AFFINITY OF CEFONICID, A LONG-ACTING CEPHALOSPORIN, FOR THE PENICILLIN-BINDING PROTEINS OF *ESCHERICHIA COLI* K-12

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The binding of cefonicid (SK&F 75073), a new parenteral cephalosporin, to the penicillin-binding proteins (PBPs) of *Escherichia coli* K-12 (strain KN-126) was determined by competitive binding studies *versus* benzyl[^{14}C]penicillin. Cefonicid showed its greatest affinity for PBPs 1a>3>1b, bound with low affinity to PBPs 4>2, and did not bind to PBPs 5 and 6. Provisional affinity constants (cefonicid concentration that gave 50% inhibition of [^{14}C]penicillin binding) were determined: PBP 1a, <0.25 μg/ml; PBP 3, 0.7 μg/ml; PBP 1b, 10 μg/ml; PBP 4, 26 μg/ml; PBP 2, 90 μg/ml; PBPs 5 and 6 >256 μg/ml. Direct binding studies with [^{14}C]-cefonicid confirmed this pattern of binding. Subinhibitory concentrations of cefonicid (MIC, broth 0.2 μg/ml, agar 0.4 μg/ml) induced filamentation of *E. coli* KN-126. This implies that PBP 3 is the primary inhibitory site despite the higher affinity of PBP 1a for this cephalosporin.

The molecular targets of β-lactam antibiotics are intrinsic membrane proteins which catalyze the final stage of peptidoglycan biosynthesis by the formation of peptide crosslinks, and which recognize β-lactam antibiotics as suicide substrates. The product of the reaction with a β-lactam antibiotic is a β-lactamoylserine ester which blocks the enzyme active site.[1] These target enzymes which have the property of forming stable covalent complexes with β-lactams, are termed penicillin-binding proteins (PBPs) and are readily detected *via* labelling with radioactive β-lactams.[2] The higher molecular weight PBPs of *Escherichia coli* (numbers 1a, 1b, 2 and 3) have been shown to be lethal targets of β-lactams, whereas the lower molecular weight PBPs (numbers 4, 5, 6) appear to be unrelated to β-lactam activity.[3]

Cefonicid (SK&F 75073) is a new cephalosporin structurally related to cefamandole, but with a methane sulfonic acid moiety replacing the *N*-methyl on the 3-tetrazole substituent. Cefonicid has a broad spectrum of activity and an unusually long serum half-life[4] which contributes to *in vivo* efficacy. We have examined the binding of cefonicid to the PBPs of *E. coli* K-12, and have correlated that binding pattern with the effect of cefonicid on the morphology of *E. coli* cells.

Materials and Methods

Membranes of *E. coli* K-12 (strain KN-126; gift of Dr. B. SPRATT) were prepared and PBP affinities assayed by slab gel electrophoresis using the techniques of SPRATT[2], utilizing a 60: 1 ratio of acrylamide to bis acrylamide[3], and a 10% acrylamide separating gel. After electrophoresis gels were stained with Coomassie blue, destained, photographed, impregnated with Enhance R autoradiography enhancer (NEN), dried under vacuum, and fluorographed at −72°C on pre-flashed Kodak X-Omat R film[5]. Films were exposed for 10 to 15 weeks. Binding of radiolabel was quantitated by scanning densitometry using an EC Apparatus densitometer and Hewlett-Packard 3390A intergrator. Binding of radiolabel was also determined by sectioning slab gels after electrophoresis into individual lanes, and then into 1 mm slices, using a Bio-Rad model 190 gel slicing apparatus modified for slab gels. The bound activity was eluted into 10 ml of Econofluor+5% Protosol (NEN) at 55°C for 18 hours. Activity was determined

by liquid scintillation counting using a Tracor Mark III counter. To obtain adequate counting precision, 100-minute count times were required with [^{14}C]cefonicid-labeled samples. Benzyl[^{14}C]penicillin (58 mCi/mmol) was obtained from Amersham; [^{14}C]cefonicid (15.7 mCi/mmol) was synthesized by the SK&F Radiochemistry Laboratory. Both were stored as lyophilized aliquots under nitrogen at −20°C. Fresh solutions of unlabeled β-lactams were prepared daily. Minimum inhibitory concentrations (MIC) were determined by agar dilution on Trypticase Soy Agar (BBL) using a Steers replicator[6]. Morphogenic effects were determined by phase contrast examination of broth cultures (antibiotic assay medium #3, Difco) at antibiotic dilutions near the broth dilution MIC.

Results and Discussion

Cefonicid competed with benzyl[^{14}C]penicillin for binding to several PBPs of *E. coli* KN-126 (Fig. 1). Cefonicid completely inhibited binding of [^{14}C]penicillin to PBP 1a at 0.25 μg/ml (lowest concentration tested). With increasing concentrations of cefonicid, PBPs 3, 1b, 4, 2 and 5/6 were inhibited in rank order.

Bound [^{14}C]penicillin was quantitated by scanning densitometry of the fluorograms and the concentration of cefonicid causing 50% reduction of benzyl[^{14}C]penicillin binding (I_{50}) was determined. Those values are compared with values for other β-lactams with *E. coli* K-12 previously reported by CURTIS[7] in Table 1. Of the four essential PBPs, PBP 1a is exquisitely sensitive to cefonicid, and PBP 3

Fig. 1. Titration of *E. coli* KN-126 PBPs by cefonicid.

Fluorograms of samples treated with the indicated concentration of cefonicid were quantitated by scanning densitometry, normalized to the density of the band in the absence of cefonicid, and plotted. The I_{50} was taken as the point at which the line of best fit crossed 50% of control.

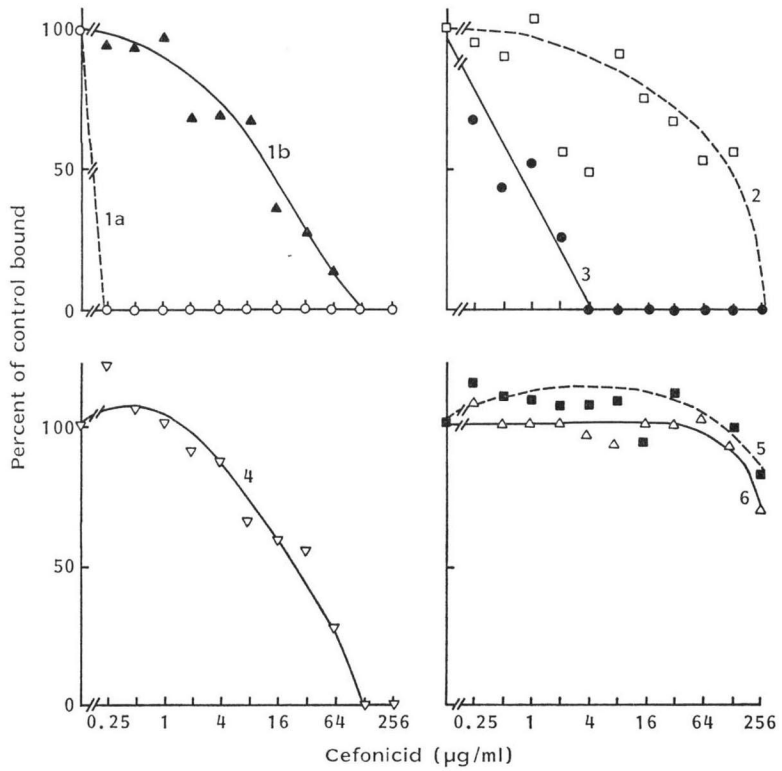
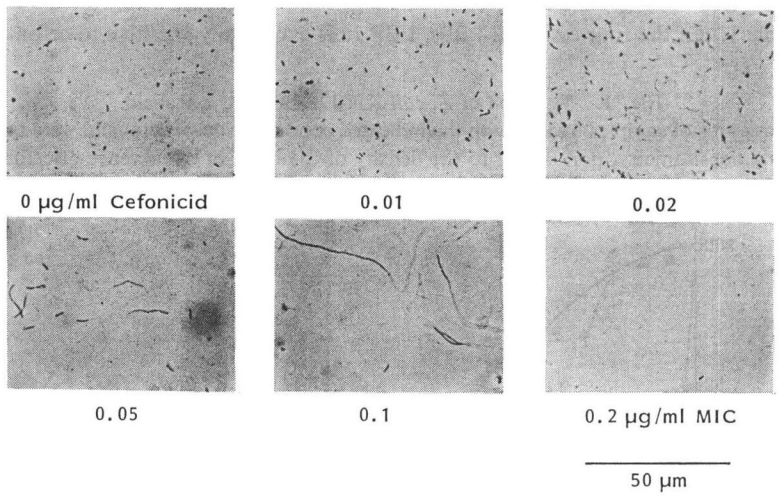


Table 1. Penicillin-binding protein affinities, MIC, and morphogenic effect of selected β-lactams upon *E. coli* K-12.

Antibiotic	MIC (μg/ml)	PBP I_{50}, μg/ml							Morphological effects[b]
		1a	1b	2	3	4	5	6	
Cefonicid	0.4	<0.25	10	90	0.7	26	>256	>256	fil
Cephalexin[a]	8	4	240	>250	8	30	>250	>250	fil
Cefamandole[a]	1.6	<0.25	2.9	61	<0.25	38	>250	>250	fil
Cephapirin[a]	6.4	<0.25	10	17	3.3	120	>250	>250	fil
Cefotaxime[a]	0.08	0.05	0.7	5	<0.05	30	>50	>50	fil
Cephaloridine[a]	2	0.25	2.5	50	8	17	>250	>250	sph
Benzylpenicillin[a]	12.5	0.5	3.0	0.8	0.9	1.0	24	19	fil+sph+rb

[a] Data from ref 7.
[b] fil = filaments
sph = spheroplasts
rb = round bodies

Fig. 2. Morphogenic effect of cefonicid at and below the broth dilution MIC. Phase contrast microscopy of 18-hour cultures in TSB.



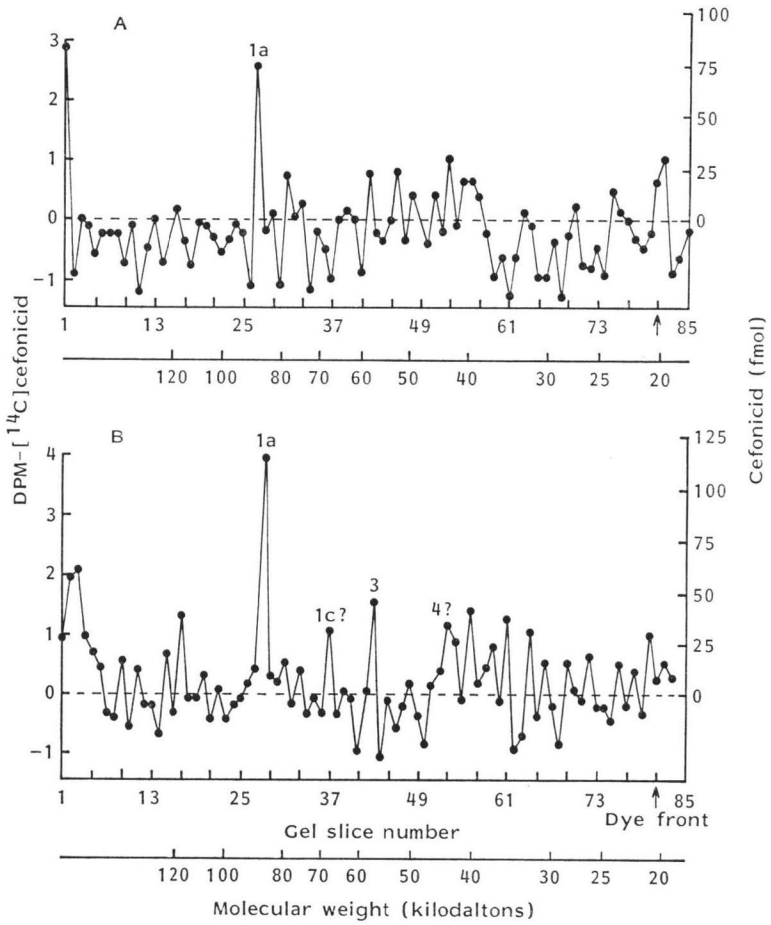
is also highly sensitive but the other two essential PBPs, 1b and 2, are much less sensitive. Of the inessential PBPs, PBP 4 shows moderate sensitivity, and PBPs 5 and 6 show little binding of cefonicid, even at very high concentrations. This pattern is similar to that seen with a variety of other cephalosporins and resembles the binding pattern observed with cefamandole (Table 1).

Inhibition of the essential PBPs leads to distinct morphogenic effects: PBPs 1a plus 1b gives spheroplasting and prompt lysis, PBP 2 gives osmotically stable round cells, and PBP 3 gives osmotically stable aseptate filaments.[7] Thus the morphological effects of β-lactam antibiotics at or just below the MIC are indicators of which target sites are most sensitive.[7] Cefonicid induces filamentation of *E. coli* KN-126 at or below the MIC (Fig. 2), implying that PBP 3 is the primary inhibitory site under those conditions. In *Salmonella typhimurium*, binding to PBP 3 alone has been reported not to be a lethal event, since concomitant binding to PBP 1a is required for lethality[11]. At the MIC, PBP 1a of *E. coli* KN-126 is fully saturated, thus cefonicid should be bactericidal as well as inhibitory. Our results are consistent with that model since for a variety of *E. coli* strains the MICs and MBCs (minimum bactericidal

concentration) of cefonicid are equal (data not shown). At much higher cefonicid concentration (*i.e.* 8~16 μg/ml) cefonicid causes prompt lysis, consistent with the hypothesis that both PBPs 1a and 1b must be inhibited simultaneously to cause prompt osmotic killing[8].

Direct binding assays with [^{14}C]cefonicid were performed to verify the competition assay results above. The specific activity of the synthesized material was too low to give useable fluorograms, even after 30 week exposure. However, using gel-slicing and scintillation counting, the binding of [^{14}C]-cefonicid was significantly detectable above background (Fig. 3). At 0.25 μg/ml cefonicid, only binding to PBP 1a was detected, whereas at 1 μg/ml (above the MIC) binding to PBP 3 is also evident. At 1 μg/ml binding was also evident to several other components. Notable was a component migrating between the positions of PBPs 1b and 2 (as determined by simultaneous assay of [^{14}C]penicillin binding, data not shown), labeled PBP 1c. There also was activity detected at *circa* 120 kilodaltons and below 40 kilodaltons. It is instructive to note that several other studies using direct binding of radiolabeled cephalosporins have detected a protein migrating in the same position as the PBP 1c identified here[9,10]. The significance, if any, of these observations is unknown. The possibility, however, of targets for ce-

Fig. 3. Direct binding of [^{14}C]cefonicid, assayed by gel-slice scintillation counting.
Curve A, 0.25 μg/ml [^{14}C]cefonicid; curve B, 1.0 μg/ml. Activity peaks are labeled as to PBP number based on comparison with a simultaneously run [^{14}C]penicillin sample (not shown).



phalosporins which do not appreciably bind penicillins, and are thus not detectable by [^{14}C]penicillin competition assay cannot be excluded.

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