INHIBITION OF PEPTIDOGLYCAN TRANSPEPTIDASE BY BETA-LACTAM ANTIBIOTICS: STRUCTURE-ACTIVITY RELATIONSHIPS

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The inhibitory activities of representative β-lactam compounds, such as penicillins G and N, cephalosporins C and G, clavulanic acid, nocardicin A and thienamycin against *Escherichia coli* KN-126 and *Bacillus megaterium* KM peptidoglycan transpeptidases were studied. Their modes of action against *E. coli* are discussed on the basis of the results and the published binding data for penicillin binding proteins. The effects of modifications at position 3 and 7 of the cephalosporin and those at α-carbon of the benzyl side-chain of cephalosporin G and penicillin G were studied. The introduction of an amino group at this position in cephalosporin G together with the removal of an acetoxy group from the acetoxymethyl group at position 3 reduced the inhibitory activity against *E. coli* transpeptidase considerably. The activity was restored by the replacement of the methyl group at position 3 of cephalexin with chlorine. The restoration was accompanied by about 15-fold increase in the lytic activity of cephachlor against *E. coli*.

Peptidoglycan transpeptidase (TPase) catalyzes the formation of cross-links at the final stage of bacterial cell wall synthesis[1,2,3]. It is one of the possible lethal targets of beta-lactam antibiotics in *Escherichia coli*[1,2,4]. β-Lactam antibiotics exert their physiological effects by binding to membrane proteins involved in morphogenesis[5~7]. At least six penicillin binding proteins (PBPs) were found in the inner membrane of *E. coli* K-12. Among them the following PBPs are thought to be essential for growth[6,8,9]. PBPs 1A and 1Bs are involved in cell elongation and are interchangeable in functions. Their joint inhibition by β-lactams results in cell lysis and spheroplast formation. PBPs 2 and 3 are involved in the maintenance of shape and in the cell division process, respectively. The TPase detectable in the membrane fraction from *E. coli* was shown to correspond to PBP 1Bs[4,9,10].

Since the demonstration of the inhibition of *E. coli* TPase by β-lactam antibiotics[1,2] many workers have reported the inhibition in different strains of *E. coli* and in other species of bacteria. However, most of these studies are either not extensive or are unreliable because of the assay methods used for the determinations of cross-linking[10,11]. We have studied the effect of many natural and semi-synthetic β-lactam antibiotics on the *in vitro* TPase in *E. coli* KN-126 and *Bacillus megaterium* KM with our improved method[10,12]. The results will be reported here and discussed in relation to the structure-activity relationships.

Methods and Materials

Organisms, growth and preparation of particulate fractions

E. coli KN-126 and *B. megaterium* were grown, harvested and stored as described previously[10,12]. Particulate fractions were prepared from the frozen cells by alumina grinding as previously reported[10].

Assay of polymerization and cross-linking

The standard reaction mixture contained 0.125 M tris(hydroxymethyl) aminomethane-hydrochloride

(pH 8.5), 0.0125 M $MgCl_2$, 2.8×10^{-5} M [^{14}C]-UDP-N-acetylglucosamine (150 μCi/μmol), 1.5×10^{-4} M UDP-N-acetylmuramylpentapeptide (prepared as described[10]), 16.7% (vol/vol) glycerol, and particulate fractions (*ca* 170 μg as protein for *E. coli* and 30~60 μg for *B. megaterium*). The mixture was incubated at 25°C for 15 or 30 minutes with *E. coli* particulate fraction and for 10 minutes with *B. megaterium* particulate fraction. The assay of polymerization and cross-linking were done as described[10]. The degree of cross-linking is expressed as the percentage of cross-linked disaccharide-peptide(s).

Materials

The following antibiotics were used: penicillin G (Meiji Seika, Tokyo), penicillin N (Division Labs., Michigan Department of Health, Lansing), cephalosporin C, A-16884, A-16886 I, A-16886 II, 7-mandelo aminocephalosporanic acid, 3 N-methyltetrazolyl cephalosporin G, cephachlor, cefamandole (Lilly Research Labs., Indianapolis), nocardicin (Fujisawa Pharmaceuticals Co., Osaka), clavulanic acid, 87/359 (Glaxo Group Laboratories Ltd., Greenford), thienamycin, cefoxitin, cephamycin C (Merck Sharp & Dohme Research Labs., Rahway), cephalosporin G (kindly prepared by Dr. C. M. CIMARUSTI of E. R. Squibb & Sons (Princeton, New Jersey) and by Mr. A. SATO of this institution). UDP-N-acetyl-D-[U-^{14}C]glucosamine (300 mCi/mmol) was purchased from Radiochemical Centre (Amersham, England).

Results and Discussion

Effects of Representative β-Lactam Compounds

Whereas all β-lactam compounds studied except nocardicin are moderate to very strong inhibitors of *B. megaterium* transpeptidase, only penicillin G (Pen G) and thienamycin are strong inhibitors of *E. coli* TPase. Clavulanic acid was a poor inhibitor of *E. coli* TPase and strong inhibitor of *B. megaterium* TPase. The hydrophilic α-aminoadipyl side-chain reduced considerably the inhibitory activity of both penicillin N and cephalosporin C toward *B. megaterium* TPase. It had little effect on the inhibitory activity of the cephalosporin nucleus to *E. coli* TPase (Table 1).

The above results together with published data for the penicillin binding proteins in *E. coli* KN-126[7,13] indicate that the action of the representative β-lactam compounds can be characterized as follows. Pen G has a nearly equal affinity toward the three possible lethal targets in *E. coli*, namely PBP 1Bs, PBP 2 and PBP 3. Cephalosporin G (CP-G) has the highest affinity toward PBP 3. Its high affinity toward PBP 1 (later resolved into 1A and 1Bs[14]) can be explained by the affinity toward PBP 1A, which is functionally interchangeable with 1Bs but has higher affinity toward β-lactams. The affinity toward PBP 2 is the least, (This situation will be described as follows: 3>1Bs>2). Like mecillinam, clavulanic acid is a specific inhibitor of PBP 2 function(s)[13] and its affinities toward other PBPs will not contribute to its antimicrobial action against *E. coli* (2≫1Bs, 3). Thienamycin has the highest affinity toward PBP 2, but its high inhibitory activity against the *in vitro* TPase (1Bs) may contribute to its antimicrobial activity (2>1Bs≫3).

Table 1 Effects of representative β-lactam compounds on peptidoglycan cross-linking *in vitro*.

β-Lactam	Concentrations required to inhibit cross-linking 50 % (μg/ml)	
	E. coli	*B. megaterium*
Penicillin G	4.0	0.052
N	48.0	0.7
Cephalosporin G	60	0.33
C	60	16
Clavulanic acid	460	0.76
Nocardicin A	80	125
Thienamycin	2.4	0.024

Effects of the Introduction of a 7-α-Methoxy Group

The introduction of a 7-methoxy group into cephalosporins increased the inhibitory

activity toward *E. coli* TPase as reported previously[15] (Table 2). The introduction did not affect the activity toward *B. megaterium* TPase. Since an increased inhibitory activity against *E. coli* TPase was not always reflected in the antimicrobial activity, a poor permeability was assumed[15]. Recently CURTIS *et al.* have studied the affinities and antibacterial activities of several 7-methoxycephalosporins in *E. coli* K-12 (DCO) and its permeability mutant DC2 and have concluded that the decrease in the antibacterial activity is due to a reduced affinity for an essential PBP and not to less favourable permeability properties[16]. Thus the situation seems to be complex. In some cases the introduction of a 7-methoxy group increases the affinity toward PBP 1Bs (the *in vitro* TPase) with a considerable decrease in the affinity toward PBP 3. In other cases, the introduction decreases the affinities toward both PBPs, but the decrease in the affinity toward PBP 1Bs is less[16]. In rare cases it increases the affinity toward PBP 1Bs with little or no decrease in the affinity toward PBP 3. Whichever the case, the ratio of the affinity toward PBP 1Bs to that of PBP 3 will be increased by the introduction of a methoxy group to cephalosporins. Consequently the lower concentration range where elongation is mainly observed with corresponding 7-H cephalosporins becomes narrower or disappears and lysis is observed as the earliest event.

Table 2. Effects of introduction of a 7-α-methoxy group into cephalosporins.

β-Lactam	Concentrations required to inhibit cross-linking 50 % (μg/ml)	
	E. coli	*B. megaterium*
Cephalosporin C	60	16
A-16884	8	17
A-16886 II	30	2
I	8	1.5
87/359	12.5	—
Cefoxitin	4.1	0.15

A-16886 I=cephamycin C (7-(5-amino-5-carboxyvaleramido)-7-methoxy-3-carbamoyloxymethyl-3-cephem-4-carboxylic acid).
Each pair differs only by the presence of a 7-methoxy group.
—, not determined.

Effects of Substitutions at Position 3 of Cephalosporins

Cephalosporin C (CP-C), deacetyl CP-C and deacetoxy CP-C show decreasing inhibitory activity against *E. coli* TPase in this order (Table 3). The substitution of the acetyl group with a carbamoyl group increases the inhibitory activity slightly against *E. coli* TPase and significantly against *B. megaterium* TPase. This relationship will also hold true for the substitution at position 3 of cephamycins

Table 3. Effects of substitutions at C-3 position of cephalosporins.

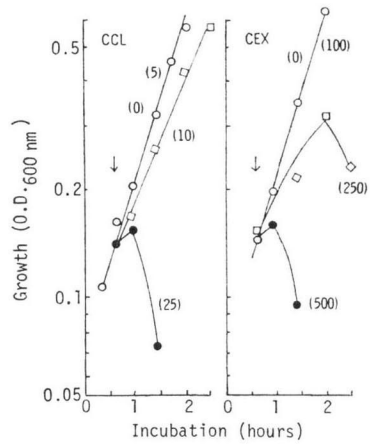
β-Lactam	Functional group at position 3	Concentrations required to inhibit cross-linking 50 % (μg/ml)	
		E. coli	*B. megaterium*
Cephalosporin C	acetoxymethyl	60	16
Deacetyl CP-C	hydroxymethyl	360	12.5
Deacetoxy CP-C	methyl	720	20
A-16886 II	carbamoyloxymethyl	30	2
Cephaloglycin	acetoxymethyl	167	0.5
Cephalexin	methyl	500	1
Cephachlor	chloro	12	0.09
Cephalosporin G	acetoxymethyl	60	0.33
CP-G: 3-tetrazolyl thiomethyl	N-methyltetrazolylthiomethyl	3.9	—

CP-G: 3-tetrazolylthiomethyl, 7-phenylacetoamido-3-N-methyltetrazolylthiomethyl-3-cephem-4-carboxylic acid. —, not determined.

(data not shown). The substitution of the acetoxymethyl group with chlorine (cephachlor) or a N-methyl tetrazolyl-thiomethyl group considerably increased the inhibitory activity against *E. coli* TPase.

Fig. 1. Effect of cephalexin (CEX) and cephachlor (CCL) on growth of *E. coli* KN-126.

Cells were grown at 37°C in a bouillon medium containing 2% dehydrated nutrient broth (Kyokuto Pharmaceuticals Co., Tokyo) and 0.5% yeast extract (pH 7). Numbers show the amount of antibiotics (μg/ml) added at times indicated by the arrow.



Effects of Substitutions at the α-Carbon of the Benzyl Side-Chain in CP-G and Pen G

The introduction of an α-amino group to the benzyl side chain of both a penicillin and a cephalosporin resulted in a reduction of the inhibitory activity against both *E. coli* and *B. megaterium* TPases (Table 4). Also, the introduction of a carboxyl or sulfonyl group into Pen G at this position reduced the inhibitory activity against TPases from both organisms. The introduction of a hydroxy group into the corresponding position of CP-G slightly decreased the inhibitory activity against *E. coli* TPase. Thus the high inhibitory activity of cephamandole against *E. coli* TPase[10] can be solely explained by the presence of a N-methyltetrazolylthiomethyl group at position 3. The introduction of an α-amino group into the benzyl side-chain of Pen G to give ampicillin did not affect or only slightly improved the affinity toward PBP 3, which is the most sensitive target toward ampicillin[7,17]. Thus by this criterion the intrinsic activity of ampicillin against *E. coli* is more or less similar to that of Pen G, though their minimum inhibitory concentrations (MIC) differ widely.

Comparison of Cephachlor with Cephalexin

The introduction of an α-amino group into the benzyl side-chain of CP-G together with the removal of an acetoxy group from the acetoxymethyl group at position 3 of CP-G considerably reduced the inhibitory activity of the resultant compound (cephalexin) against *E. coli* TPase (Table 3). These modifications did not impair the affinity toward PBP 3 and might increase it. Thus cephalexin remains

Table 4. Effects of substitutions at the α-carbon of the benzyl side-chain.

β-Lactam	Substitution at benzylic carbon	Concentrations required to inhibit cross-linking 50% (μg/ml)	
		E. coli	*B. megaterium*
Penicillin G		4.0	0.052
Ampicillin	NH_2	10	0.7
Carbenicillin	COOH	23.2	0.42
Sulbenicillin	SO_3H	29	0.32
Cephalosporin G		60	0.33
Cephaloglycin	NH_2	167	0.5
Mandelo 7-ACA	OH	96	—

Mandelo-7-ACA, 7-phenylhydroxyacetoamido-3-acetoxymethyl-3-cephem-4-carboxylic acid.

a potent antimicrobial agent against *E. coli*. The substitution of the methyl group at position 3 of cephalexin with chlorine increased the inhibitory activity against *E. coli* TPase fortyfold and *B. megaterium* TPase tenfold. The affinities toward PBP 1Bs and 3 were increased 55-fold and 4-fold respectively[17]. With these changes, the antibacterial activity increased only twofold or so in terms of MIC[17]. However, cells of growing *E. coli* KN-126 lysed rapidly at 25 μg of cephachlor or less per ml, whereas they lysed at 250 μg of cephalexin per ml (Fig. 1). ROLINSON[18] observed lysis of filamentous cells of *E. coli* at low concentrations of cephalexin (2~50×MIC) and rapid lysis of short cells at higher concentrations (around 200×MIC). He suggested that the lysis of these two forms of *E. coli* might be due to two different processes. Indeed, the rapid lysis can be explained by the inhibition of the *in vitro* TPase (PBP 1Bs), while the lysis at low concentrations will be ascribed to the inhibition of PBP 3 function(s) (septum formation). How the improvement in the inhibitory activity of cephachlor against *E. coli* TPase is reflected in the clinical situation remains to be seen.

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