Stability of cefdinir (CI-983, FK482) to extended-spectrum plasmid-mediated β -lactamases

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Summary. Fourteen plasmid-encoded extended-spectrum β -lactamases were purified from *Escherichia coli* transconjugants of original clinical isolates. The $V_{\max}$, K_m and $V_{\max}/K_m$ were each determined for ampicillin, carbenicillin, cephaloridine, cephalixin, cefuroxime, cefixime, cefdinir, ceftazidime and cefotaxime as substrates with eight of these enzymes and with the narrow-spectrum β -lactamase, TEM-1. The relative rates of hydrolysis of ampicillin, cephaloridine, cephalixin, cefuroxime, cefixime, cefdinir, ceftazidime and cefotaxime were also determined for the remaining six enzymes. Cefdinir had $V_{\max}/K_m$ or relative rates of hydrolysis values either equal to or lower than ampicillin, cephaloridine, cephalixin and cefotaxime for all the enzymes tested. Overall, cefdinir was more stable to the 15 β -lactamases tested than either cefuroxime or cefixime; however, ceftazidime was more stable than cefdinir to hydrolysis by eight of the enzymes tested.

Introduction

Very few oral cephalosporins have been manufactured. The early oral cephalosporins, cephadrine and cephalixin, have proved effective in certain infections; however, they are susceptible to hydrolysis by many β -lactamases. A similar drug, cefaclor, is also susceptible, but has improved activity against *Haemophilus influenzae*. As these cephalosporins are susceptible to hydrolysis by many β -lactamases, they fall within the so-called "first generation". Cefdinir is an aminothiazolyl hydroxyimino-cephalosporin with a C3 vinyl group and this combination makes it suitable for oral administration.

A limited study by Neu *et al.*¹ examined the minimum inhibitory concentrations of a series of β -lactam antibiotics and demonstrated the ability of cefdinir to resist β -lactamase hydrolysis differentially. We investigated the β -lactamase stability of cefdinir and other cephalosporins to plasmid-encoded extended-spectrum β -lactamases.² These enzymes are capable of hydrolysing the later-developed extended-spectrum cephalosporins such as ceftazidime and cefotaxime. The β -lactamases examined in the present study were TEM-1, TEM-3, TEM-4, TEM-5, TEM-7, TEM-10, TEM-E1, TEM-E2, TEM-E3, TEM-E4, SHV-2, SHV-3, SHV-5 and BIL-1. This array of enzymes includes at least one β -lactamase from each group of extended-spectrum β -lactamases.²

Materials and methods

Bacterial strains

The β -lactamase preparations used in the stability assays were obtained from the strains of *Escherichia coli* listed in table I.

β -Lactamase preparation

The β -lactamase samples were prepared from 1-L cultures of bacteria grown overnight in nutrient broth at 37°C. The cells were harvested by centrifugation at 6000 *g* for 15 min at 4°C. The resultant pellet was washed in 25 mM sodium phosphate buffer (pH 7.0) and the centrifugation was repeated. The final pellet was resuspended in 1 ml of 25 mM sodium phosphate buffer (pH 7.0) and subjected to two 30-s treatments of ultrasonication (8 μ m) at 0°C, with a 1-min cooling period between treatments. The resultant lysate was cleared by centrifugation at 32 000 *g* for 30 min at 4°C. This crude β -lactamase preparation was applied to a Sephadex G-75 filtration column (2 cm² $\times$ 90 cm) and eluted with 25 mM sodium phosphate buffer (pH 7.0) at 15 ml/h.³ The fractions with the greatest β -lactamase activity were pooled and used in the kinetic measurements.

Iso-electric focusing

The integrity of the β -lactamase preparations was checked by analytical iso-electric focusing.⁴ The enzymes were examined on polyacrylamide gels employing a 1:1 ratio of pH 3.5–10 ampholines and either pH 9–11 or pH 4–6 ampholines depending on

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Table I. Strains producing the β -lactamases used in this study

Strain	Plasmid-encoded	β -lactamase
<i>E. coli</i> K-12	J53-2	TEM-1
<i>E. coli</i> K-12	C600	TEM-3
<i>E. coli</i> K-12	J53-2	TEM-4
<i>E. coli</i> K-12	J53-2	TEM-5
<i>E. coli</i> K-12	J53-2	TEM-6
<i>E. coli</i>	BM694	TEM-7
<i>E. coli</i> K-12	J53-2	TEM-10
<i>E. coli</i> K-12	J53-2	TEM-E1
<i>E. coli</i> K-12	J53-2	TEM-E2
<i>E. coli</i> K-12	J53-2	TEM-E3
<i>E. coli</i> K-12	J53-2	TEM-E4
<i>E. coli</i> K-12	J53-2	SHV-2
<i>E. coli</i> K-12	J53-2	SHV-3
<i>E. coli</i> K-12	J53-2	SHV-5
<i>E. coli</i> K-12	J53-2	BIL-1

the expected pI of the purified β -lactamase. The purified β -lactamases were focused alongside known standard β -lactamase samples.

Assay of β -lactamase

The β -lactamase activities were measured by the decrease of absorbance of the β -lactam substrate in a Perkin-Elmer Lambda 2 spectrophotometer. In the normal assay procedure, a test and a control cuvette were set up. The test cuvette contained 0.3 ml of the substrate solution plus 2.6 ml of 50 mM sodium phosphate buffer, pH 7.0. The control cuvette contained 2.9 ml of the same buffer. The assay was started by the introduction of 0.1 ml of the enzyme solution to both cuvettes. The fall in absorbance was measured at a suitable wavelength for each substrate, usually at the λ max. The fall in absorbance was then related to the decrease in substrate concentration. The TEM-1 β -lactamase was used as a control.

Measurement of substrate profile

The substrate profiles of β -lactamases were determined by measuring the rates of hydrolysis of eight β -

lactams at a fixed concentration (0.1 mM). The rates were expressed relative to ampicillin.

Measurement of V_{max} , K_m and relative efficiency (V_{max}/K_m)

The rates of hydrolysis of cephalosporins were measured at concentrations between 0.01 mM and 0.1 mM, and the rates of hydrolysis of penicillins were measured at concentrations between 0.1 mM and 1 mM. The K_m and V_{max} values for the hydrolysis of a β -lactam by a β -lactamase were then obtained by the method of Lineweaver-Burk.⁵ The results were expressed as efficiency of hydrolysis values (V_{max}/K_m) and normalised with respect to ampicillin as outlined by Bush and Sykes.⁶

Results

All the β -lactamases were purified and each purified enzyme had a pI value corresponding to that previously reported. There was no evidence of the presence of chromosomal β -lactamase. V_{max} is a measure of the ability of an enzyme to hydrolyse a drug that has bound to its active site. V_{max} values, relative to that obtained with ampicillin, are shown for nine β -lactam substrates with eight extended-spectrum β -lactamases (table II) and the narrow spectrum TEM-1 β -lactamase (control). The V_{max} values for cefdinir were always lower than the V_{max} values for ampicillin and cephaloridine. For most enzymes, the V_{max} value for cefdinir was higher than that for carbenicillin, cephalexin, cefixime or ceftazidime in only one instance. These lower V_{max} values for cefdinir suggest that cefdinir would be less likely than the other drugs tested to be hydrolysed by the β -lactamases shown in table II.

The rate of hydrolysis only measures the activity of the enzyme once the drug has bound. The K_m value establishes the affinity of the substrate bound to the enzyme; the higher the value the less affinity there is for the substrate. There was no general pattern to the

Table II. Relative* V_{max} values of extended spectrum β -lactamases for nine β -lactam antibiotics

β -lactamase	Relative V_{max} value* for								
	Ampicillin	Carbenicillin	Cephaloridine	Cephalexin	Cefuroxime	Cefixime	Cefdinir	Ceftazidime	Cefotaxime
TEM-1 (control)	100	11	23	0.72	UM	UM	UM	UM	0.06
TEM-3	100	...	454	151	95	284	21	9.1	536
TEM-5	100	51	410	37	102	124	96	135	26
TEM-10	100	48	58	3.0	2.0	12	1.5	30	2.2
TEM-E2	100	23	87	3.19	UM	13	0.23	0.87	0.84
TEM-E3	100	51	61	5.3	95	28	2.4	44	1.9
TEM-E4	100	38	98	12.5	1.3	0.37	4.2	6.3	2.1
SHV-3	100	15	119	11.2	5.6	2.1	0.5	1.0	12
BIL-1	UM	UM	50	100	UM	UM	UM	UM	UM

UM, unmeasurable as hydrolysis was too low.

..., not done.

*Expressed as a percentage of the value for ampicillin except with BIL-1, which is related to the nitrocefin value.

Table III. Km values of extended spectrum β -lactamases for nine β -lactam antibiotics

β -lactamase	Km value (μ M) for								
	Ampicillin	Carbenicillin	Cephaloridine	Cephalexin	Cefuroxime	Cefixime	Cefdinir	Ceftazidime	Cefotaxime
TEM-1 (control)	167	100	167	166	UM	UM	UM	UM	286
TEM-3	63	...	100	333	250	250	118	167	11
TEM-5	69	38	143	77	250	125	200	330	21
TEM-10	111	39	100	200	167	66	200	100	15
TEM-E2	119	83	500	333	UM	1000	77	500	181
TEM-E3	91	38	91	333	250	250	118	167	11
TEM-E4	29	72	80	200	200	17	500	1500	77
SHV-3	83	111	59	30	500	100	16.7	500	25
BIL-1	UM	UM	166	118	UM	UM	UM	UM	UM

UM, unmeasurable as hydrolysis was too low.
 not done.

Table IV. Relative efficiencies ($V_{\max}/K_m$) of hydrolysis of nine β -lactam antibiotics by extended spectrum β -lactamases

β -lactamase	Relative efficiency ($V_{\max}/K_m$)* of hydrolysis of								
	Ampicillin	Carbenicillin	Cephaloridine	Cephalexin	Cefuroxime	Cefixime	Cefdinir	Ceftazidime	Cefotaxime
TEM-1	100	18	23	0.87	UM	UM	UM	UM	0.04
TEM-3	100	...	285	38	48	89	27	7.4	269
TEM-5	100	93	198	37	28	77	37	28	85
TEM-10	100	137	64	1.7	1.3	12	0.83	33	16
TEM-E2	100	32	20	1.1	UM	0.37	0.36	0.21	0.55
TEM-E3	100	122	61	5.7	1.0	31	5.7	24	16
TEM-E4	100	15	36	1.84	0.19	0.65	0.25	0.12	0.79
SHV-3	100	11	168	31	34	1.76	1.8	0.17	38
BIL-1	UM	UM	60	54	UM	UM	UM	UM	UM

*Value expressed as a percentage of the value for ampicillin except for BIL-1 which is related to cephalexin.
 UM, unmeasurable as hydrolysis was too low.
 not done.

Table V. Relative rates of hydrolysis of eight β -lactam antibiotics by six extended-spectrum β -lactamases

β -lactamase	Relative rate of hydrolysis* of							
	Ampicillin	Cephaloridine	Cephalexin	Cefuroxime	Cefixime	Cefdinir	Ceftazidime	Cefotaxime
TEM-4	100	114	83	19	21	8.8	3.4	39
TEM-6	100	180	19	7.7	9.5	1.8	19	13
TEM-7	100	59	1.5	0.7	0.8	0.44	1.5	2.2
TEM-E1	100	123	26	5.4	12	5.3	2.0	5.8
SHV-2	100	55	11	5.4	1.8	0.63	UM	1.1
SHV-5	100	34	25	3.8	3.9	1.1	5.0	14

*Value expressed as a percentage of the value for ampicillin.
 UM, unmeasurable as hydrolysis was too low.

results (table III). The K_m values for cefdinir were higher than those of the other drugs tested for some enzymes but lower for other enzymes. Generally, the affinity of the enzymes for cefdinir was low.

The relative efficiency ($V_{\max}/K_m$) takes both values into account. An enzyme may have a high efficiency because either the $V_{\max}$ is high or the enzyme has a particular affinity for binding the drug (low K_m). The relative efficiency results (table IV) show that all enzymes were less or equally efficient at hydrolysing cefdinir than they were with ampicillin, carbenicillin, cephaloridine, cephalexin and cefo-

taxime. Cefdinir was also more stable than cefixime in the majority of cases. Ceftazidime and cefuroxime were more stable than cefdinir to at least four of the nine β -lactamases tested.

The relative rates of hydrolysis at fixed substrate concentrations (10^{-4} M for cephalosporins and 10^{-3} M for penicillins) were determined for a supplementary range of six extended-spectrum β -lactamases (table V). These values are not as accurate as the $V_{\max}$ determinations in table II; however, they give an indication of the abilities of the enzymes to hydrolyse the substrates. Cefdinir was more stable than ampicillin, cephalo-

ridine, cephalexin, cefuroxime, cefotaxime or cefixime to all the β -lactamases listed in table V. Also, cefdinir had lower relative rates of hydrolysis than ceftazidime with three of the six β -lactamases tested. The results strongly support those shown in table II, that cefdinir was a less susceptible substrate for extended-spectrum β -lactamases than the later generation cephalosporins.

Discussion

In this study, the stabilities of at least eight different β -lactams were tested against 14 partially purified extended-spectrum β -lactamases and the narrow-spectrum β -lactamase TEM-1. Tables IV and V show that, overall, cefdinir was more (or equally) stable to the β -lactamases than ampicillin, cephaloridine, cephalexin or cefotaxime. Also, of the 128 stability values listed in tables IV and V there are only 13 instances in which other compounds are more stable (i.e. gave a lower value) than cefdinir. In the majority of cases, cefdinir was more stable than cefuroxime or cefixime to each of the 15 β -lactamases. Ceftazidime was more stable than cefdinir to eight β -lactamases. Of all the β -lactams

examined, cefdinir was the most stable compound to TEM-6, 7 and 10 and to SHV-5.

A recent study by Wise *et al.*⁷ on the stability of cefdinir and cefixime to crude β preparations of TEM-3 and TEM-5 showed that in both cases cefixime was more stable than cefdinir. However, our data suggest that cefdinir was more stable than cefixime to both of these two extended-spectrum β -lactamases. This observation illustrates how kinetic data determined by different laboratories can vary; however, in this particular case, the discrepancies may have occurred for two reasons. Firstly, the kinetics in the previous study⁷ were determined with crude cell lysates, whereas the kinetics in this study were derived from partially purified β -lactamases. Secondly, and because of this, the TEM-3 and TEM-5 preparations used in the previous study⁷ presumably still contained the *E. coli* and the *Klebsiella aerogenes* chromosomal β -lactamase, respectively. Both these factors would have a considerable contributory effect on the constants derived from kinetic experimentation.

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