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

I. INHIBITION OF CHROMOSOMALLY AND R-FACTOR-MEDIATED β-LACTAMASES

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6-Acetylmethylenepenicillanic acid (Ro 15-1903) was seen to be a powerful inhibitor of various β-lactamases. Nearly all of the chromosomally and R-factor-mediated β-lactamases which were studied were inhibited at much lower concentrations than required with clavulanic acid or sulbactam. Ro 15-1903 protected β-lactamase-labile penicillins and cephalosporins from hydrolysis. The compound itself was stable against hydrolysis and inhibition of β-lactamase was irreversible.

Penicillins and cephalosporins specifically interfere with the synthesis of the cell envelope in bacteria — a structure not present in higher animals and man — and thus belong to the best tolerated antimicrobial agents available. Their use is, however, increasingly restricted by the prevalence of organisms harboring β-lactamases able to inactivate these drugs. Although newly developed cephalosporins or relatedcompounds are much more stable to hydrolysis than older ones, there is no completely stable β-lactam compound available. An alternative strategy for overcoming the problems provoked by β-lactamases has therefore been pursued[1]. The search for potent agents inhibiting β-lactamases and thus able to protect labile antibiotics in β-lactamase-producing strains has led to the discovery or to the synthesis of a variety of compounds exhibiting the desired properties[2]. Up to now only two β-lactamase inhibitors have been evaluated clinically[2] and only clavulanic acid is now being used in therapy[3]. Since all inhibitors either have gaps in their spectrum of activity or suffer from other disadvantages, the search for better β-lactamase inhibitors is continuing. We have recently found that 6-acylmethylenepenicillanic acid derivatives are very potent inhibitors covering an unusually large spectrum of different enzymes. Among many compounds synthesized in this class, 6-acetylmethylenepenicillanic acid (Ro 15-1903) evolved as the most active and this paper describes some of its properties as an inhibitor of β-lactamases.

Materials and Methods

Chemicals

Ro 15-1903 and its pivaloyloxymethylester were prepared in the Roche Laboratories, Basel[4]. Sulbactam was synthesized by known procedures. We thank Beecham Pharmaceuticals (Betchworth, U. K.) for the supply of clavulanic acid and Glaxo Group Research (Greenford, U. K.) for nitrocefin. Other reagents and antibiotics were obtained commercially.

β-Lactamase Preparations

In most instances crude extracts, obtained by sonically disrupting cells, were used as the enzyme

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source[5]. Some of the organisms producing chromosomally mediated β-lactamases have been described[5,6] and were taken from our culture collection. The enzymes produced were characterized by their substrate profile and classified according to RICHMOND and SYKES[7]. R-Factor carrying strains were gratefully obtained from Prof. B. WIEDEMANN (University of Bonn); the respective β-lactamases and the R-factors specifying these enzymes have been described[8]. TEM 1 β-lactamase was purified to homogeneity by ion exchange chromatography, gel filtration and hydroxylapatite chromatography.

β-Lactamase Assays

The spectrophotometric method with nitrocefin as the substrate was used to measure β-lactamase activity[5,9]. The final volume of one ml contained 100 μM nitrocefin, 50 mM phosphate buffer pH 7.0 and enough enzyme to yield a difference in OD_{482} of 0.1 per minute at 37°C. The TEM 1 β-lactamase was also assayed with benzylpenicillin as the substrate, using $\Delta\varepsilon$ at 240 nm of 660. As an alternative, the iodometric determination of β-lactamase activity, using different substrates, and as described by SAWAI *et al.*[10] was also employed. To evaluate the inhibition of β-lactamases, several inhibitor concentrations were tested and the concentration which reduced the control reaction by 50% is recorded as the IC_{50}. Since progressive inhibition was observed, a 15-minute preincubation time of enzyme and inhibitor was used in these instances.

Measurement of the Number of Inhibitor Molecules Transformed before Inactivation of One Molecule of Enzyme

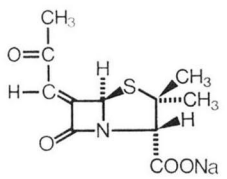
Ten microlitres of 16 μM TEM 1 β-lactamase was mixed with the same volume of solutions containing an appropriate concentration of inhibitor. After 150-minute incubation at 37°C 5 μl of the reaction mixture was diluted with 1 ml of sodium phosphate buffer (100 mM, pH 7.0). A 10 μl aliquot was then transferred to a cuvette containing 1 ml of 2 mM benzylpenicillin and activity was measured spectrophotometrically. The minimal ratio of inhibitor molecules to enzyme molecules needed to completely inhibit the enzyme was obtained by graphical extrapolation, from which the turnover number could be obtained.

Results

Inhibition of Chromosomally Mediated β-Lactamases

The chemical structure of Ro 15-1903 is given in Fig. 1. Its inhibitory properties as compared to sulbactam and clavulanic acid for a variety of chromosomally determined β-lactamases are shown in Table 1. Against the most common cephalosporinases, as produced *e.g.* in *E. cloacae*, sulbactam is much more active than clavulanic acid, both, however, exhibit a moderate degree of inhibition. Ro 15-1903 is the most active compound in all instances, especially against the cephalosporinase from *Citrobacter*. *P. vulgaris* has been shown to produce a distinctly different enzyme (cefuroximase), sensitive to clavulanic acid[11]. Against the β-lactamase from *P. vulgaris* 1028, Ro 15-1903 was found to be 8 times more active than clavulanic acid. The penicillinases produced in *Klebsiellae* are extremely sensitive to inhibition by Ro 15-1903.

Fig. 1. Structure of sodium 6-acetylmethylenepenicillanic acid (Ro 15-1903).



Inhibition of R-Factor-mediated β-Lactamases

Similarly, as observed in the chromosomally mediated β-lactamases, Ro 15-1903 was found to be much more active than the other two inhibitors in nearly all of the R-factor-mediated β-lactamases (Table 2). In general these enzymes are well inhibited by clavulanic acid, sulbactam being mostly inferior. Only the PSE 3 enzyme was found to be less well inhibited by Ro 15-1903 than by clavulanic acid.

Table 1. Inhibitory properties of sulbactam, clavulanic acid and Ro 15-1903 for chromosomally determined β-lactamases.

Organism	β-Lactamase type	IC_{50} (μM)		
		Sulbactam	Clavulanic acid	Ro 15-1903
E. cloacae 908	Ia	8.5	105	6.3
E. cloacae P99	Ia	7.2	140	4.7
C. freundii 43	I	7.5	235	0.1
E. coli 1024	I	11.2	85	1.7
P. vulgaris 1028	Ic	0.1	0.03	0.004
P. aeruginosa 143738	I	10.5	750	1.8
P. aeruginosa 18SH	Id	6.5	650	2.2
K. pneumoniae NCTC 418	II	4.5	0.02	0.005
K. pneumoniae 2/9	II	0.8	0.01	0.0008
K. oxytoca 1082 E	IV	4.6	0.08	0.006

Table 2. Inhibitory properties of sulbactam, clavulanic acid and Ro 15-1903 for R-factor-mediated β-lactamases.

Organism	β-Lactamase type	IC_{50} (μM)		
		Sulbactam	Clavulanic acid	Ro 15-1903
E. coli TEM 1	TEM 1	1.0	0.04	0.0007
E. coli TEM 2	TEM 2	1.3	0.08	0.001
E. coli 1527 E	OXA 1	4.7	1.5	0.004
E. coli 1573 E	OXA 2	0.3	1.3	0.02
E. coli 1894 E	OXA 3	12	55	4.6
E. coli 2008 E	SHV 1	2.7	0.04	0.002
E. coli HMS I	HMS 1	3.6	0.2	0.1
P. aeruginosa 1973 E	PSE 1	30	1.0	0.04
P. aeruginosa 1937 E	PSE 2	3.4	0.04	0.01
P. aeruginosa 1920 E	PSE 3	5.5	0.02	0.06
P. aeruginosa 1559 E	PSE 4	3.2	0.07	0.02
S. aureus 887	Staphylococcal	3.6	0.1	0.006

Table 3. Comparison of the inhibitory properties of Ro 15-1903 and its pivaloyloxymethylester (Ro 15-1315).

Organism	β-Lactamase type	IC_{50}	
		Ro 15-1903	Ro 15-1315
P. vulgaris 1028	Ic	0.004	0.16
K. pneumoniae NCTC 418	II	0.005	0.19
E. coli 1024	I	1.7	32
E. coli TEM 1	IIIa	0.0007	0.02

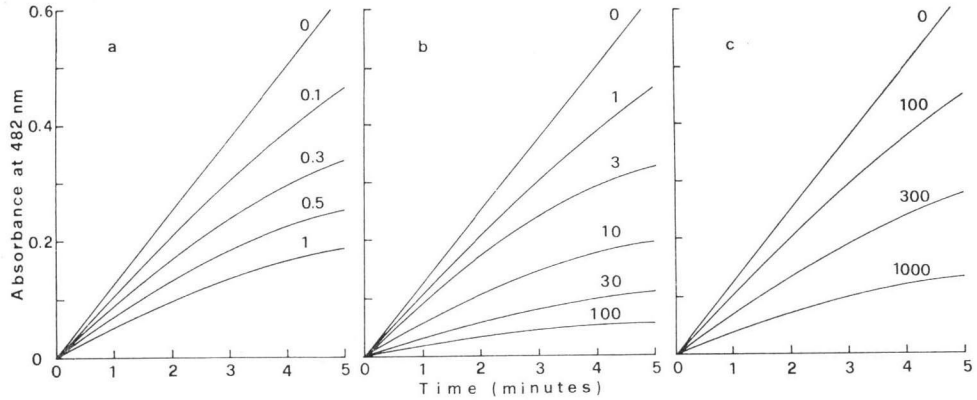
Activity of the Pivaloyloxymethylester of Ro 15-1903

Ro 15-1903 (as well as other compounds in this class of β-lactams) was originally prepared as the pivaloyloxymethylester. The ester is more easily accessible chemically, more stable, and of potential interest for oral absorption. Its inhibitory activity, however, is 10 to 100 times weaker than that of the free acid, as shown in Table 3.

Irreversible Inhibition

Similarly as known from clavulanic acid[2], Ro 15-1903 was seen to provoke a progressive inhibition. This is shown in Fig. 2 for the β-lactamase from *S. aureus* 887, but was also observed with other types of β-lactamases. The comparison with

Fig. 2. Progressive inhibition of the β-lactamase from *S. aureus* 887.
a) Ro 15-1903. b) Clavulanic acid. c) Sulbactam.
Figures indicate concentrations (μM) of inhibitor, added at time zero.



clavulanic acid and sulbactam again showed Ro 15-1903 to be the most active inhibitor.

Stability against Hydrolysis

During incubation of Ro 15-1903 with purified TEM 1 β-lactamase (3.2 nM pure enzyme), no hydrolysis could be detected. The stability of Ro 15-1903 to the enzyme was reinforced by the number of Ro 15-1903 molecules transformed before complete inactivation. One mole of β-lactamase was inactivated by 1.04 moles of Ro 15-1903, whilst 110 and 2,900 moles were necessary with clavulanic acid and sulbactam respectively.

Protection of β-Lactamase-substrates against Hydrolysis

It has already been shown that Ro 15-1903 protected nitrocefin from hydrolysis by various β-lactamases at lower concentrations than necessary with clavulanic acid or sulbactam. Other substrates are

Fig. 3. Inhibition by Ro 15-1903 (5, 10, 20, 50 μM) of the hydrolysis of 20 mM ampicillin by the β-lactamase from *K. oxytoca* 1082 E.
Reaction started by the addition of enzyme and hydrolysis determined iodometrically.

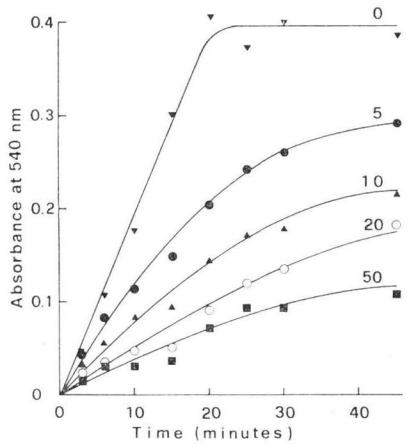
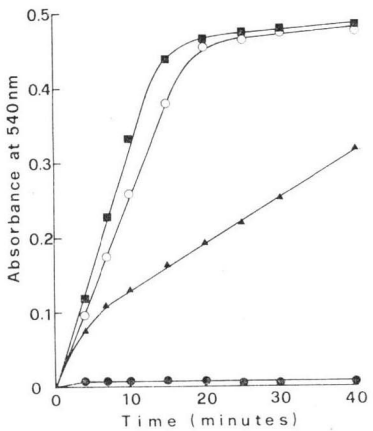


Fig. 4. Hydrolysis of 20 mM ceftriaxone by the β-lactamase from *K. oxytoca* 1082 E (○), and its inhibition by 10 μM sulbactam (■), clavulanic acid (▲), and Ro 15-1903 (●).



protected in a similar way. This is shown for ampicillin against the broad-spectrum β-lactamase from *K. oxytoca* 1082 in Fig. 3. The iodometric assay was used in this case and the reaction was started with enzyme. The same is true for ceftriaxone, a third-generation cephalosporin, also hydrolyzed by this enzyme. Clavulanic acid and sulbactam did not prevent its hydrolysis at concentrations of 1 μM, whereas Ro 15-1903 exhibited partial protection. With 10 μM Ro 15-1903, ceftriaxone was completely protected from hydrolysis (Fig. 4).

Discussion

6-Acetylmethylenepenicillanic acid (Ro 15-1903) seems to belong to the most potent inhibitors so far described for a wide variety of different β-lactamases. In its broad spectrum of inhibitory activity, which includes the chromosomally mediated cephalosporinases, this compound resembles members of the family of olivanic acids, some of which being very potent inhibitors[2,12]. Unlike these, however, Ro 15-1903 is practically devoid of antibacterial activity[13]. Although the chromosomally mediated cephalosporinases are the least well inhibited enzymes, Ro 15-1903 is more active than clavulanic acid and sulbactam. Distinct differences, however, exist in this class of enzymes, as also described, *e.g.* for the β-lactamase from *P. vulgaris*[11] or from *C. freundii*[14]. These two enzymes respond highly sensitively to Ro 15-1903. Nearly all of the penicillinases, whether chromosomally- or R-factor-determined, are very susceptible to Ro 15-1903, which is again more active than clavulanic acid or sulbactam. Since quite distinct differences with respect to inhibition were found, *e.g.* among the PSE-enzymes, it seems that this different response to different types of inhibitors may help to classify and characterize such enzymes. This has also been suggested recently by TODA *et al.*[15].

Similarly, as found for clavulanic acid, Ro 15-1903 seems irreversibly to inhibit β-lactamases. The progressive time course of inhibition is an indication of this. A detailed kinetic study of the mechanism of inhibition has been carried out and will shortly be reported elsewhere. The potentiating activity of Ro 15-1903 in combination with ampicillin is described in the accompanying paper[13].

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