Note

A NOVEL CHARACTER OF ACYLAMINOBENZYLPENICILLIN APALCILLIN, IN BINDING TO PENICILLIN-BINDING PROTEINS OF *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

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This short communication involves a brief revision to the affinity of an acylaminobenzylpenicillin apalcillin[1] to penicillin-binding proteins of *Escherichia coli* and *Pseudomonas aeruginosa* previously reported[2,8] and an indication to a method of rapid screening of related groups of β-lactam antibiotics.

β-Lactam antibiotics kill bacteria by binding to penicillin-binding proteins (PBPs) mainly causing inhibition of transpeptidase activities, *i.e.*, peptidoglycan-crosslinking activities of these proteins, to form a supramolecular network of the compound[4].

The most reliable way to estimate the activity of a β-lactam antibiotic against its targets, penicillin-binding proteins, may be to measure the 50% inhibition concentrations of their transpeptidase activities. However, the transpeptidase assay system has only recently been established for *E. coli* penicillin-binding proteins 1A, 1B, 2 and 3[5~8] but it is still difficult to assay most penicillin-binding proteins of other bacteria. Therefore, estimation of affinities of penicillin-binding proteins[9] has been and still is the most convenient and practical way to identify the target proteins of β-lactam antibiotics. The binding affinities of an antibiotic for PBPs are theoretically not the same as the affinities to inhibit enzymatic (transpeptidase) activities, as the former step may not always be necessary for the expression of the latter activity. The affinity of PBP-binding provides rather qualitative index, whereas measuring the inhibition of the transpeptidases is expected to provide a much more quantitative evaluation.

There are three methods to estimate the binding affinities to PBPs: (1) direct binding of labeled antibiotics, (2) inhibition of the binding of labeled benzylpenicillin by prebinding with unlabeled antibiotics, and (3) simultaneous competition of unlabeled antibiotic with labeled benzylpenicillin for binding. Theoretically the first and the second methods are expected to provide similar results, as long as a PBP does not have a β-lactamase activity that may remove the bound sensitive antibiotic, which would seriously confuse the results, especially of the second method, measurement of inhibition by prebinding.

In this report we describe the peculiar character of an antipseudomonal, acylated aminobenzylpenicillin apalcillin[1], in binding to and inhibiting penicillin-binding proteins. This antibiotic shows high binding affinity to most essential PBPs of *E. coli*, PBP-1B, 2 and 3 in all three assay methods described above, but shows extremely low binding affinity to PBP-1A in the simultaneous competition assay with benzylpenicillin (Reference 8 and Table 1, E, F). In the two other assays, binding of apalcillin to *E. coli* PBP-1A was as high as, or even slightly higher than benzylpenicillin (Table 1, A~D). In this table, 50% binding concentrations of apalcillin and benzylpenicillin to *E. coli* PBPs are shown, measured with the three assay methods as described before.

The binding assay methods using membranes of usual strains do not seem to provide enough accurate results for minor *E. coli* PBPs such as

Table 1. Comparison of binding affinities of apalcillin and benzylpenicillin to *E. coli* PBPs measured by three assay methods and inhibition of their enzymatic activities.

PBP	Binding of ^{14}C-labeled compound[a]			Competition with [^{14}C]benzylpenicillin						Enzyme inhibition[d]		
				Pretreatment with unlabeled compound[b]			Simultaneous addition of unlabeled compound[c]					
	A (APPC)	B (PCG)	A/B	C (APPC)	D (PCG)	C/D	E (APPC)	F (PCG)	E/F	G (APPC)	H (PCG)	G/H
1A	3.1	4.9	0.6	1.3	2.7	0.5	450	150	3.0	0.4	0.3	1.3
1Bs	5.2	19	0.3	2.9	14	0.2	34	89	0.4	5	5	1
2	3.6	11	0.3	0.5	6.5	0.08	25	65	0.4	nd[e]	nd	—
3	1.4	9.7	0.14	0.04	6.5	0.005	8.5	81	0.1	<0.2	0.5	<0.4
4	15	2.2	6.3	18	1.9	9	>2,000	89	>22	0.9	0.01	90
5	2.9			7.2	140	0.05				0.4	1	0.4
		5.7					270	350	0.8			
6	18			34	49	0.7				nd	nd	—

[a] Membranes of *E. coli* strain JE1011 were prepared as described previously[2,3]. Binding affinities were assayed by the following procedure: 33 μl of membrane preparation (600 μg protein in 50 mM sodium phosphate buffer, pH 7.0 containing 10 mM $MgCl_2$) was incubated at 30°C for 10 minutes in the presence of increasing concentrations (0.5 to 50 μM) of ^{14}C-labeled apalcillin (APPC, sodium salt, labeled in the hydroxy-1,5-naphthylidine moiety, specific activity of 3.67 Ci/mol, prepared by Sumitomo Chemical Co., Ltd.) or benzylpenicillin (PCG, potassium salt, labeled in the 6-phenyl-1-[^{14}C]acetamido moiety, specific activity of 60 Ci/mol, purchased from Radiochemical Centre, Amersham, England). Then the reaction was stopped by addition of excess homologous unlabeled antibiotic (to a final concentration of 2,600 μg/ml) and Sarkosyl (final concentration, 1%) and the protein-^{14}C-antibiotic complexes were separated by sodium dodecylsulfate/acrylamide gel electrophoresis and detected by fluorography as described previously[2,3]. Radioactivity in isolated protein-^{14}C-antibiotic complexes was measured by densitometrical tracing of the X-ray film. Data represent concentrations in μM required for 50% binding to PBP.

[b] 30 μl of membrane preparation (see above) was incubated with 3 μl of solution of increasing concentration of unlabeled antibiotic to a final concentration of 0.018~180 μM apalcillin or 0.027~270 μM benzylpenicillin for 10 minutes at 30°C; after addition of 3 μl of [^{14}C]benzylpenicillin (to a final concentration of 70 μM), incubation was continued for 10 minutes at 30°C. Further manipulations were as in the direct binding method. Data represent concentrations in μM required for 50% inhibition of the binding of [^{14}C]benzylpenicillin to PBP.

[c] Concentration in μM calculated from data in the previous paper[3].

[d] Assay of the transpeptidase activities (PBPs 1A, 1Bs and 3, purified proteins) was performed as previously described[5,6,8]. Data represent apparent 50% inhibition concentrations in μM. Data for DD-carboxypeptidase activities (PBPs 4 and 5 in membranes) were from the previous paper[5].

[e] Not determined.

Table 2. Comparison of binding affinities of apalcillin and benzylpenicillin to *P. aeruginosa* PBPs measured by three assay methods.

PBP	Binding of ^{14}C-labeled compound[a]			Competition with [^{14}C]benzylpenicillin					
				Pretreatment with unlabeled compound[b]			Simultaneous addition of unlabeled compound[c]		
	A (APPC)	B (PCG)	A/B	C (APPC)	D (PCG)	C/D	E (APPC)	F (PCG)	E/F
1A	1.1	4.9	0.2	0.4	0.5	0.6	25	57	0.4
1B	2.0	10	0.2	1.8	2.4	0.7	220	65	3
2	16	>80	<0.2	1.6	>27	<0.06	17	89	0.2
3	1.1	41	0.03	0.04	0.5	0.06	<8.5	57	<0.1
4	0.9	2.2	0.4	0.09	0.08	1.0	850	138	6
5	1.3	10	0.1	47	>270	<0.2	67	97	0.6

[a~c] Membranes of *P. aeruginosa* NCTC10490 were used[2,8]. For binding assays and numbers see the legend to Table 1.

PBP-2, 3 and 4. Some differences between the 50%-binding concentrations for PBP-2 and 3 measured in the first two binding assays (Table 1, A~D) were observed. This may be due to a certain extent to the inaccuracy of estimation of the radioactivity in the minor PBPs. Use of membranes from strains overproducing these PBPs[7,8,10] may be necessary for a more exact determination of each of these minor PBPs.

Apparent 50% inhibitory concentrations of apalcillin for the transpeptidase activities of *E. coli* PBPs 1A, 1B and 3 (for assay methods see ref 5, 6, 8) were found to be 0.4, 5 and <0.2 μM respectively. For benzylpenicillin the values were 0.3, 5 and 0.5 μM. These results indicate that apalcillin is a strong inhibitor of PBP-3, the septum-peptidoglycan transpeptidase, and also of PBP-1A and 1B, the cell wall-peptidoglycan transpeptidases, in *E. coli*. These results are apparent values because the transpeptidase activities of the above-mentioned PBPs could only be measured when a transglycosylase reaction by the respective PBP takes place simultaneously. These PBPs are bifunctional peptidoglycan synthetases carrying the dual enzyme activities of transglycosylase and transpeptidase[5,6,8]. These results indicate that apalcillin inhibits the transpeptidase activities of *E. coli* PBP-1A, 1B and 3 and also binds to these proteins as effectively as or more effectively than benzylpenicillin.

Apalcillin shows an extremely low binding affinity for *E. coli* PBP-4 in all three assay methods (Table 1, A~F), but especially in the third method (Table 1, E, F). However, the inhibition of the D-alanine carboxypeptidase activity of *E. coli* PBP-4 was also very low (0.9 μM, compared with 0.01 μM for benzylpenicillin[2]).

In *P. aeruginosa* the PBPs are similar to those of *E. coli*, but the order of the mobilities of PBP-1A and 1B seems to be the reverse of the *E. coli* PBPs-1A and 1B in sodium dodecylsulfate polyacrylamide gel electrophoresis[3]. Apalcillin bound to the pseudomonal PBPs-1B and -4 very well compared with benzylpenicillin in both the direct binding assay and the inhibition after prebinding assay (Table 2, A~D). As in the case of the *E. coli* PBPs-1A and -4, the binding of apalcillin to the pseudomonal PBPs-1B and -4 in the simultaneous competition assay was also very poor (Table 2, E, F).

The difference of the apparent affinities of apalcillin to the above mentioned PBPs measured in the presence and absence of benzylpenicillin is remarkable and probably is due to an antagonistic effect between the two antibiotics concerned with these PBPs.

Similar phenomena have also been observed with another acylaminobenzylpenicillin, piperacillin (M. FUKASAWA and H. NOGUCHI, unpublished experiments), and acylureidopenicillins, mezlocillin and azlocillin[11]. Although the reason for the peculiar behavior of these penicillins in binding to *E. coli* PBPs 1A and 4 (and *P. aeruginosa* PBPs 1B and 4) is still unknown, *i.e.* binding very weakly in the presence of benzylpenicillin but much more strongly in its absence, these characteristics may provide a useful method to distinguish acylaminobenzylpenicillins and acylureidopenicillins among unknown antibiotics easily.

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