RU 29 246, THE ACTIVE COMPOUND OF THE CEPHALOSPORIN-PRODRUG-ESTER HR 916

II. STABILITY TO β-LACTAMASES AND AFFINITY FOR PENICILLIN-BINDING PROTEINS

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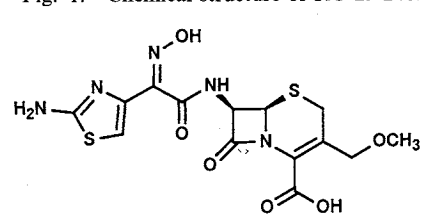
The aminothiazolyl-cephalosporin RU 29 246, the active metabolite of the prodrug-ester HR 916, is active against strains producing the widespread plasmid-encoded TEM-1, TEM-2 and SHV-1 β-lactamases. Except for TEM-7 the activity of RU 29 246 against strains producing extended broad spectrum β-lactamases (TEM-3, TEM-5, TEM-6, SHV-2, SHV-4, SHV-5, CMY-1, CTX-M), however, is low. Relative hydrolysis rates of RU 29 246 are comparable with those of cefpodoxime, the active metabolite of CS-807, and are extremely low for the TEM-1 and SHV-1 β-lactamases. The compound demonstrates remarkable inhibitory activity against the chromosomal β-lactamase of *Enterobacter cloacae* P99. In the presence of 1.7 μM this enzyme loses 50% of its activity. At concentrations of 0.43, 0.003 and 0.01 μg/ml the compound binds preferentially to the penicillin-binding protein (PBP) 3 of *Escherichia coli* K12, to the PBPs 2x and 3 of *Streptococcus pneumoniae* R6 and to PBP 1 of *Staphylococcus aureus* SG 511, respectively.

RU 29 246 (7-β-[[2-(2-aminothiazol-4-yl)-2-*syn*-oximino]acetamido]ceph-3-em-3-methoxymethyl-4-carboxylic acid, Fig. 1), a new cephem antibiotic, is active against a broad spectrum of clinically important pathogens including many strains of Staphylococci, Streptococci and Enterobacteriaceae[1].

Stability against β-lactamases is a major parameter for new cephalosporins and the compound was therefore further investigated for its stability against various chromosomally- and plasmid-encoded enzymes including extended broad spectrum β-lactamases described recently. In addition, the ability of RU 29 246 to inhibit the activity of β-lactamase was investigated as well as its affinity for penicillin-binding proteins.

Fig. 1. Chemical structure of RU 29 246.



Materials and Methods

Antibiotics

RU 29 246 was provided by Hoechst AG, the other compounds (cefpodoxime, cefixime, cefdinir,

ceftibuten, cefaclor, cefuroxime and cephaloridine) were obtained from the manufacturers.

Bacterial Strains and Antibiotic Susceptibility Tests

The strains producing the appropriate β-lactamases were *Escherichia coli* V9458 (TEM-1), *E. coli* V7381 (TEM-2), *Klebsiella pneumoniae* CF104 (TEM-3), *E. coli* CF604 (TEM-5), *E. coli* HB80-251 (TEM-6)[2], *E. coli* (TEM-7), *E. coli* 2141E (SHV-1), *K. pneumoniae* SH122 (SHV-2)[3], *K. pneumoniae* 197 (SHV-4), *K. pneumoniae* 160 (SHV-5), *K. pneumoniae* CHO (CMY-1)[4], *K. pneumoniae* Athen (CMY-2), *E. coli* GRI (CTX-M)[5], *Enterobacter cloacae* P99, *K. pneumoniae* 1082E (K-1), and *Citrobacter freundii* J20.

All strains producing plasmid-encoded β-lactamases were tested by the agar dilution technique using Mueller-Hinton agar (Difco). An inoculum of 10^4 cfu/spot was delivered by a multipoint-inoculator to agar plates which contained 2-fold antibiotic dilutions. The plates were incubated for 16 hours in ambient air at 35°C. After incubation for 16 hours the MIC of each antibiotic was determined. The MIC was defined as the lowest concentration of antibiotic at which no visible growth or growth of ≤ 3 colonies was observed.

Purification of β-Lactamases

For the purification of TEM-1, TEM-2, SHV-1, P99, K-1 and J20 β-lactamases, cells were grown in 1 liter Mueller-Hinton broth at 37°C to late logarithmic phase, sedimented at $6,000 \times g$ for 30 minutes and resuspended in 5 ml 0.1 M potassium phosphate buffer, pH 7.0. The cell suspensions were sonicated (7 minutes, pulsed 50%) and centrifuged at $15,000 \times g$ for 30 minutes. The crude extracts were applied to Sephacryl S-200 superfine (Pharmacia), the column was eluted with buffer and the enzymes were detected by spectrophotometric measurement with pyridinium-2-azo-*p*-dimethylaniline chromophore (PADAC, Calbiochem) as substrate.

For the purification of the TEM-6 of CTX-M enzymes, cells were grown in 900 ml of tryptic soy broth for 6 hours at 35°C, sedimented at $6,000 \times g$ for 20 minutes and resuspended in 4.5 ml of 1% glycine. The suspensions were sonicated (5 minutes, pulsed 60~70%) and centrifuged at $15,000 \times g$ for 30 minutes. The supernatants were dialyzed twice for 5 hours against 500 ml of 1% glycine. Purification of the β-lactamases was achieved by electrofocusing. Approximately 6 ml of the crude enzyme preparations were loaded onto a Multiphor preparative flatbed electrofocusing unit (LKB). Focusing was performed lengthwise at a constant power of 16 W (4°C) for 16 hours. Ultradex granulated gel containing 5% ampholytes pH 7~11 (LKB) was used. After 16 hours the gel bed was fractionated in equally large compartments and β-lactamase activity was estimated spectrophotometrically using cefotaxime (CTX-M) or ceftazidime (TEM-6) as substrates. The gel fractions containing the β-lactamases were loaded onto polypropylene columns with nylon nets (0.01 mm) and eluted with 6 ml of 0.05 M phosphate buffer, pH 7.0.

Stability against β-Lactamases

Hydrolytic stability of RU 29 246, cefpodoxime and cefuroxime in the presence of β-lactamases obtained from different bacteria was determined by the spectrophotometric method according to ROSS and co-workers[6] at 258, 255 and 275 nm, respectively. The time necessary to hydrolyse 50 nmol/ml of antibiotic at 22°C was measured and compared with the hydrolysis time of cephaloridine (255 nm).

Inhibition of β-Lactamase Activity

In a total volume of 1 ml potassium phosphate buffer (0.1 M, pH 7.0) the reaction mixtures contained 20 μM PADAC and RU 29 246, cefpodoxime or cefuroxime in various concentrations (0.05~500 μM). The reactions were started by the addition of appropriate amounts of enzyme and were followed spectrophotometrically at 578 nm and 22°C. The degradation of PADAC without inhibitor was taken as control and set as 100%.

Affinity for Penicillin-binding Proteins (PBPs)

The binding of RU 29 246 and cefpodoxime to the PBPs of *Escherichia coli* K12 was determined using [^{125}I]-ampicillin according to SCHWARZ and co-workers[7].

The binding to the PBPs of *Streptococcus pneumoniae* R6 and *Staphylococcus aureus* SG 511 was determined using [^{3}H]-propionylampicillin as described previously[8].

Results

Activity of RU 29 246 against Strains Producing Extended Broad Spectrum β-Lactamases

RU 29 246 was found to be active against strains producing common plasmid-encoded β-lactamases *e.g.* TEM-1, TEM-2 or SHV-1 (Table 1) as well as TEM-7 among the more recently discovered enzymes. The MICs of RU 29 246 against strains known to synthesize these enzymes ranged between 0.125 and 0.5 μg/ml. The activity of RU 29 246 against isolates producing other extended broad spectrum β-lactamases is low as is also the case for the majority of the other orally absorbable cephalosporins included in the table. MIC-values of RU 29 246 against these isolates ranged between 8 and 1,024 μg/ml.

Relative Stability of RU 29 246 against β-Lactamases

In the presence of all enzymes tested hydrolysis rates of RU 29 246 were much lower than those of cephaloridine (relative hydrolysis rate = 100%) and almost equal to those of cefpodoxime (Table 2). RU 29 246 was more stable than cefuroxime especially against the cephalosporinases produced by *Klebsiella* and *Citrobacter*. Against the TEM-1 and SHV-1 enzymes RU 29 246 was completely stable, while the plasmid- and chromosomally-encoded extended spectrum enzymes investigated showed some hydrolysis. The relative hydrolysis rates were highest for CTX-M among the plasmid-encoded β-lactamases and for the β-lactamase produced by *Citrobacter freundii* J20 amont the chromosomally-encoded enzymes (both 13%).

Table 1. Antibacterial spectrum of RU 29 246 (MIC, μg/ml) in comparison with other oral cephalosporins against strains producing plasmid-encoded β-lactamases.

Strain (β-lactamase)	MIC (μg/ml)						
	RU 29 246	Cefpodoxime	Cefuroxime	Cefdinir	Cefixime	Ceftibuten	Cefaclor
Escherichia coli V9458 (TEM-1)	0.5	0.5	8	0.25	0.5	0.25	1
E. coli V7381 (TEM-2)	0.25	0.5	4	0.13	0.25	0.13	1
Klebsiella pneumoniae CF104 (TEM-3)	32	32	64	4	8	0.25	32
E. coli CF604 (TEM-5)	16	16	16	8	32	1	32
E. coli HB80-251 (TEM-6)	8	16	16	1	2	1	8
E. coli (TEM-7)	0.5	16	8	0.5	0.5	0.13	4
E. coli 2141E (SHV-1)	0.125	0.125	1	—	—	—	—
K. pneumoniae SH122 (SHV-2)	128	64	16	8	0.25	0.25	16
K. pneumoniae 197 (SHV-4)	128	64	64	8	>64	8	>64
K. pneumoniae 160 (SHV-5)	128	64	64	16	>64	8	>64
K. pneumoniae CHO (CMY-1)	128	>64	>64	64	>64	>64	>64
K. pneumoniae Athen (CMY-2)	256	>64	32	32	>64	>64	>64
E. coli GRI (CTX-M)	1,024	64	>64	64	16	1	>64

Table 2. Stability of RU 29 246, cefpodoxime and cefuroxime against plasmid- and chromosomally-encoded β-lactamases.

Strain	β-Lactamase	Relative rate of hydrolysis[a] (%)		
		RU 29 246	Cefpodoxime	Cefuroxime
	Plasmid-encoded			
Escherichia coli	TEM-1	<0.1	<0.1	<0.1
E. coli	TEM-2	5.0	0.9	<0.1
E. coli	TEM-6	10.0	—	—
E. coli	SHV-1	<0.1	<0.1	<0.1
E. coli	CTX-M	13.0	—	—
	Chromosomally-encoded			
Enterobacter cloacae	P99	5.0	2.5	<0.1
Klebsiella sp. 1082E	K-1	8.0	7.3	51.6
Citrobacter freundii	J20	13.0	17.8	84.8

[a] Relative hydrolysis rate of cephaloridine = 100%.

Table 3. Comparative inhibition of enzymatic catalyzed hydrolysis of PADAC in the presence of RU 29 246, cefpodoxime, and cefuroxime.

Strain	β-Lactamase	IC_{50} (μM)		
		RU 29 246	Cefpodoxime	Cefuroxime
Escherichia coli	TEM-1	>500.0	>500.0	234.6
Enterobacter cloacae	P99	1.7	3.8	11.4
Klebsiella sp.	K-1	221.8	>500.0	26.1
Citrobacter freundii	J20	101.7	294.2	68.6

Inhibition of β-Lactamase Activity

As indicated in Table 3, both RU 29 246 and cefpodoxime did not inhibit the plasmid-encoded TEM-1 enzyme of *Escherichia coli* and only reduced the activity of the chromosomally-encoded *Klebsiella* and *Citrobacter* enzymes by 50% at concentrations >200 or >100 μM, respectively. High inhibitory activity, however, could be observed with the enzyme of *Enterobacter cloacae* P99. In the presence of 1.7 μM RU 29 246 this enzyme lost 50% of its activity.

Affinity for PBPs

The results of a competition assay with [^{125}I]-ampicillin show that RU 29 246 had most affinity for the high molecular weight PBPs in *Escherichia coli* K12 (Table 4). This is in agreement with the results for cefpodoxime and other third generation cephalosporins described in the literature. The primary target was PBP 3 which was 50% saturated at concentrations of 0.43 and 0.17 μg/ml by RU 29 246 and cefpodoxime, respectively. These values correlated well with corresponding MICs (0.4 and 0.2 μg/ml) determined against this strain in Mueller-Hinton broth. At a concentration of 0.69 μg/ml, RU 29 246 saturated 50% of PBPs 1A/B.

In the presence of RU 29 246 (1/2 or 1/4 the MIC) the growth of *E. coli* K12 was filamentous.

As shown in Table 5, RU 29 246 had the highest affinity for the PBPs 2x and 3 of *Streptococcus pneumoniae* R6 (ID_{50} = 0.003 μg/ml) and showed the strongest binding to PBP 1 of *Staphylococcus aureus* SG 511 (ID_{50} = 0.01 μg/ml).

Table 4. Affinities of RU 29 246 and cefpodoxime for PBPs of *Escherichia coli* K12 using a competition assay with [^{125}I]-ampicillin.

Antibiotic	ID_{50} (μg/ml)					MIC (μg/ml)
	1A/B	2	3	4	5/6	
RU 29 246	0.69	1.92	0.43	>10	>10	0.4
Cefpodoxime	0.37	2.37	0.17	>10	>10	0.2

Table 5. Affinities of RU 29 246 for PBPs of *Streptococcus pneumoniae* R6 and *Staphylococcus aureus* SG 511 using a competition assay with [^{3}H]-propionylampicillin.

Strain	ID_{50} (μg/ml)					
	1a	1b	2x	2a	2b	3
Streptococcus pneumoniae R6	0.03	0.01	0.003	0.03	10	0.003

Strain	ID_{50} (μg/ml)			
	1	2	3	4
Staphylococcus aureus SG 511	0.01	0.1	0.3	10

Discussion

It is important that a cephalosporin should be stable against β-lactamases. The stability of a compound depends on the type of enzyme to which it is exposed. The chromosomally-encoded enzymes produced by many Enterobacteriaceae are active against the majority of cephalosporins. So, the MIC_{90}-values of RU 29 246 are equal to or above 128 μg/ml for *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Serratia marcescens* and *Serratia liquefaciens*[1].

It has been shown recently that RU 29 246 is highly stable in the presence of a number of common plasmid-encoded enzymes which contributes to the low MIC-values determined against many of these enzyme producers[9]. Until recently plasmid-encoded enzymes hydrolyzing third generation cephalosporins were rarely produced by *Escherichia coli* or *Klebsiella* spp. Now a large number of extended broad spectrum β-lactamases have been described[10]. The stability of RU 29 246 and that of other orally absorbable cephalosporins to the novel enzymes is lower than that against the widespread TEM-1 or SHV-1 β-lactamases. Terefore the MIC-values of these compounds are accordingly high for strains producing extended broad spectrum enzymes such as TEM-3, TEM-4, TEM-5, TEM-6, SHV-2, SHV-4, SHV-5, the cephamycinases CMY-1, CMY-2 or the new type of cefotaximase CTX-M.

RU 29 246 is not an inhibitor for the TEM-1 β-lactamase, but inhibits the chromosomally-encoded enzyme of *Enterobacter cloacae* P99 at fairly low concentrations. In this respect, its properties are similar to those of other third generation cephalosporins of the cefotaxime-class but differ from recently developed aminothiazol-cephalosporins with quaternary substituents in the 3-position like cefpirome which does not inhibit this enzyme[11].

The affinity of RU 29 246 for the PBPs of *Escherichia coli* K12 is very similar to that of cefpodoxime. The high binding to PBP 3 results in the formation of filaments with subsequent lysis of the cells. The high affinity of the compound to PBP 1 of *Staphylococcus aureus* SG 511 might be one of the reasons for its high antibacterial activity against Staphylococci[1] which is a desirable property for a new oral cephalosporin.

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