

CEFPIROME (HR 810): LACK OF SELECTION OF β -LACTAMASE OVERPRODUCING VARIANTS

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With respect to the selection of β -lactam-resistant variants marked discrepancies between the recently developed cephalosporin HR 810 and other recently developed cephalosporins could be observed: β -lactam resistant subpopulations did not emerge during a 16-hour culture in the presence of the 20-fold minimal inhibitory concentration in a clinical *Enterobacter cloacae* isolate (2240/81) in contrast to cefoperazone, cefotaxime, ceftriaxone and ceftazidime. Breakdown of the antibacterial agents during the 16-hour period as evaluated by monitoring their levels in the medium was not responsible for selection of β -lactam-resistant subpopulations. The study of affinity of various cephalosporins to the chromosomally mediated β -lactamase of *E. cloacae* strain 2240/81 revealed low affinity of HR 810 to the enzyme (K_i amounted 4.4×10^{-3} M). It is suggested that the low affinity of this new agent to the *E. cloacae* enzyme plays a major role in the lack of selection of resistant subpopulations.

The overproduction of chromosomally mediated β -lactamases, particularly in strains of *Enterobacter cloacae* appears to play a significant role in the resistance of these organisms to the newer cephalosporins^{1,2}. Such β -lactamase overproducing variants can be easily detected on agar plates containing β -lactam antibiotics in concentrations exceeding the minimal inhibitory concentration (MIC)^{3,4}. One might assume that breakdown of the antibiotic in the medium due to β -lactamase might contribute to the selection of resistant subpopulations, thus being responsible for high MIC's. Consequently, antibiotic levels in the medium during the incubation period along with β -lactamase production of the wild strain as well as that of resistant subpopulations have to be monitored to answer this question. In a previous paper we demonstrated that β -lactamase production of resistant subpopulations is strongly influenced by the media employed which is due to the induction potency of bicyclic molecules unrelated to the β -lactam nucleus such as tryptophane, folic acid, or thiamine^{3,5}. Therefore, it cannot be considered sufficient to employ only a single medium for monitoring enzyme production and to assume enzyme production in these resistant variants constitutive³. As imipenem is known to be a potent inducer of *E. cloacae* β -lactamase, enzyme production was evaluated in the presence of this agent, too.

Recent studies by LIVERMORE pointed to a relation between the affinity of a β -lactam compound to the inducible Id enzyme of *Pseudomonas aeruginosa* and emerging resistance⁶. In this study we investigated the affinity of various cephalosporins to the chromosomally mediated *E. cloacae* β -lactamase in an attempt to answer the question, as to whether the affinity of a β -lactam contributes to the selection of resistant variants.

Materials and Methods

Strain

The *E. cloacae* wild strain 2240/81 was isolated from a clinical specimen; identification was performed with the API 20E system (API Bio-Mérieux, Nürtingen FRG).

Minimal Inhibitory Concentrations (MIC's)

MICs were determined in Mueller-Hinton broth by serial two-fold dilutions maintaining an inoculum of 5×10^8 cfu/ml (microdilution procedures).

Killing Curves

Killing curves were recorded by monitoring the colony-forming units per milliliter (cfu/ml). Bacteria were grown overnight and diluted to 10^8 cfu/ml into Mueller-Hinton broth. Antibiotics were added to the cultures in the early exponential growth phase after 1 hour. All experiments were carried out in a shaking incubator at 37°C (140 rpm/minute). Every 1 or 2 hours aliquots were plated onto Isosensitest agar (Oxoid, Basingstoke England) to determine the number of cfu/ml. Prior to plating antibiotics present in the Mueller-Hinton broth were inactivated by addition of a so-called broad-spectrum β -lactamase; the enzyme hydrolyzed not only penicillins and the older cephalosporins, but also the imino-cephalosporins and to less extent cefoperazone⁷⁾.

Monitoring of β -Lactamase Induction

Isosensitest broth and Trypticase soy broth were obtained from Oxoid Ltd., Basingstoke England, minimal salts medium was prepared as described by KNIPPERS (containing 12 g/liter Tris adjusted with HCl to pH 7.5, 5 g/liter KCl, 1 g/liter NaCl, 0.5 g/liter Na_2HPO_4 , 0.2 g/liter MgSO_4 , 0.1 g/liter CaCl_2 , 1.1 g/liter NH_4Cl , and 1 g/liter glucose)⁸⁾. One batch was used throughout the experiments. For the study of enzyme induction overnight cultures of the wild strain and the resistant subpopulations were diluted 100-fold and incubated at 37°C with shaking (140 rpm). In one assay series 0.25 mg/liter imipenem was added to the Isosensitest broth.

Enzyme induction was monitored for 24 hours (samples were taken after 2, 3, 4, 6, 8, 12 and 24 hours). Cells were harvested at the above mentioned time-intervals by centrifugation at $5,000 \times g$ for 10 minutes (Christ Minifuge, Osterode FRG) and washed once with NaCl (9 g/liter). The cells were resuspended in a corresponding volume of distilled water and subjected to ultrasonic disruption (Branson Sonic Power-Ultrasonics, Plainview, NY, USA) for 2 minutes at 4°C. Cell debris was removed by centrifugation at $5,000 \times g$ for 15 minutes at 4°C. The extracts were immediately frozen at -20°C and analyzed within 10 days.

β -Lactamase Assay

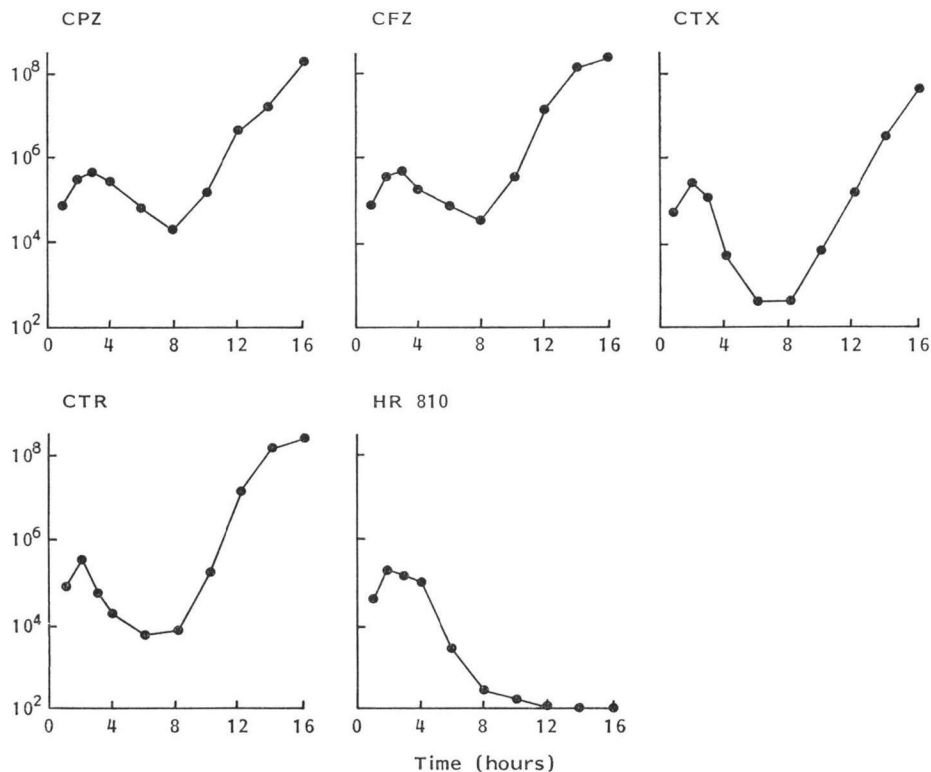
Enzyme activity was determined by means of the chromogenic cephalosporin compound Centa (Hoechst A.G., Frankfurt FRG); its sensitivity is comparable to that of nitrocefin, but it is less affected by pH and the protein matrix than nitrocefin. Increase in absorbance was monitored at 412 nm for 2 minutes. All experiments were carried out in 0.05 mol/liter triethanolamine buffer adjusted to pH 7.6 with a final substrate concentration of 3.3×10^{-4} mol/liter at 37°C. Total protein content of the cell-free supernatants was determined by the method of MARKWELL *et al.*⁹⁾. Enzyme activity was expressed in units/mg protein; one enzyme unit is defined as the amount of enzyme hydrolyzing 1 μmol substrate/minute.

Isoelectric Focusing

Isoelectric focusing was performed as previously described (CULLMANN *et al.*¹⁰⁾. Gel sheets containing Ampholine pH 3.5~9.5 were used (LKB-Produkter, Stockholm Sweden). Enzyme activity was detected with the chromogenic cephalosporin nitrocefin (obtained from Cascan-Glaxo, Wiesbaden FRG). In the experiments strains were grown for 4 hours. TEM-1 enzyme (*Escherichia coli* K12 R6K), OXA-1 enzyme (*E. coli* K12 pRGN 238), and PSE-2 enzyme (*P. aeruginosa* pMG 33) served as known marker enzymes¹¹⁾. The affinity of the β -lactam compounds included in this study was evaluated by means of enzyme kinetics using highly purified enzyme. Preparation of the enzyme from *E. cloacae*

Fig. 1. Killing curves of the *Enterobacter cloacae* wild strain 2240/81 in the presence of 20-fold the MIC of cefoperazone (CPZ), ceftazidime (CFZ), ceftriaxone (CTR), cefotaxime (CTX) and HR 810.

After 8 hours regrowth occurred in all cultures with the single exception of the HR 810 containing culture.



2240/81 and experimental details were previously reported¹²⁾. K_m and K_i values were calculated graphically from Eadie-Hofstee or Dixon plots, respectively¹³⁾.

Reagents and Antibiotics

β -Lactam compounds were supplied by the following manufacturers: cephalothin, cefotaxime, HR 810 and the chromogenic compounds Centa and PADAC from Hoechst A.G. (Frankfurt, FRG); cefoxitin and imipenem from Merck Sharp & Dohme (Munich, FRG); cefoperazone from Pfizer, (Karlsruhe, FRG); ceftazidime from Cascan-Glaxo (Wiesbaden, FRG); ceftriaxone from Hoffmann-La Roche (Basel, Switzerland); latamoxef from Eli Lilly (Giessen, FRG); cefacedone from E. Merck (Darmstadt, FRG). If not specified otherwise, all reagents and media were obtained from E. Merck (Darmstadt, FRG).

Results

Killing Curves

The *E. cloacae* wild strain 2240/81 was grown in the presence of the 20-fold MIC of each of the antibiotics (cefoperazone, cefotaxime, ceftriaxone, ceftazidime and HR 810). The addition of the antibiotic resulted in a reduction of the initial inoculum of about 99%; regrowth occurred after about 8 hours with the exception of HR 810 (Fig. 1). Antibiotic concentrations at 16 hours remained in excess of the MIC by at least 10-fold with the exception of cefoperazone (Table 1), control assays

Table 1. Inactivation of β -lactam compounds after a 16-hour culture with the *Enterobacter cloacae* (wild strain).

Compound	Initial concentration (mg/liter)	Final concentration (after 16 hours)	
		(mg/liter)	(%)
Cefoperazone	7.8	<0.4	<5
Cefotaxime	25	20.5	82
Ceftriaxone	31	17.5	57
Ceftazidime	3.9	1.9	49
HR 810	12.5	8.5	68

containing only the antibiotic in Mueller-Hinton broth revealed a recovery of the agents of at least 95% during a 16-hour incubation at 37°C (details not shown) *i.e.* the antibiotics are not subjected to slow inactivation due to chemical instability.

β -Lactamase Production of the Wild Strain and the 16-Hour Subpopulations

In the wild strain 2240/81 β -lactamase production was barely detectable (Fig. 2); on the contrary addition of 0.25 mg/liter imipenem resulted in a marked induction of the chromosomally mediated enzyme (Fig. 2). The 16-hour subpopulations did not show a uniform behavior; cefoperazone and ceftriaxone subpopulations exhibited the highest levels of enzyme production when further grown in Isosensitest medium, which were not enhanced by addition of 0.25 mg/liter imipenem (Fig. 3a). Spontaneous enzyme production among the cefotaxime and ceftazidime 16-hour subpopulations was marginal, whereas both exhibited a marked increase in their spontaneous enzyme production after addition of 0.25 mg/liter imipenem (Fig. 3b). It is evident that enzyme production depends on the media employed *i.e.* minimal medium, Isosensitest or Trypticase soy broth in the resistant subpopulations. The most striking result, however, was obtained for the HR 810 16-hour subpopulation, properties were identical as compared to the parent strain 2240/81. Enzymes produced by the parent strain and the 16 hour-subpopulations proved to be identical as revealed by isoelectric focusing (data not shown).

Fig. 2. Kinetics of β -lactamase induction in the *E. cloacae* wild strain 2240/81.

Spontaneous enzyme production was barely detectable, considerable enzyme production could be observed only after induction with 0.25 mg/liter imipenem.

● Minimal salts medium, ○ Trypticase soy broth, ▲ Isosensitest broth, □ Isosensitest broth with 0.25 mg/liter imipenem.

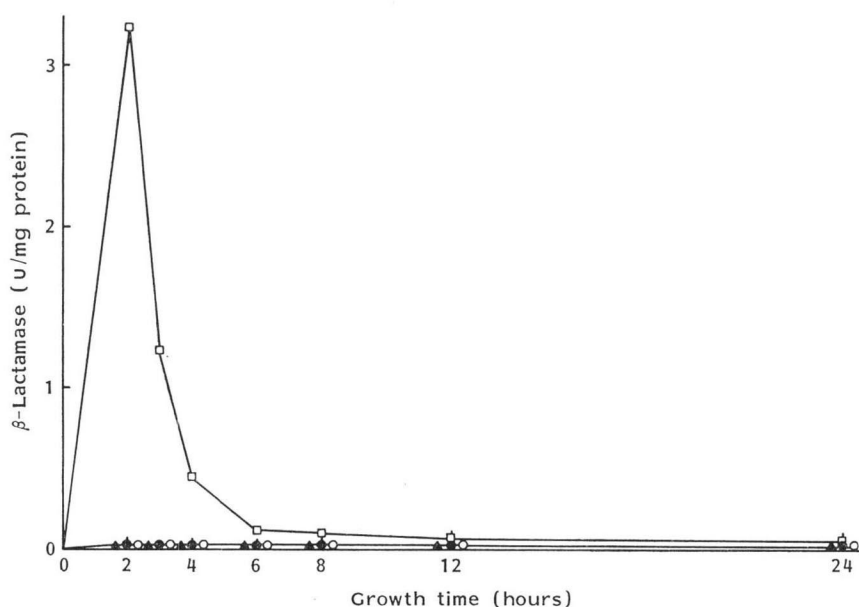


Fig. 3a. Kinetics of β -lactamase production of the 16-hour cefoperazone subpopulation; 0.25 mg/liter imipenem did not enhance enzyme production; details see Fig. 2.

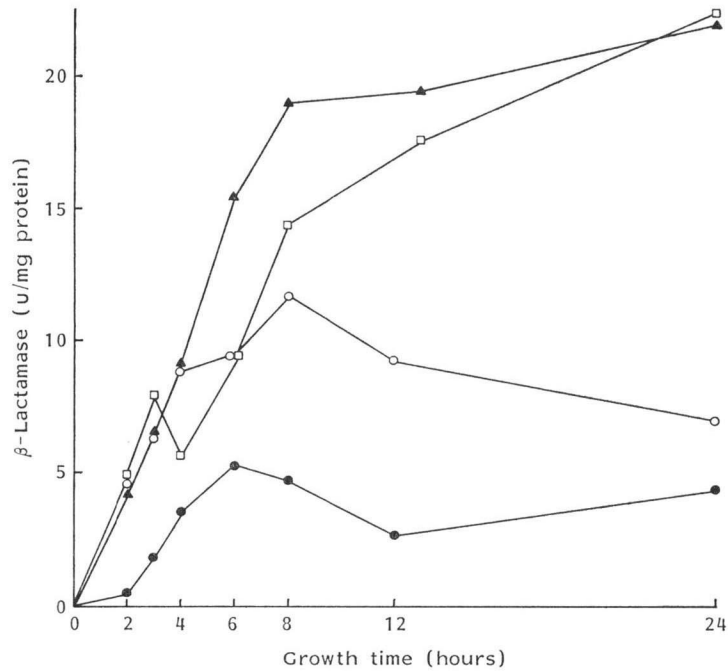


Fig. 3b. In the 16-hour cefotaxime subpopulation spontaneous enzyme production was marginal, yet increased markedly after induction with 0.25 mg/liter imipenem; details see Fig. 2.

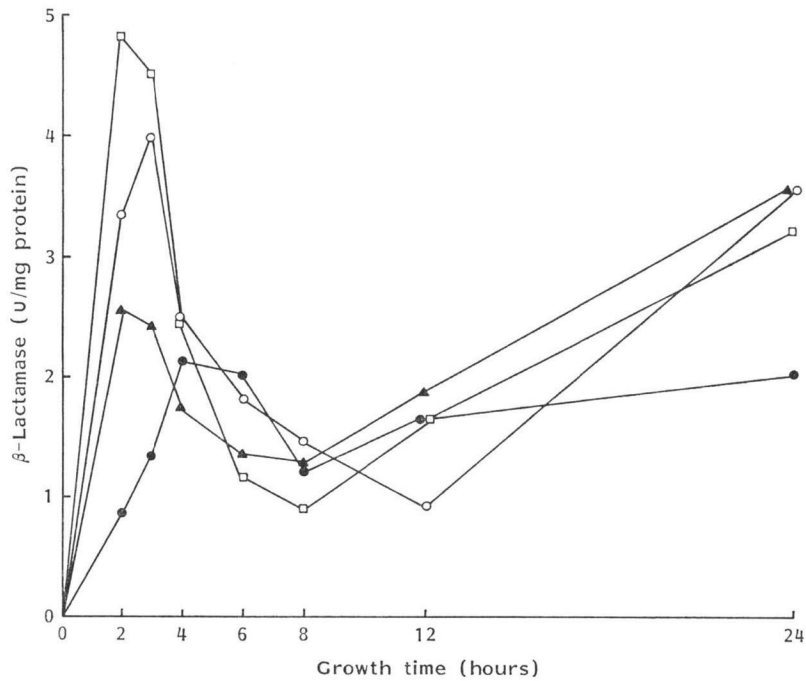


Table 2. Minimal inhibitory concentrations (in mg/liter) of *Enterobacter cloacae* wild strain 2240/81 and the resistant subpopulations against various β -lactam compounds.

Strain	Cephalothin	Cefoxitin	Cefoperazone	Cefotaxime
2240/Wild strain	1,024	128	0.125	0.25
2240/Cefoperazone	1,024	256	256	128
2240/Cefotaxime	1,024	128	128	128
2240/Ceftazidime	1,024	128	128	128
2240/Ceftriaxone	1,024	256	256	128
2240/HR 810	1,024	128	0.25	0.25

Strain	Ceftriaxone	Ceftazidime	HR 810	Latamoxef	Imipenem
2240/Wild strain	0.25	0.25	0.25	0.25	1
2240/Cefoperazone	512	128	128	16	2
2240/Cefotaxime	128	64	16	8	2
2240/Ceftazidime	256	64	8	8	1
2240/Ceftriaxone	512	128	128	8	1
2240/HR 810	0.25	0.25	0.25	0.25	1

Table 3. Affinity of various β -lactam compounds to the chromosomally mediated enzyme.

β -Lactam compound	K_m (M)	K_i (M)	
		Cephalothin	Cefacedone as the substrate
Cefoperazone	6×10^{-5}	1×10^{-5}	n.d.
Cefotaxime	n.d.	5×10^{-8}	1.3×10^{-8}
Ceftriaxone	n.d.	1.2×10^{-8}	1×10^{-8}
Ceftazidime	n.d.	8×10^{-8}	3×10^{-8}
HR 810	n.d.	4.4×10^{-3}	(with PADAC as the substrate)

n.d. Not detected.

The enzyme focused at pH 8.5 as calculated from the known marker enzymes. Satellite bands were observed in those subpopulations exhibiting the production of large amounts of enzyme. However, no further enzymes appeared.

MIC's of the Wild Strain and the 16 Hour-subpopulations

MIC's are collated in Table 2. It is evident that MIC's against latamoxef and HR 810 are less affected in the resistant subpopulations than those of other related agents. No discrepancies occurred for the imipenem MIC's between the parent strain and the related cephalosporin-resistant subpopulations.

Enzyme Kinetics

Cephalothin and cefacedone served as the substrates for determination of the K_i values which were considered a criterion for the affinity of the newer cephalosporins to the *E. cloacae* enzyme. With cefacedone as the substrate K_i values were somewhat lower as compared to cephalothin as the substrate. Due to the low affinity of HR 810 to the enzyme, cephalothin and cefacedone could not be employed as substrates because of spectral interference; this was why studies with HR 810 were carried out with the chromogenic compound PADAC as the substrate for K_i determination. For cefoperazone K_m exceeded K_i somewhat but not striking, in most cases this has to be attributed to the experimental conditions. Cefotaxime and ceftriaxone exhibited the highest affinity among the com-

pounds included in this study (Table 3). The Dixon plot revealed a merely competitive type of inhibition for each of the cephalosporins.

Discussion

The following conclusions can be drawn from the results shown above:

a) With respect to the selection of resistant subpopulations, there are striking discrepancies between HR 810 and other recently developed cephalosporins. In the presence of HR 810 no resistant variants could be selected which is consistent with previous observations⁴⁾.

b) The slow breakdown of the antibiotic in the 16-hour cultures cannot be made responsible for the selection of resistant subpopulations.

c) Contrary to results obtained with the newer cephalosporins, HR 810 exhibits low affinity to the chromosomally mediated β -lactamase from *E. cloacae* 2240/81.

An excellent overview on this problem was recently given by SANDERS²⁾. Resistant variants can not only be obtained under laboratory conditions; but have been implicated in therapeutic failure¹⁴⁻¹⁷⁾. Thus new compounds like HR 810 offer promise in this regard.

In this study we investigated as to whether resistant subpopulations are selected in the presence of the 20-fold MIC. Further exploration of this problem revealed that in 10 clinical *E. cloacae* isolates (all strains exhibited the production of a cefoxitin inducible β -lactamase) that resistant subpopulations could be selected in concentrations of the newer cephalosporins exceeding the MIC's up to 100-fold. On the contrary, HR 810 concentrations exceeding 8-fold the MIC were sufficient to rule out the emergence of resistant subpopulations (CULLMANN & DICK, manuscript in preparation).

With respect to the penicillin binding proteins, HR 810 binds preferentially to PBP 3 (like cefotaxime and other related compounds)¹⁸⁾. Compared with cefotaxime the affinity of HR 810 to PBP 3 is lowered (50% saturation of PBP 3a requires 0.09 mg/liter HR 810, but only 0.002 mg/liter cefotaxime¹⁸⁾). One might therefore assume that the behavior of HR 810 towards the penicillin binding proteins is not involved in the above described mechanism of resistance *i.e.* the selection of resistant subpopulations.

These considerations emphasize the low affinity of HR 810 to the *E. cloacae* cephalosporinase. The relevance of low affinity of β -lactam antibiotics to chromosomally mediated β -lactamases was pointed out recently by LIVERMORE; cefsulodin, a compound that lacks binding to the Id enzyme, is still active even in the case of resistance against other cephalosporins⁹⁾. In the case of extremely low affinity to the β -lactamase, affinity to the penicillin binding proteins will still exceed that of the β -lactamase *i.e.* binding to the target is much less affected by the periplasmatically located β -lactamase.

The resistant subpopulations did not exhibit a uniform behavior and exhibited discrepancies in their enzyme production due to the media employed. We already described that discrepancies exist between single resistant subpopulations between spontaneous and total amount of enzyme production⁹⁾. This phenomenon can be attributed to the induction of β -lactamases by compounds unrelated to the β -lactam nucleus such as amino acids or vitamins⁹⁾. Nevertheless, resistance against the newer cephalosporins of resistant subpopulations is due to the overproduction of chromosomally mediated β -lactamase, which cannot be considered constitutively produced; the inducible part should be taken into account in any case.

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