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Factors involved in the enhanced efficacy against Gram-negative bacteria of fourth generation cephalosporins

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The fourth generation cephalosporins, cefpirome and cefepime, demonstrate better activity against strains of *Enterobacter cloacae* with derepressed β-lactamase than the third generation compounds cefotaxime and ceftriaxone. Several methodological refinements were used to measure the parameters, predicted by the Zimmermann–Rosselet equation to be important in the efficacy of β-lactams. Outer membrane permeability was measured by a novel HPLC method. The kinetics of interaction of purified β-lactamase with β-lactams were estimated to calculate the inhibition and catalytic constants. The periplasmic concentration of β-lactams leading to growth inhibition of cells was determined by substituting the above parameters into the Zimmermann–Rosselet equation. Consideration of these three factors allowed accurate prediction of MICs in isogenic *E. cloacae* strains with differing porin or β-lactamase contents. The fourth generation cephalosporins had markedly reduced affinity for β-lactamase and increased outer membrane permeability when compared to the third generation cephalosporins. Such advantages were only partly offset by a lower stability of complexes with β-lactamase and reduced affinity for their targets.

Introduction

In recent years, pharmaceutical companies have attempted to improve the efficacy and spectrum of β-lactams by introducing novel substitutions on to cephalosporin molecules. The most recent commercial cephalosporins are those of the third generation, including cefotaxime, ceftriaxone, and ceftazidime which feature a methoxyimyl 5-aminothiazol moiety at the 7α position of the cephem ring (Rolinson, 1986). This results in increased stability to β-lactamases. However, the rapid development of mutant, plasmid-encoded TEM β-lactamases, which include third generation antibiotics as potential substrates, and the ineffectiveness of these compounds against strains of *Enterobacter cloacae* and *Pseudomonas aeruginosa* with derepressed chromosomal β-lactamase, have somewhat limited their clinical success. Recently, a fourth generation of compounds, including cefpirome, cefepime and E1040, have been introduced. These agents contain a positively charged quaternary nitrogen at the 3 position (Rolinson, 1986). As a consequence, these compounds have better activity than that of third generation compounds against β-lactamase derepressed mutants of *P. aeruginosa* and enteric bacteria (Phelps *et al.*, 1986; Sanders & Sanders, 1986). While several studies have examined the role of β-lactamase affinity and stability in determining the MIC of these antibiotics, studies on their outer membrane permeability and target

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affinity have received less attention. Recently, we reported on a novel HPLC based method for measuring outer membrane permeability in intact cells of *E. cloacae* (Bellido, Pechere & Hancock, 1991*a*). This method, as well as refinements in measurement of β-lactamase kinetic parameters, allowed estimation of all of the factors influenced by the structural differences between third and fourth generation cephalosporins (Bellido, Pechere & Hancock, 1991*b*). We review and expand here the findings of that study.

Assay of outer membrane permeability

The Zimmermann & Rosselet (1977) hypothesis indicates that outer membrane permeability is rate-limiting for hydrolysis of externally added β-lactam by β-lactamase in the periplasm. Thus, in our studies, an equilibrium rate of diffusion of β-lactam across the outer membrane was balanced and maintained by an equal rate of hydrolysis in the periplasm. Since most of the compounds used were poorly hydrolysed by *E. cloacae* β-lactamase, measurable rates of hydrolysis were only achieved when sufficient β-lactamase was present in the periplasm (due to the constitutive expression of β-lactamase in the *E. cloacae* strain R1 utilized here) and when sufficiently high levels of compound were present externally. However, the high sensitivity of the HPLC outer membrane penetration assay procedure (as described in Bellido *et al.*, 1991*a*) permitted external antibiotic concentrations equal to or less than the MIC to be utilized.

As clearly shown in Figure 1 and Table I, the fourth generation compounds, cefepime and cefpirome, despite being slightly bulkier than the third generation cephalosporins, cefotaxime and ceftriaxone, permeate the outer membrane (i.e. disappeared from the supernatant) 5–7 times more rapidly. This is consistent with an extra positive charge on the fourth generation compounds (Rolinson, 1986) and the selectivity of OmpF porins (the outer membrane β-lactam channel (Figure 3)) for cations over anions (as determined in *Escherichia coli*) (Benz, Schmid & Hancock, 1985). Since the fourth generation cephalosporins are bullet-shaped (as determined by molecular modelling using the Alchemy program (Tripos Associates Inc., St Louis, MO, USA)) and too wide to go through porins sideways, the positive charge would serve to orient the β-lactam at the mouth of the porin. However, charge is not the only important

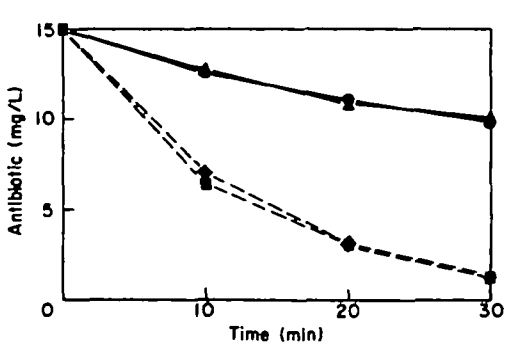

Figure 1. Uptake of β-lactam antibiotics into cells assessed by HPLC determination of disappearance of β-lactam from the supernatant. The concentration gradient across the outer membrane was maintained by hydrolysis of β-lactam by periplasmic β-lactamase (see text). Third generation cephalosporins, cefotaxime (●) and ceftriaxone (▲). Fourth generation cephalosporins, cefpirome (◆) and cefepime (■).

Table I. Factors influencing β-lactam activity in *E. cloacae* strain R1

Antibiotic	Outer membrane permeability parameter P[a] (nm/s)	β-Lactamase kinetic constants[b] K_m (μM)	V_{max} (pmol/mg/s)	Antibiotic *concentration* in periplasm at the MIC[c] S_i (nM)
Cephaloridine	960	140	1,650,000	>2180
Ceftriaxone	5·1	0·1	32	17
Cefotaxime	5·6	0·2	55	13
Cefpirome	37	140	250	200
Cefepime	29	180	216	250

[a]Assessed from the measured rate (V) of disappearance of antibiotic from the medium using an HPLC assay (Bellido *et al.*, 1991*a*) and Fick's Law of diffusion $V = P \times A \times (S_o - S_p)$ where A = the total surface area of cells in the assay, S_o = the external antibiotic concentration and S_p = the periplasmic antibiotic concentration.

[b]Determined using concentrated suspensions of purified β-lactamase. When K_m was low it was calculated as K_i and $V_{max} = K_{cat} \times E$ (see Bellido *et al.*, 1991*b* for details).

[c]Calculated as described in the text. Because of the extremely high V_{max}, S_i for cephaloridine can be considered rather imprecise.

determinant of good penetration. The smaller, zwitterionic, first generation cephalosporin, cephaloridine, permeates the outer membrane at rates substantially higher than the bulkier fourth generation compounds.

β-Lactam hydrolysis and calculated periplasmic inhibitory concentrations

We confirmed previous observations (Phelps *et al.*, 1986; Then *et al.*, 1988; Nikaido, Liu & Rosenberg, 1990) that the fourth generation cephalosporins had 4–8 fold higher V_{max} values but dramatically reduced affinities for *E. cloacae* chromosomal β-lactamase. Indeed K_m values were nearly four orders of magnitude higher for cefpirome and cefepime than for cefotaxime and ceftriaxone (Table I). All four of these β-lactams formed much more stable complexes with β-lactamase than the first generation cephalosporin, cephaloridine, as revealed by their relative V_{max} values (Table I), which are related to the stability (i.e. the rate of breakdown into products) of the enzyme-substrate complexes.

Calculation of the actual rate at which the substrate was being hydrolysed at the MIC revealed that these large differences in K_m somewhat overestimated the importance of enzyme kinetics in determining the MIC of strain R1. Thus despite a 40-fold difference in MICs between the third and fourth generation cephalosporins (Table II), the actual rates of hydrolysis in the periplasm at the MIC concentrations (calculated from the K_m, V_{max} and S_i values in Table I) were only around ten-fold higher for the third generation cephalosporins (Bellido *et al.*, 1991*b*).

The periplasmic concentration (S_i) of β-lactam when an external concentration (S_o) equivalent to the MIC was present outside the cell was calculated from $MIC = S_i [1 + V_{max}/P \times A \times (K_m + S_i)]$, a simple rearrangement of the Zimmermann & Rosselet (1977) equation. Since the periplasm is adjacent to the β-lactam binding sites of the target penicillin binding proteins (PBP), this S_i value represents the actual minimal concentration of β-lactam required *in situ* to inhibit these PBPs sufficiently to prevent

Table II. MIC predictions, for *E. cloacae* 218 strains S and R2, based on factors influencing β-lactam activity and the Zimmermann–Rosselet equation, $MIC = S_i\,[1 + V_{max}/P \times A \times (K_m + S_i)]$

Antibiotic	MIC (mg/L) for[a] Strain S Predicted	Strain S Observed	Strain R1 Observed	Strain R2 Predicted	Strain R2 Observed
Cephaloridine	>400[b]	>400	>400	>400	>400
Ceftriaxone	0·1[b]	0·1	20	100	100
Cefotaxime	0·1[b]	0·1	20	90	100
Cefpirome	0·05	0·1	0·5	1·4	2
Cefepime	0·05	0·1	0·5	1·4	2

[a]MIC predictions were not made for strain R1 since the S_i values used in MIC predictions for strains S and R2 were calculated by substituting measured values for MIC, V_{max}, K_m, P and A into the above equation. Extrapolation of this S_i value was possible since S, R1 and R2 are isogenic, S being the wild type, R1 being derepressed for chromosomal β-lactamase, and R2 being both derepressed for chromosomal β-lactamase and having an additional porin defect.

[b]An additional factor, β-lactamase induction by the antibiotic (Minami *et al.*, 1980), was included in these predictions.

bacterial growth. Interestingly, the results indicated that cefpirome and cefepime, in addition to having a lower affinity for β-lactamase compared to cefotaxime and ceftriaxone, also had a 10–20 fold lower affinity for penicillin binding proteins (Table II). This is consistent with the observed similarity of the β-lactam binding pockets (active sites) of β-lactamase and penicillin binding proteins (Frere & Jors, 1985).

Prediction of MICs

If the values reported in Table I were correctly determined, they should permit accurate predictions to be made. Since in strain R1 we had measured three of these parameters (P, K_m, V_{max}) and estimated one other (S_i), as described above, we reasoned that the four parameters could be used to predict MIC values for strains S and R2 which differed in the measured parameters (using the above rearrangement of the Zimmermann & Rosselet equation). Predicted MIC values were in every case within one dilution of the observed values (Table II).

The accuracy of MIC predictions (Table II) indicates that all parameters measured and all assumptions made were probably correct. Thus, fourth generation cephalosporins have two substantial advantages and two disadvantages relative to the third generation cephalosporins. The advantages are a 10,000 fold reduction in affinity for chromosomal β-lactamase of *E. cloacae* and a 5 to 7 fold increase in permeation rate across the outer membrane. The disadvantages are a 4–8 fold higher rate of conversion to products after association with β-lactamase, as reflected by the magnitude of V_{max}, and a 10–20 fold lower affinity for their PBP targets. Overall, these changes result in a class of compounds that demonstrate excellent activity against bacteria with derepressed chromosomal β-lactamase, including those with lowered outer membrane permeability like *E. cloacae* strain R2 (Table II) or *P. aeruginosa* (Sanders & Sanders, 1986). In contrast, the third generation compounds are ineffective against such β-lactamase-hyperproducing strains which are being encountered with increasing frequency in the clinic.

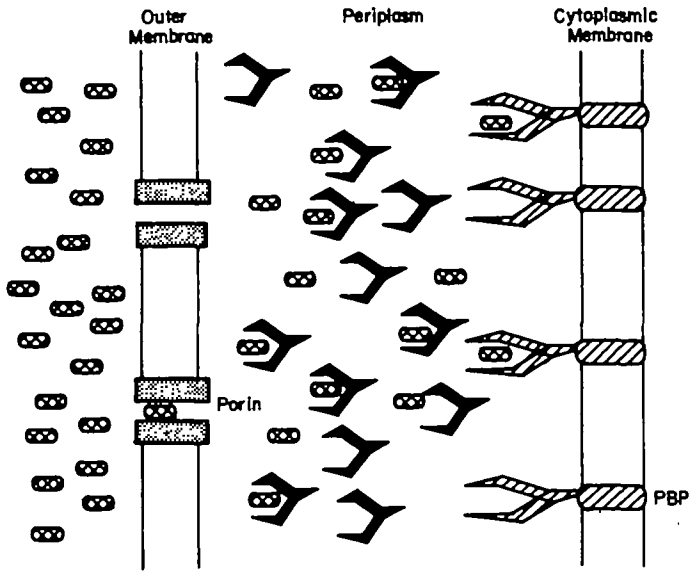

Figure 2. Identity and location of factors determining the efficacy of β-lactams (⊂⊃) in Gram-negative bacteria namely: permeation through porins in the outer membrane, periplasmic β-lactamase (⊃) hydrolysis kinetics (K_m, V_{max}) and amounts, and binding affinity for cytoplasmic membrane penicillin binding proteins (PBP).

These results point out that the interplay of a finite number of parameters determines the efficacy of β-lactams (Figure 2). The ideal β-lactam would have rapid outer membrane penetration, high stability when complexed to β-lactamase and poor affinity for all β-lactamases, and the ability to inhibit bacterial cell wall synthesis at low periplasmic concentrations. Some of these factors, especially the latter two, may be incompatible. However, we are now in a position to realistically assess structure/activity relationship for each of these parameters. In the meantime, the fourth generation structures would appear to be an excellent compromise.

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