STUDIES ON THE MECHANISM OF ACTION OF IMIPENEM (*N*-FORMIMIDOYLTHIENAMYCIN) *IN VITRO*: BINDING TO THE PENICILLIN-BINDING PROTEINS (PBPs) IN *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*, AND INHIBITION OF ENZYME ACTIVITIES DUE TO THE PBPs IN *E. COLI*

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The binding affinities of imipenem (*N*-formimidoylthienamycin) to penicillin-binding proteins (PBSs) of *Escherichia coli* and *Pseudomonas aeruginosa* were determined by two different methods in which competition with [^{14}C]benzylpenicillin for the binding sites was measured. By both methods imipenem was shown to have very high binding affinities to PBPs-2 and -4 in *E. coli* and *P. aeruginosa*, and appreciable affinities to most of their other major PBPs. But higher concentrations of imipenem were required for binding to the PBPs-3 in these bacteria.

More direct information about the antibacterial activity of imipenem was obtained by measuring its inhibition of the peptidoglycan-synthetic enzyme activities of *E. coli* PBPs. The results of enzyme inhibitions were compatible with those obtained in binding experiments. The antibiotic inhibited the transpeptidase activities of PBPs-1A, -1B and -2, and the D-alanine carboxypeptidase activities of PBPs-4 and -5. The antibiotic also seemed to cause strong inhibition of the transglycosylase activity of PBP-1A by some unknown mechanism. It inhibited the transpeptidase activity of PBP-3 only weakly, which is consistent with the findings that it had low binding affinity to PBP-3 and did not inhibit septum formation by the cells.

Imipenem (formerly named imipemide, *N*-formimidoylthienamycin, or MK-0787) was obtained by chemical modification of thienamycin, a β-lactamase-resistant carbapenem with broad and high antibacterial activities, to overcome the instability of the latter compound in aqueous solution[1]. Imipenem is as resistant as the parent compound to β-lactamases, and even shows higher bactericidal activities than the parent compound *in vitro* and *in vivo* toward a variety of Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa*[2,3].

The binding affinities of the parent compound, thienamycin, to the penicillin-binding proteins (PBPs) of *Escherichia coli* and its effect on the morphology of the cells have been studied by SPRATT *et al.*[4]. It has been established that in *E. coli*, PBPs play essential roles in cell duplication[5], functioning in the biosynthesis of the peptidoglycan sacculus[6]. The PBPs in other bacteria are presumed to have similar roles.

This paper reports the affinities of imipenem to PBPs of two Gram-negative bacilli, *E. coli* and *P. aeruginosa*, and its inhibition of the peptidoglycan-synthetic enzyme activities of PBPs in *E. coli* were also presented.

Materials and Methods

Abbreviations

A_2pm, 2,6-diaminopimelic acid; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; SDS, sodium dodecylsulfate.

Antibiotics

Imipenem was supplied by Merck Sharp & Dohme Research Laboratories, Rahway, N.J. Benzylpenicillin potassium salt was obtained from Takeda Chemical Industries Co., Ltd., Osaka, Japan, and [^{14}C]benzylpenicillin potassium salt (59.5 Ci/mol) from the Radiochemical Centre, Amersham, England.

Bacterial Strains and Growth of Cells

E. coli K12 strain JE1011 and *P. aeruginosa* IFO12689 were used in assays of binding with the PBPs. *E. coli* mutant strains, the *dacA* mutant strain JE11191[7] lacking D-alanine carboxypeptidase Ia (PBP-5), the *dacB* mutant strain JE10064[8] lacking D-alanine carboxypeptidase Ib (PBP-4) and JST9753λd*lip5c*I-857Qam73[9], were used as enzyme sources in assays of the activities of PBPs-4, -5 and -2, respectively. The *E. coli* strains were cultured at 30°C in broth adjusted to pH 7.2 and containing 10 g of Polypepton, 5 g of yeast extract, 5 g of sodium chloride, 1 g of glucose and 20 mg of thymine per liter and were harvested in the late log phase. *P. aeruginosa* was cultured in brain heart infusion broth (Difco) and was also harvested in the late log phase.

Assay for Binding Affinity to PBPs

Membrane fractions were prepared by sonication of the cells, differential centrifugation of the homogenate and suspension of fractions in 50 mM sodium phosphate buffer, pH 7.0, as described previously[10]. The binding of [^{14}C]benzylpenicillin to PBPs, separation of PBP-[^{14}C]benzylpenicillin complexes by SDS/polyacrylamide gel electrophoresis and their detection by fluorography were as described previously[11]. The binding affinities of imipenem to PBPs were assayed by measuring inhibition of the binding of [^{14}C]benzylpenicillin (83 μM, 31 μg/ml, final concentration) by the unlabeled antibiotic.

In the "simultaneous competition assays", imipenem at five-fold increasing concentrations of 3.3 μM to 2.08 mM (*E. coli*), or of 16.6 μM to 415 μM (*P. aeruginosa*) or in control experiments of benzylpenicillin, at 83 μM or 166 μM (both bacteria) were added together with [^{14}C]benzylpenicillin to the membranes and the binding reaction was carried out for 10 minutes at 30°C. In "competition assays with prebound unlabeled antibiotic", membrane fractions were incubated with imipenem at five-fold increasing concentrations of 0.4 μM to 45.5 μM (*E. coli* and *P. aeruginosa*) or in control experiments with benzylpenicillin at concentrations of 1.8 μM to 45.5 μM (*E. coli*), or 1.8 μM to 8.3 μM (*P. aeruginosa*) for 10 minutes at 30°C before addition of [^{14}C]benzylpenicillin. After addition of [^{14}C]benzylpenicillin the tubes were incubated for an additional 10 minutes at 30°C.

Enzymes and Substrates

Purified preparations of *E. coli* PBPs-1A[12], -1B[13] and -3[14] that appeared homogeneous on SDS/polyacrylamide gel-electrophoresis, membranes for assays of PBPs-2[9], -4[8] and -5[7] and the ^{14}C-labeled precursors for cell wall peptidoglycan synthesis were prepared by the standard methods described previously.

Assay of Enzyme Inhibitions

Effects on peptidoglycan synthetase activities (transglycosylase and transpeptidase) associated with *E. coli* PBPs-1A, -1B and -3 were measured with purified preparations of the proteins as described previously[12~14]. The peptidoglycan synthetase activities of the PBP-2—*rod*A system[9] and D-alanine carboxypeptidases Ib and Ia, which are associated with PBPs-4[8] and -5[7], respectively, were measured with crude membrane preparations.

Results

Affinity of Imipenem for Penicillin-binding Proteins of *E. coli* and *P. aeruginosa*

Since no radioactive imipenem was available for testing its covalent binding to PBPs the assay was

Table 1. Affinity of imipenem to PBPs of *E. coli* and *P. aeruginosa* measured by competition with [^{14}C]-benzylpenicillin.

Strain[a]	PBP	Concentration of antibiotic (μg/ml) for 50% inhibition					
		Simultaneous competition assay[b]			Competition assay with prebinding		
		Imipenem (A)	Benzyl-penicillin (B)	(A)/(B)	Imipenem (C)	Benzyl-penicillin (D)	(C)/(D)
E. coli JE1011	1A	24	59	0.4	0.2	1.4	0.1
	1B	32	40	0.8	0.6	4.7	0.1
	2	5.3	31	0.2	<0.1	3.7	<0.03
	3	>660	43	>15	9.8	1.7	5.8
	4	11	40	0.3	<0.1	1.0	<0.1
	5	2.6	37	0.1	0.3	25	0.01
	6	11	34	0.3	0.6	6.8	0.1
P. aeruginosa IFO12689	1A	26	34	0.8	0.3	<0.7	>0.4
	1B	32	28	1.1	0.6	0.7	0.7
	2	<5.3	31	<0.2	<0.6	4.1	<0.1
	3	>132	31	>4.3	4.0	0.7	5.7
	4	7.9	34	0.2	<0.1	0.7	<0.1
	5	13	34	0.4	2.3	>3.4	<0.7

[a] The minimum inhibitory concentrations of imipenem for *E. coli* strain JE1011 and *P. aeruginosa* strain IFO 12689 were 0.1 μg/ml and 3.1 μg/ml, respectively, as measured by the agar dilution method.

[b] The values in this assay are not absolute concentrations, but values relative to that of benzylpenicillin present simultaneously in the reaction mixture.

carried out by two competition methods (Table 1). In the first method, which is called "simultaneous competition assay" in this report, membranes were incubated with ^{14}C-labeled benzylpenicillin (83 μM) in the presence of different concentrations of unlabeled imipenem (A) or benzylpenicillin (B), and inhibition of binding of [^{14}C]benzylpenicillin to PBPs by the unlabeled antibiotics was measured. By this method, PBPs were exposed simultaneously to ^{14}C-labeled benzylpenicillin and unlabeled antibiotics, and the concentration of the antibiotics required to inhibit binding of [^{14}C]benzylpenicillin depended on the concentration of the latter. The calculation of (A)/(B) ratios (see Table 1) was necessary to compare the affinity of imipenem with that of benzylpenicillin.

In the second method, which is called here "competition assay with prebound unlabeled antibiotic", the extent of binding of unlabeled antibiotics to membranes during preincubation was estimated by measurement of the residual amounts of PBPs available for binding by [^{14}C]benzylpenicillin added after the preincubation. The concentrations of antibiotics required for 50% inhibition of the binding of [^{14}C]-benzylpenicillin were theoretically independent of the concentration of [^{14}C]benzylpenicillin within a certain range of experimental conditions. The ratios (C)/(D) (see Table 1) in the "competition assay with prebinding", were calculated and compared with the (A)/(B) ratios in a simultaneous competition assay. The (C)/(D) ratios were mostly smaller than the (A)/(B) ratios, but there was a fairly good correlation between the two series of ratios (A)/(B) and (C)/(D).

Imipenem showed very high binding affinities for PBPs-2, -4, and -5 in both *E. coli* and *P. aeruginosa* and for PBP-6 in *E. coli*. These affinities were much higher than those of benzylpenicillin. In contrast, imipenem showed low affinities for the two PBPs-3 of Gram-negative bacilli. Its affinities for PBPs-1A and -1B were intermediate between those for PBPs-2 and -3 but similar to those of benzylpenicillin or higher. Its very low ratios of (A)/(B) and (C)/(D) for *E. coli* PBPs-5 and -6 may be partly due to its

resistance to the β-lactamase activities of these proteins. Other experiments not shown in this report indicated that the imipenem bound to PBPs-5 and -6 was not liberated by prolonged incubation of the complexes, whereas benzylpenicillin was removed rapidly from complexes with PBPs[15].

Effect of Imipenem on Enzymatic Activities of *E. coli* PBPs

The effect of imipenem on enzymatic synthesis of peptidoglycan by purified *E. coli* PBPs-1A and -1B are shown in Table 2. Both the transglycosylase activity (formation of peptidoglycan) and transpeptidase activity (crosslinking) of PBP-1A were strongly inhibited by imipenem; about 0.06 μg/ml was required for 50% inhibition of transglycosylase activity and 0.05 μg/ml for 50% inhibition of transpeptidase activity. The strong inhibition of the transglycosylase activity of PBP-1A is an unusual property of this antibiotic, because most other β-lactam antibiotics tested, such as benzylpenicillin, had only a slight effect on this reaction[12]. In contrast, the transglycosylase reaction catalysed by PBP-1B was apparently enhanced by imipenem under the reaction conditions given in Table 2, in which the transpeptidase activity of this PBP was fully inhibited. The mechanism of this enhancement is unknown; no enhancement was observed when the reaction conditions were slightly changed (for instance, by increasing of the methanol concentration; data not shown). The concentration of imipenem for 50% inhibition of transpeptidase activity of PBP-1B was about 0.8 μg/ml.

Table 2. Effects of imipenem on peptidoglycan synthetase activities in purified preparations of *E. coli* PBP-1A and PBP-1B[a].

PBP responsible for enzyme activity	Concentration of imipenem (μg/ml)	Formation of peptidoglycan (transglycosylase activity)[b]	Extent of crosslinkage (transpeptidase activity)[c]
A	0	28.4	13.7
	0.03	16.0	8.2
	0.1	12.9	4.3
	0.3	11.1	3.0
B	0	8.6	16.0
	0.1	11.2	15.8
	1	11.5	7.5
	10	15.1	0.1

[a] Assay conditions: (PBP-1A) Reaction mixtures contained, in a final volume of 35 μl, 14 mM Tris-HCl buffer, pH 7.6, 0.9 mM $MgCl_2$, 11% glycerol, 14% methanol, 0.4% Triton X-100, 60 pmol lipid-linked precursor, [^{14}C]GlcNAc-MurNAc-(L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala)-diphosphorylundecaprenol and purified *E. coli* PBP-1A (*ca.* 0.2 μg protein). Imipenem was added at various concentrations to the reaction mixture. Then the mixtures were incubated for 60 minutes at 37°C, mixed with 10 μl of isobutyric acid, spotted on Whatman No. 3 MM paper and subjected to paper chromatography with isobutyric acid - 1 M ammonia (3 : 3) as solvent. The radioactivity at the origin of the paper (peptidoglycan) was counted in a liquid scintillation counter (transglycosylase activity) and then the product at the origin was digested with lysozyme and subjected to paper chromatography. The extent of crosslinkage was estimated from the ratio of radioactivity of bis(disaccharide-tetrapeptide) to that of [bis(disaccharide-tetrapeptide)+ disaccharide-tetrapeptide] giving the percent crosslinkage (transpeptidase reaction).

(PBP-1B) Reaction mixtures contained, in a final volume of 35 μl, 10 mM sodium phosphate buffer, pH 7.0, 33 mM $MgCl_2$, 14% methanol, 160 pmol lipid-linked precursor labeled with *meso*-[^{14}C]A_2pm and purified *E. coli* PBP-1B (*ca.* 6 μg protein). Purified PBP-1B was preincubated with imipenem in reaction mixture without the radioactive substrate for 10 minutes at 37°C and then the substrate was added and the reaction mixture was incubated for an additional 15 minutes at 37°C. The reaction product was then treated as described above for PBP-1A.

[b] pmol repeating unit incorporated.

[c] % crosslinkage in formed peptidoglycan.

Table 3. Concentrations of imipenem and benzylpenicillin required for 50% inhibition of peptidoglycan-crosslinking activities and D-alanine carboxypeptidase activities in *E. coli* PBPs[a].

PBP responsible for enzyme activity	Enzyme activity	50% Inhibitory concentration (μg/ml)	
		Imipenem	Benzylpenicillin
1A	Transpeptidase	0.05	<0.1
1B	Transpeptidase	0.8	1
2	Transpeptidase	1	1.6
3	Transpeptidase	>3	0.1~0.3
4	D-Alanine carboxypeptidase	0.003	0.04
5	D-Alanine carboxypeptidase	0.034	5.5

[a] Values for PBPs-1A and -1B were calculated from Table 2 (imipenem) and unpublished experiments (benzylpenicillin). Assay conditions for PBPs-2, -3, -4 and -5 were as follows.

(PBP-2) The reaction mixture, in a final volume of 36 μl, contained 56 mM Tris-HCl buffer, pH 8.5, 28 mM $MgCl_2$, 14 mM EDTA, 150 pmol lipid-linked precursor labeled in *meso*-[^{14}C]A_2pm (specific activity, 44 Ci/mol), 280 μM UDP-GlcNAc, 340 μg (as protein) of particulate membranes and β-lactam antibiotic. The reaction was performed for 60 minutes at 37°C, and was stopped by boiling for 1 minute. The reaction product was then treated as described in the legend to Table 2.

(PBP-3) The reaction mixture, in a final volume of 35 μl, contained 25 mM Tris-HCl buffer, pH 7.6, 5 mM $MgCl_2$, 3 mM sodium ethylenediaminetetraacetate, 0.077% Triton X-100, 11% glycerol, 14% methanol, 60 pmol lipid-linked precursor labeled in *meso*-[1,7-^{14}C]A_2pm (44 Ci/mol, Radiochemical Centre, Amersham, England), purified PBP-3 from *E. coli* strain JST975srev61/pLC26-6[14] (*ca.* 0.2 μg protein), and β-lactam antibiotic. The reaction was performed for 30 minutes at 37°C, and was stopped by addition of 10 μl of isobutyric acid. The reaction product was then treated as described in the legend to Table 2.

(PBPs-4 and -5) The reaction mixtures, in a final volume of 30 μl, contained 10 mM Tris-HCl buffer, pH 7.5, 19 μM lipid-linked precursor labeled in D-alanyl-D-alanine, 33 mM $MgCl_2$ (only for D-alanine carboxypeptidase Ib assay), 0.1% Triton X-100 and 95~100 μg (as protein) crude membrane fraction from strain JE11191 (*dacA*) for assay of D-alanine carboxypeptidase Ib (PBP-4), or from strain JE10064 (*dacB*) for assay of D-alanine carboxypeptidase Ia (PBP-5). The reaction mixtures were incubated for 60 minutes at 30°C and the release of D-[^{14}C]alanine was determined by counting the radioactivity after separation of other compounds by paper chromatography. The D-alanine carboxypeptidase activity of PBP-6[15] was not measured.

Methods for assay of the enzymatic activities of PBPs-2 and -3 have been developed very recently[9,14]. Results of preliminary measurements of the inhibitions of PBPs-2 and -3 activities by these methods are shown in Table 3. The concentrations of imipenem and benzylpenicillin required for 50% inhibition of the D-alanine carboxypeptidase activities of PBPs-4 and -5 were also determined and are summarized in Table 3.

Discussion

According to the working hypothesis of TIPPER and STROMINGER[16], β-lactam antibiotics exert their antibacterial action by formation of covalent bonds between their carbonyl carbon and a nucleophilic residue at the active center of the target enzymes, *i.e.*, peptidoglycan transpeptidases. On the basis of this hypothesis, the affinity of β-lactam antibiotics to PBPs has been used routinely to study their mechanism of action. Of the seven major PBPs in *E. coli*[10,11], PBPs-1A and -1B are presumed to function in peptidoglycan synthesis during cell-elongation[5,6,12,13]. PBPs-2 and -3 function in the process which determines the rod-shape[5,9,10], and in septum-formation[5,14], respectively, during cell duplication. Binding to one or two of these important PBPs will cause lysis of the cells after a change in the cellular phenotype that depends on the PBP bound. When an appropriate radioactive compound is not available, there are two main methods for assay of the binding potencies of β-lactam antibiotics to PBPs: "simultaneous competition assay" and "competition assay with prebound unlabeled antibiotic", as

described in this paper. The results obtained by the former method indicate the affinity of imipenem compared with that of benzylpenicillin. The two assay methods gave similar results for most β-lactam antibiotics. However, for some unknown reason, certain β-lactam antibiotics, such as acylaminobenzylpenicillins and acylureidopenicillins, showed exceedingly low competitive binding activity to PBP-1A of *E. coli* in "simultaneous competition assay" but fairly high activity for the same PBP in "competition assay with prebound unlabeled antibiotic"[17] (H. NOGUCHI, M. FUKAZAWA, T. KOMATSU, F. ISHINO, M. MATSUHASHI and S. MITSUHASHI, manuscript in preparation). Both competition assays were therefore carried out to estimate the binding affinities of imipenem to the PBPs of *E. coli* and *Pseudomonas*.

The introduction of a *N*-formimidoyl group caused approximately two-fold reduction of minimum inhibitory concentration of thienamycin against the bacteria including *E. coli* and *P. aeruginosa*[3], but it did not seem to effect the binding pattern of this carbapenem to *E. coli* PBPs significantly (*cf.* ref 4). However, if we compared our results of the binding affinities of *N*-formimidoyl derivative to those of the parent reported by SPRATT *et al.*[4], the former compound seemed to have obtained an appreciable progress in the affinities to PBPs.

The high binding affinity of imipenem to the PBPs of *E. coli* and *P. aeruginosa* may explain the very high anti-*E. coli* and antipseudomonal potency of this antibiotic. The binding of this antibiotic to PBPs-1A, -1B and -2 is probably the main reason for its bactericidal action, because the functions of the low molecular weight PBPs-4, -5 and -6 are supposed to be less important. Moreover, its strong binding to PBP-2 and low binding to PBP-3 are consistent with the morphological changes that it induces in these bacteria, namely rounding of the cells at a subinhibitory concentration and lysis at higher concentration, but no formation of unseptated, filamentous cells at any concentration.

However, inhibition of the enzymic activities of the target proteins (PBPs) might not be caused solely by the formation of a covalent linkage between the protein and the β-lactam. For this reason, measurements were made of the inhibition by imipenem of the activities of PBPs in peptidoglycan synthesis. The inhibitory activities of imipenem on *E. coli* PBP transpeptidases were found to correlate fairly well with the binding affinities. The inhibition of PBP-2-transpeptidase by this antibiotic was weaker than expected from its binding affinity, but the conditions for assay of the transpeptidase activity of PBP-2, such as, a high pH with Tris-HCl buffer, were unfavorable for the action of this antibiotic.

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