*, and *Proteus vulgaris* (Table 2). The β-lactamases from the two latter organisms are chromosomally determined enzymes[7]. Mo-

Table 1. Affinity of Ro 15-1903, clavulanic acid, and sulbactam for the penicillin binding proteins of *E. coli* W 3110.

Compound	IC_{50} (μg/ml) for PBP						
	1a	1b	2	3	4	5	6
Ro 15-1903	14	240	350	390	37	85	>500
Clavulanic acid	190	>500	3.4	>500	75	160	110
Sulbactam	17	500	67	410	>500	>500	500

Table 2. *In vitro* activity of Ro 15-1903 and clavulanic acid alone and in combination with ampicillin.*

Strain	β-Lactamase type	MIC (μg/ml)				
		Ampicillin	Ro 15-1903	Clavulanic acid	Ro 15-1903 +Ampicillin**	Clavulanic acid +Ampicillin**
S. aureus 887	Staphylococcal	>50	>50	>50	0.4+ 0.4	0.8+ 0.8
E. coli TEM 1	TEM 1	>50	>50	50	12.5+12.5	6.3+6.3
E. coli TEM 2	TEM 2	>50	>50	50	25+25	12.5+12.5
E. coli 1527E	OXA 1	>50	>50	50	12.5+12.5	12.5+12.5
E. coli 1573E	OXA 2	>50	>50	50	3.1+3.1	3.1+3.1
E. coli 1894E	OXA 3	>50	>50	>50	6.3+6.3	6.3+6.3
E. coli 2008E	SHV 1	>50	>50	50	12.5+12.5	6.3+6.3
E. coli HMS 1	HMS 1	>50	>50	25	12.5+12.5	6.3+6.3
E. coli 1024	I	>50	>50	>50	25+25	50+50
S. marcescens 69438	I	>50	>50	>50	>50+50	>50+50
E. cloacae P99	Ia	>50	>50	25	>50+50	25+25
E. cloacae 908	Ia	>50	>50	>50	>50+50	>50+50
P. vulgaris 1028	Ic	>50	>50	>50	1.6+1.6	1.6+1.6
K. pneumoniae 418	II	>50	>50	25	3.1+3.1	0.8+3.1
K. oxytoca 1082 E	IV	>50	>50	50	50+25	12.5+12.5

* Inoculum, 10^5 CFU/ml.

** Figures presented were selected on the basis of minimal total drug concentration required for inhibition.

Table 3. *In vitro* activity of piperacillin in the presence of Ro 15-1903 or sulbactam against high inocula of *P. aeruginosa**.

Strain	Type of β-lactamase	MIC (μg/ml)		
		Piperacillin	Piperacillin +Ro 15-1903**	Piperacillin +Sulbactam**
1973 E	PSE 1	>200	200+200	>200+200
1937 E	PSE 2	>200	100+100	100+100
1920 E	PSE 3	200	50+50	50+50
1559 E	PSE 4	>200	>200+200	>200+200
18 SH	Id	200	25+25	25+25

* Inoculum, 10^7 CFU/ml.

** MIC Ro 15-1903, >200 μg/ml.
MIC Sulbactam, >200 μg/ml.

Table 4. Synergistic effect of Ro 15-1903 and sulbactam on the activity of ceftriaxone against *B. fragilis*.

Strain	MIC (μg/ml)				
	Ceftriaxone	Ro 15-1903	Sulbactam	Ro 15-1903+ Ceftriaxone	Sulbactam+ Ceftriaxone
6	100	>50	25	6.3+6.3	1.6+1.6
35	12.5	>50	12.5	0.8+0.8	0.2+0.2
36	25	>50	12.5	3.1+3.1	3.1+3.1
43	25	>50	12.5	12.5+12.5	6.3+6.3
44	1.6	>50	3.1	0.2+0.2	0.1+0.1
47	25	>50	12.5	0.8+0.8	0.2+0.2
57	6.3	>50	12.5	0.8+0.8	0.2+0.2
65	100	>50	25	6.3+6.3	1.6+1.6
67	50	>50	>50	6.3+6.3	1.6+1.6
69	50	>50	25	6.3+6.3	1.6+1.6
87	3.1	>50	3.1	0.8+0.8	0.2+0.2
88	12.5	>50	25	0.8+0.8	0.2+0.2
91	6.3	>50	25	3.1+3.1	3.1+3.1
92	6.3	>50	12.5	1.6+1.6	0.2+0.2
96	100	>50	25	12.5+12.5	6.3+6.3
106	6.3	>50	12.5	1.6+1.6	0.2+0.2

derate activity with the combination of Ro 15-1903 and ampicillin was found against strains of *E. coli* producing plasmid-mediated β-lactamases of type TEM-1, TEM-2, OXA-1, SHV-1, and HMS-1. *Enterobacter cloacae* and *Serratia marcescens* were not inhibited by Ro 15-1903 in combination with ampicillin. Similar data as with Ro 15-1903 were obtained with clavulanic acid. No synergy between Ro 15-1903 and ampicillin occurred against strains of *S. aureus* and *E. coli* that did not produce measurable amounts of β-lactamase.

The activity of piperacillin against high inocula of *P. aeruginosa* could be enhanced by the addition of Ro 15-1903 or sulbactam in several strains. The combined MIC values were, however, relatively high (Table 3).

Ceftriaxone and Ro 15-1903 showed marked synergy against 14 of 16 strains of *B. fragilis* (Table 4). This combination prevented growth of all 16 strains at concentrations equal to or lower than 12.5+12.5 μg/ml, while the MICs of ceftriaxone by itself against half of the strains were equal to or higher than 25 μg/ml. Unlike Ro 15-1903, sulbactam by itself showed some activity against most strains of *B. fragilis*. When combined with ceftriaxone, sulbactam was more effective than Ro 15-1903.

In Vivo Activity

In keeping with the *in vitro* results, Ro 15-1903 was inactive by itself *in vivo* (Table 5). Subcutaneous administration of Ro 15-1903 combined with ampicillin in a 1:1 ratio protected mice against systemic infections with *S. aureus, K. pneumoniae, Proteus mirabilis, Proteus rettgeri* and *P. vulgaris*. No therapeutic effect was observed against infections with *E. cloacae, S. marcescens, E. coli* and *P. aeruginosa*. In supplementary experiments Ro 15-1903 also failed to enhance the therapeutic efficacy of cefazolin against *E. cloacae* 908 and *S. marcescens* 69438. Lack of protective activity of Ro 15-1903 combined with ampicillin against *S. aureus* 887 after oral dosing of 50 mg/kg each suggests either poor absorption of Ro 15-1903 from the gastrointestinal tract or inactivation of the drug.

Table 5. *In vivo* activity of Ro 15-1903 and ampicillin alone and combined in a fixed 1:1 ratio against bacteria producing β-lactamase.

Organism	ED_{50} (mg/kg, s.c.)		
	Ro 15-1903	Ampicillin	Ro 15-1903 +Ampicillin
S. aureus 887	>50	>50	8.3+8.3
E. coli 1024	>50	>50	>50+50
E. coli RTEM	>50	>50	>50+50
K. pneumoniae 418	>50	>50	28+28
E. cloacae 908	>50	>50	>50+50
S. marcescens 69438	>50	>50	>50+50
P. mirabilis 2117	>50	>50	8.4+8.4
P. vulgaris 1028	>50	>50	21+21
P. rettgeri R31	>50	>50	17+17
P. aeruginosa BA	>50	>100	>100+100

The combination of sulbactam and ampicillin showed a similar spectrum as that of Ro 15-1903 and ampicillin, and there was no distinct difference in activity between these two combinations in the mouse.

Discussion

Ro 15-1903 is a strong β-lactamase inhibitor. The activity of this compound against various types of β-lactamase exceeds that of well-known inhibitors such as clavulanic acid and sulbactam[7]. It, therefore, seems to result that Ro 15-1903 is capable of protecting β-lactam antibiotics against hydrolysis mediated by β-lactamases. Our *in vitro* and *in vivo* data demonstrate indeed synergy between Ro 15-1903 and ampicillin, piperacillin and ceftriaxone against some organisms producing β-lactamases. Since Ro 15-1903 by itself showed no antibacterial activity and only poor affinity for PBPs, inhibition of β-lactamase by Ro 15-1903 is likely to be the sole reason for the synergy observed. The various bacterial species differed in susceptibility to combinations between Ro 15-1903 and the β-lactam antibiotics used. Ro 15-1903 resembled clavulanic acid in showing pronounced synergy with ampicillin against *S. aureus, K. pneumoniae, P. vulgaris* and *P. mirabilis*, but only weak or no synergy at all against *S. marcescens* and *E. cloacae*. These *in vitro* findings have been confirmed in mice. Marked synergy against *B. fragilis* occurred between Ro 15-1903 and ceftriaxone, a modern cephalosporin with high stability against β-lactamases except those from *B. fragilis*. Dense populations of *P. aeruginosa* could be inhibited by piperacillin to a certain extent when this antibiotic was combined with Ro 15-1903 or sulbactam.

Under the test conditions used, Ro 15-1903 did not show more pronounced synergy against bacterial cells than sulbactam or clavulanic acid, although it is a considerably stronger inhibitor of isolated β-lactamases[7]. Reasons for the relatively weak ampicillin-protecting activity of Ro 15-1903 against whole bacteria could be poor access to the target enzymes or insufficient stability of the compound when tested *in vitro* and in animals. The various possibilities are being investigated.

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