CEFONICID: A STABLE β-LACTAMASE INHIBITOR

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The stability of cefonicid (SK&F 75073) towards representatives of six major classes of β-lactamases was determined using a spectrophotometric assay. Cefonicid was stable to hydrolysis by the Type I enzyme from *Enterobacter cloacae* and by the enzyme from the anaerobe, *Bacteroides fragilis*. It was 6 to 7 times more stable than cefamandole to the Type IIIA and B enzymes from *Escherichia coli*, a little less stable than this antibiotic to the Type V enzyme from *E. coli*, and of equal stability to the Type IV enzyme from *Klebsiella aerogenes*. Cefonicid was a non-competitive inhibitor (Ki of 0.8×10^{-6} M) of cephalothin hydrolysis by the Type I enzyme.

Cefonicid, SK&F 75073 (Fig. 1), is a long-acting, semi-synthetic cephalosporin now undergoing clinical trials in the United States and Europe. The potency and antimicrobial spectrum of the β-lactam antibiotics are the resultant of their stability towards inactivation by β-lactamase enzymes, their binding specificity and their uptake by bacteria[1,2]. The β-lactamase enzymes have recently been extensively reviewed by HAMILTON-MILLER and SMITH[3].

In this paper, we report the relative stability of cefonicid, cefamandole and cephalothin towards the partially purified β-lactamases from five Gram-negative microorganisms and one anaerobe, *Bacteroides fragilis* HH154.

Materials and Methods

Antibiotics and Reagents

Cephalothin and cefamandole were kindly supplied by Eli Lilly and Co., Indianapolis, Indiana, U.S.A. and cefonicid was synthesized at SK&F. All other reagents were purchased from commercial sources.

Microorganisms and Culture Conditions

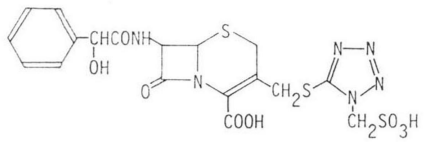
Enterobacter cloacae P99, *Escherichia coli* TEM, *E. coli* RPI, *Klebsiella aerogenes* KI, and *E. coli* RGN 238, which produce the class I, IIIA, IIIB, IV, and V β-lactamases[1,2], respectively, were kindly provided by Dr. R. B. SYKES (E.R. Squibb, Princeton, NJ, U.S.A.). The anaerobe, *B. fragilis* HH154 was from our own culture collection and was originally a clinical isolate from Hahnemann Hospital, Philadelphia, PA, U.S.A.

The aerobic microorganisms were grown in a defined medium of the following composition: Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; glycerol, 4 g; deionized water to 1 liter. Cultures were incubated at 37°C on a rotary shaker and the cells harvested at mid-log phase. The β-lactamases of *E. coli* TEM and *E. coli* RGN238 were induced by addition of sterile cephalothin at 1 g/liter two hours before harvesting. *B. fragilis* was grown in SCHAEDLER medium at 37°C for 6 hours as previously described[4].

Enzyme Isolation

The type I, IIIB, IV and *B. fragilis* enzymes were isolated from mid-log phase cells recovered by centrifugation at 10,000 *g* for 20 minutes at 4°C. After washing the cells with 10 mM potassium phos-

Fig. 1. Chemical structure of cefonicid.



SK&F 75073

phate buffer, pH 7.0 containing 5 mM dithioerythritol, an equal volume of 25 micron ballotini was added to the pellet and the mixture suspended at a ratio of 1 : 5 cells to buffer. This suspension was sonicated for 6×60 seconds with cooling in between, holding the maximum temperature to 10°C. Cell debris were removed by centrifugation at 20,000 *g* for 20 minutes at 4°C. The supernatant solution was collected and particulate material removed by centrifugation for 90 minutes at 140,000 *g* at 4°C. The supernatant solution was concentrated to 10 ml by use of an Amicon cell and a PM10 ultrafilter. This solution was aliquoted into 250 μl portions, checked for activity against the chromogenic substrate, nitrocefin[5] and frozen at −80°C. When necessary, protein concentrations were checked by the LOWRY[6] technique using the DTE-containing buffer as both diluent and blank.

The types IIIA and V enzymes were prepared in a similar manner except that the cell lysate was obtained by suspending the washed cells in phosphate buffered saline (10 mM potassium phosphate, pH 7.0, 154 mM sodium chloride) at a 1 : 2 ratio of cells to buffer and adding dropwise to liquid nitrogen. The frozen, broken cell mass was allowed to thaw at room temperature, centrifuged, ultrafiltered and stored as previously described.

Enzymatic Assays

The hydrolysis of the substrate was monitored spectrophotometrically by following the rate of decrease in absorbance at 260 nm with a Gilford 240 recording spectrophotometer equipped with a constant temperature (30°C) cuvette assembly. The reaction mixtures (total volume of 1 ml) contained suitable amounts of the enzyme preparations and various concentrations (50~200 μM) of the substrates dissolved in 100 mM potassium phosphate buffer, pH 7.0. The initial rates were calculated from tangential analysis of the progress curves. Apparent kinetic constants were determined by linear regression analyses of conventional LINEWEAVER-BURK[7] plots with regression coefficients being equal to or greater than 0.985.

Inhibition studies used one of two techniques: (a) diminution of enzyme rates at 260 nm with cephalothin as a substrate followed by determination of inhibitor constants by the use of DIXON plots[8] and (b) reduction of the rate of hydrolysis of the chromogenic substrate, nitrocefin[5].

Results

The kinetic constants for the hydrolysis of cefonicid, cefamandole and cephalothin by the six β-lactamases are shown in Table 1. Cephalothin was rapidly hydrolyzed by all the enzymes with roughly similar kinetics. Cefonicid and cefamandole were resistant to hydrolysis by the type I en-

Table 1. Kinetic constants with β-lactamases.

β-Lactamase	Source	Cephalothin			Cefonicid			Cefamandole		
		K_m[a]	V_{max}[b]	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Type I	*E. cloacae* P99	60	5.0	0.083	R	—	—	R	—	—
Type III A	*E. coli* TEM	141	9.8	0.070	120	3.7	0.030	514	26.6	0.052
Type III B	*E. coli* RPI	166	8.2	0.049	66	3.1	0.047	236	18.7	0.079
Type IV	*K. aerogenes* KI	110	11.5	0.105	95	11.4	0.120	497	10.0	0.020
Type V	*E. coli* RGN238	96	8.3	0.087	169	8.5	0.050	141	5.9	0.042
Anaerobe	*B. fragilis* HH 154	135	14.1	0.104	R	—	—	18	2.0	0.111

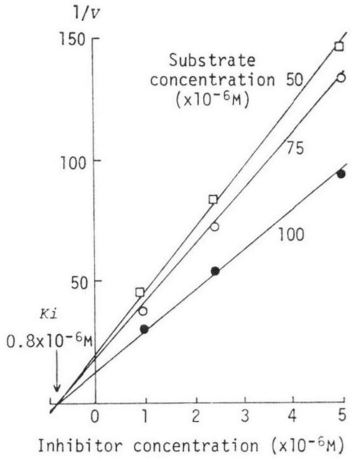
[a] The K_m is expressed as 10^{-6} moles/liter.
[b] The V_{max} is expressed as 10^{-6} moles/liter/min.
[c] R indicates resistant to hydrolysis.

Table 2. Relative hydrolytic rates[a] of cefonicid and cefamandole.

β-Lactamase	Cefonicid	Cefamandole	Cefonicid/ cefamandole
Type I	R	R	—
Type III A	38	271	0.14
Type III B	38	229	0.17
Type IV	98	88	1.11
Type V	103	71	1.45
Anaerobe	R	14	α

[a] The relative hydrolytic rates were calculated from maximal velocity (V_{max}) data corrected for extinction coefficients using cephalothin as 100 %.

Fig. 2. DIXON plot of cefonicid inhibition of cephalothin hydrolysis by the *E. cloacae* enzyme.



zyme from *E. cloacae*. Cefonicid also exhibited resistance to hydrolysis by the *B. fragilis* enzyme whereas cefamandole was hydrolyzed at a slow rate. With respect to the other four enzymes, both cefonicid and cefamandole were substrates but there were significant differences in their kinetics. For example, with the type III enzymes, both the MICHAELIS constants (Km's) and maximal velocities (Vmax) for the two substrates differed. However, with the type IV, the MICHAELIS constants differed but the maximal velocities were similar, and with the type V, the reverse occurred in that the MICHAELIS constants were similar but the maximal velocities differed.

The relative hydrolytic rates for cefonicid and cefamandole using cephalothin as 100% are shown in Table 2. Cefonicid was six to seven times more stable to hydrolysis than cefamandole with the type IIIA and IIIB enzymes, equal with the type IV, less stable with type V, and completely stable to the enzyme from the anaerobe.

During the isolation of the *E. cloacae* enzyme, we discovered that cefonicid inhibited the hydrolysis of the chromogenic cephalosporin (nitrocefin). Using cephalothin as the substrate and conventional kinetic techniques, we showed that cefonicid was a non-competitive inhibitor of cephalothin hydrolysis by the *E. cloacae* enzyme (Ki_{app} of 800×10^{-9}M, Fig. 2). Whether or not cefonicid inactivated as well as inhibited the enzyme was not studied in this series of experiments.

Discussion

In addition to its intrinsic β-lactamase stability, cefonicid was an effective inhibitor of the class I enzyme from *E. cloacae*. To our knowledge, this antibiotic is the first non-competitive inhibitor of a type I enzyme for which a Ki has been reported[9]. Neither clavulanic acid nor CP-45899, a penicillin sulfone, inhibit the class I β-lactamases[10,11,12]. Work is now underway to further delineate the structure-activity relationships in the cefonicid series.

The relative velocities used in this paper for comparison of the stabilities of the cephalosporin antibiotics were calculated from maximal velocity data corrected for differences in molar extinction coefficients of the substrates. These corrections compensate for both differences in velocity/substrate relationships (different Vmax/Km ratios) and in molar extinction coefficients. The use of uncorrected absorbance values for comparing antibiotic stabilities may distort this relationship amongst antibiotics and such a distortion could well be magnified if the substrate concentration used was close to the Km

for one enzyme, but ten times the Km for another. Such differences might well contribute to the inconsistencies observed with some compounds between *in vitro* antibacterial activity and β-lactamase stability. However, the differences are more typically due to the "permeability" of the material. For example, cefonicid is relatively inactive against *E. cloacae* P99, although it is an effective inhibitor of the β-lactamase. *E. cloacae* cells treated with an agent that enhances "permeability" are sensitive to cefonicid[12], which suggests that in this case "permeability" is the major barrier to activity.

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