COMPARISON OF TWO CARBAPENEMS, SM-7338 AND IMIPENEM: AFFINITIES FOR PENICILLIN-BINDING PROTEINS AND MORPHOLOGICAL CHANGES

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We investigated the binding affinities of SM-7338 for penicillin-binding proteins (PBPs) and the morphological changes induced by it compared with those of imipenem. Both SM-7338 and imipenem had the highest binding affinities for PBP-2 of *Escherichia coli*, which were in good agreement with the primary morphological response of spherical cell formation. SM-7338 also showed high affinities for PBP-1A, -1Bs, and -3, and imipenem showed high affinities for PBP-1A and -1Bs but not for PBP-3. At 4-fold MIC, SM-7338 induced a indeterminate form, whereas imipenem did not. This may be due to the higher affinity of SM-7338 for PBP-3 compared to that of imipenem. Against *Pseudomonas aeruginosa*, SM-7338 had very high affinities for PBP-2 and -3, and imipenem had higher affinities for PBP-2 and -1A. SM-7338 induced this organism to filamentous cells at a concentration lower than its MIC, bulge cells at 2-fold MIC, and spherical cells at 4-fold MIC, while imipenem principally induced round cell formation at each concentration. These morphological differences in *P. aeruginosa* may be due to the differences in binding profiles to PBPs. We also studied the affinities for PBPs using radioactive SM-7338. The data obtained supported these results.

Many new β-lactam antimicrobial agents have been introduced into clinical practice. Recently, non-traditional β-lactam antibiotics have been developed; one class which has been of particular interest is the carbapenems. Discovery of thienamycin[1], the antibiotic produced by *Streptomyces cattleya*, has taken the lead in opening the new era of β-lactam agents, the carbapenems. Subsequently a number of carbapenems have been discovered and some of them were chemically synthesized, however, none have been available for clinical use except imipenem, the *N*-formimidoyl derivative of thienamycin. SM-7338 a new carbapenem, which differs chemically from imipenem, has a methyl group at 4 position and carries a proline derivative on the 3-sulfur instead of the formimidoylaminoethyl group in imipenem. SM-7338 has a strong activity against Gram-positive and Gram-negative bacteria compared with other β-lactams[2~7].

The binding affinities of thienamycin and imipenem for penicillin-binding proteins (PBPs) of *Escherichia coli* and these effects on the shapes of the cells have been previously studied[8~10]. In *E. coli* PBPs have been known to play essential roles in the maintenance of rod shape, septum formation, and functioning in the peptidoglycan biosynthesis, and so on[11~14]. The PBPs in *Pseudomonas aeruginosa* are presumed to have similar roles[15].

In this paper we examined the mode of action of SM-7338 compared with imipenem from the viewpoint of affinities for PBPs and morphological changes, and we reported the difference of morphological responses between *E. coli* and *P. aeruginosa* induced by SM-7338 whereas the same morphological changes were observed by imipenem.

Materials and Methods

Antibiotics

SM-7338 was prepared in the Research Laboratories of Sumitomo Pharmaceuticals Co., Ltd.,

Osaka, Japan, and imipenem was also synthesized in our laboratory. [^{14}C]SM-7338 (27.5 mCi/mmol) was supplied by Sumitomo Chemical Co., Ltd., Osaka. Benzylpenicillin potassium salt was purchased from Meiji Seika Kaisha, Ltd., Tokyo, Japan, and [^{14}C]benzylpenicillin potassium salt (59 mCi/mmol) was from the Radiochemical Centre, Amersham, England.

Bacterial Strains and Antibiotic Susceptibility Test

E. coli K-12 strain C600 and *Pseudomonas aeruginosa* NCTC10490 were used in PBPs and morphological experiments.

The MICs were determined using a broth dilution method. Tubes containing a 1 ml sample of serial 2-fold dilutions of antibiotics in Antibiotic Medium No. 3 (Penassay Broth: Difco Laboratories, Detroit, Mich.) were inoculated with an overnight broth culture of each strain resulting in a final inoculum of 10^6 cfu/ml. The tubes were incubated at 37°C for 24 hours. The MIC was defined as the lowest concentration of the antibiotic that prevented completely visible growth.

Preparation of Membrane Fractions

E. coli and *P. aeruginosa* were grown in Penassay broth at 37°C with vigorous aeration, and were harvested in late-exponential growth phase. Membrane fractions were prepared by sonication of the cells, and centrifugation of homogenate, as previously described[11,15,16]. Cell membranes were suspended in 50 mM sodium phosphate buffer (pH 7.0) containing 10 mM $MgCl_2$ at a concentration of 20 mg/ml protein, and stored at −80°C until their use in binding experiments.

Analysis of PBPs

The first method used for PBPs analysis, a "competition assay" with [^{14}C]benzylpenicillin, was described by SPRATT[16] with some modifications[15,17]. *E. coli* and *P. aeruginosa* membranes (30 μl) were incubated with SM-7338 or imipenem (3 μl) at various concentrations for 10 minutes at 30°C, subsequently, [^{14}C]-benzylpenicillin (50 μCi/ml; 3 μl, a final concentration of 22.5 μg/ml) was added and incubation was continued for another 10 minutes. Reactions were terminated by the addition of excess non-radioactive benzylpenicillin (a final concentration of 2,600 μg/ml) and sarkosyl (a final concentration, 1%). After removal of sarkosyl-insoluble fractions by centrifugation the supernatant was used for sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The second assay was a "direct assay" with radioactive SM-7338, which was fundamentally the same procedure as the competition assay. The membranes were incubated with [^{14}C]SM-7338 at various concentrations for 10 minutes at 30°C, and reactions were stopped by addition of excess homologous unlabeled antibiotic and sarkosyl. The subsequent procedure was the same as in the competition assay.

The method for SDS-PAGE was that of LAEMMLI[18] with slight modifications. To achieve better separation of the PBPs, the separating gels consisted of 7.5% (w/v) monoacrylamide and 0.1% (w/v) *N,N'*-methylenebisacrylamide for *E. coli* and 10 and 0.13%, respectively, for *P. aeruginosa*.

After electrophoresis of the samples in acrylamide slab gels, 2,5-diphenyloxazole was incorporated into the gels and the gels were dried under vacuum with heating as described by BONNER and LASKEY[19]. Dried gels were placed on X-ray film at −80°C for a week or more.

The relative band densities on the fluorogram were determined using a scanning densitometer with peak integration. The complete binding (100%) of [^{14}C]SM-7338 to each PBP was defined as the plateau of the band densities.

Electron Microscopy

Cells were prefixed with 1% glutaraldehyde in 10 mM phosphate buffer (pH 7.0) for 1 hour at 37°C, and refixed with the same glutaraldehyde buffer for 2 hours at 4°C. The fixed cells were dehydrated with a graded series of acetone. For scanning electron microscopy, the specimens were critical point dried, coated with gold, and examined with S-430 scanning electron microscope (Hitachi Ltd., Tokyo, Japan).

Results and Discussion

The affinities of SM-7338 and imipenem for PBPs of *E. coli* and *P. aeruginosa* were investigated

using a competition assay with [^{14}C]benzylpenicillin (Table 1). Against *E. coli*, both SM-7338 and imipenem had extremely high binding affinities for PBP-2 and -4. SM-7338 also showed high affinity for PBP-3. In contrast, considerably low affinity for PBP-3 was observed by imipenem. Binding affinities of imipenem for PBP-1A, -1Bs, -5, and -6 were higher than those of SM-7338. Against *P. aeruginosa*, SM-7338 possessed strong binding affinities for PBP-2, -3, and -4 but not for PBP-5. Imipenem showed high affinities for almost all PBPs, with stronger affinity for PBP-4 and moderate affinity for PBP-5.

We also examined the affinities of SM-7338 for PBPs using a direct assay with radioactive SM-7338 (Table 2). [^{14}C]SM-7338 was strongly bound to PBP-4 and -2 of *E. coli* and PBP-4, -3, -2, and -1A of *P. aeruginosa*, in descending order of binding strength. These results were coincident with those of the competition assay. At a concentration of 30 μg/ml of [^{14}C]SM-7338, it completely bound to all PBPs. No additional PBPs were observed with radioactive SM-7338.

Theoretically, the competition assay and the direct assay are expected to provide similar results, as long as PBPs don't have a β-lactamase activity that may remove the bound antibiotic. However, there are some discrepancy in the affinities SM-7338 for PBP-5 and -6 of *E. coli* and PBP-5 of *P. aeruginosa*. SPRATT[16], NOGUCHI *et al.*[15], and LIVERMORE[20] demonstrated an activity of these PBPs which released the bound [^{14}C]benzylpenicillin. In the competition assay, it is possible that the PBP-SM-7338 complex was broken down by the addition of [^{14}C]benzylpenicillin, so that, in appearance, affinity of SM-7338 obtained by the competition assay was lower than that obtained by the direct assay. There is another discrepancy in the results of the affinity for PBP-4 of *E. coli* using those two method. SPRATT[16] also

Table 1. Affinities of SM-7338 and imipenem for PBPs of *Escherichia coli* K-12 C600 and *Pseudomonas aeruginosa* NCTC10490 using a competition assay with [^{14}C]benzylpenicillin[a].

Strain	Antibiotic	ID_{50} (μg/ml)[b]							MIC[c] (μg/ml)
		1A	1B(s)	2	3	4	5	6	
E. coli	SM-7338	0.81	0.87	0.017	0.49	0.049	1.3	17	0.025
K-12 C600	Imipenem	0.10	0.60	0.019	>16	0.053	0.44	1.5	0.20
P. aeruginosa	SM-7338	0.27	0.24	0.030	0.031	0.010	15	—	0.05
NCTC10490	Imipenem	0.15	0.46	0.11	0.28	0.011	1.8	—	0.78

[a] Membrane fractions were incubated with SM-7338 or imipenem at various concentration for 10 minutes at 30°C, subsequently, [^{14}C]benzylpenicillin was added and incubation was continued for another 10 minutes. Reactions were terminated by the addition of excess non-radioactive bezylpenicillin and sarkosyl. [^{14}C]Benzylpenicillin-protein complex was subjected to SDS-PAGE, followed by fluorography.

[b] Values indicate concentrations of antibiotics required to reduce [^{14}C]benzylpenicillin binding by 50%.

[c] The MICs were determined by the broth dilution method with final inoculation of 10^6 cfu/ml.

Table 2. Affinities of [^{14}C]SM-7338 for PBPs of *Escherichia coli* K-12 C600 and *Pseudomonas aeruginosa* NCTC10490 using a direct assay[a].

Strain	50% binding of [^{14}C]SM-7338 (μg/ml)[b]						
	1A	1B(s)	2	3	4	5	6
E. coli K-12 C600	0.56	0.64	0.015	0.21	0.0073	0.28	2.9
P. aeruginosa NCTC10490	0.087	0.28	0.084	0.049	0.0062	1.8	—

[a] Membrane fractions were incubated with [^{14}C]SM-7338 at various concentration for 10 minutes at 30°C, and reactions were terminated by the addition of excess unlabeled SM-7338 and sarkosyl. [^{14}C]SM-7338-protein complex was subjected to SDS-PAGE, followed by fluorography.

[b] Values indicate concentrations of [^{14}C]SM-7338 required for 50% binding to each PBP.

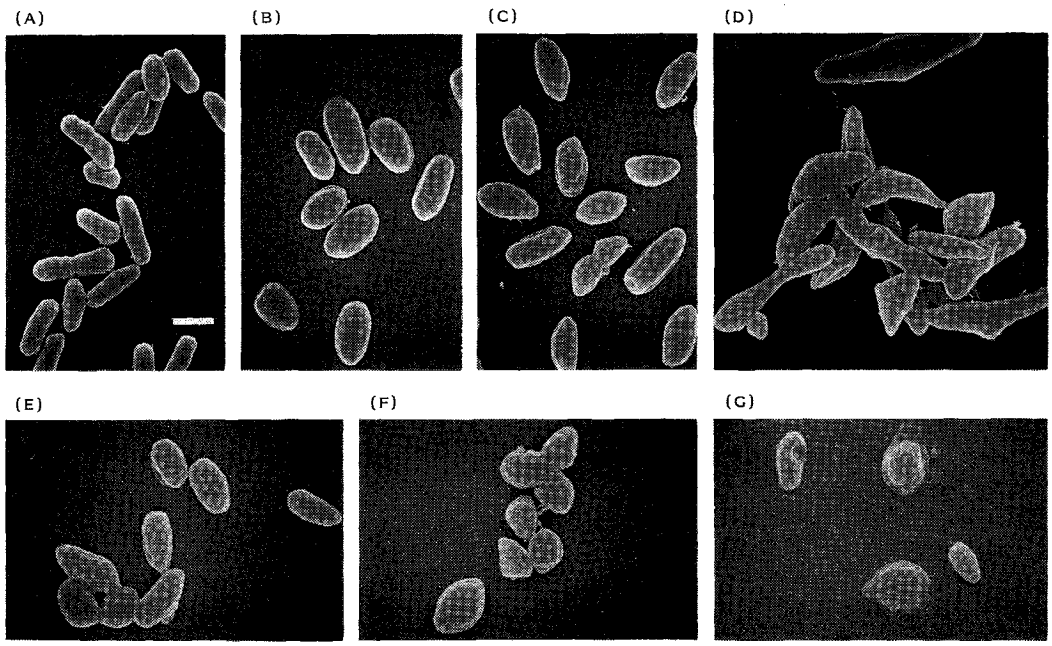
reported that this PBP didn't release the bound [^{14}C]benzylpenicillin, but released [^{14}C]cefoxitin, and it also showed biphasic binding of [^{14}C]benzylpenicillin. Binding patterns of two carbapenems for this PBP, obtained by competition assay, were like biphasic (data not shown). These properties of PBP-4 might the responsible for the difference of the data between the competition assay and the direct assay. The certain reason for this is not known.

Both SM-7338 and imipenem induced in *E. coli* a same spherical form at concentrations lower than the MIC. These primary morphological responses were in good agreement with the highest affinities of both antibiotics for PBP-2. At 4-fold MIC, however, SM-7338 induced indeterminate cells dissimilar to imipenem. This difference may be due to the higher affinity of SM-7338 for PBP-3 compared to that of imipenem. The certain reason for this indeterminate cell formation was not known (Fig. 1).

Against *P. aeruginosa*, SM-7338 induced filamentous cell formation at concentrations lower than its MIC, bulge cell formation at 2-fold MIC, and spherical cell formation at 4-fold MIC (Fig. 2). The primary morphological response of filamentous cell formation cause by SM-7338 was due to its preferential binding to PBP-3. At 2-fold MIC, SM-7338 induced a bulge form. This morphological response may be due to simultaneous binding to PBP-2 and -3. In fact, the affinities of SM-7338 for PBP-2 and -3 were similar. OTSUKI[21] demonstrated that mecillinam and cephalexin synergy induced *E. coli* to a bulge form, since mecillinam bound exclusively PBP-2 and cephalexin bound selectively to PBP-3. FUGLESANG *et al.*[22] also reported bulge formation in several Gram-negative bacteria with the combination of mecillinam and ampicillin. SM-7338 alone may inhibit both PBPs similarly to the combined effect

Fig. 1. Scanning electron micrographs of *Escherichia coli* K-12 C600 exposed for 2 hours at 37°C SM-7338, and imipenem at ¼MIC, MIC, and 4MIC, respectively.

Nothing (A), SM-7338 (B~D), imipenem (E~G).



The MICs (μg/ml) of SM-7338 and imipenem were 0.025 and 0.20, respectively, as measured by the broth dilution method. Bar in frame A indicates 1 μm. All micrographs are in the identical scale.

of those two antibiotics. At 4-fold MIC, spherical cells were formed, for cells could no longer maintain the rod shape because of complete binding of SM-7338 to PBP-2.

This interpretation was supported by the results of direct binding of [^{14}C]SM-7338. In proportion to its concentration, this compound bound PBP-3 and -2 in descending order and it bound perfectly at 4-fold MIC (data not shown). Additionally, we examined the time-course of bulge cell formation. For first 60 minutes, cells extended 3~4-fold, and for another 60 minutes, a bulge developed in the middle of elongated cells (data not shown). It seems that SM-7338 first inhibits PBP-3, and successively inhibits PBP-2.

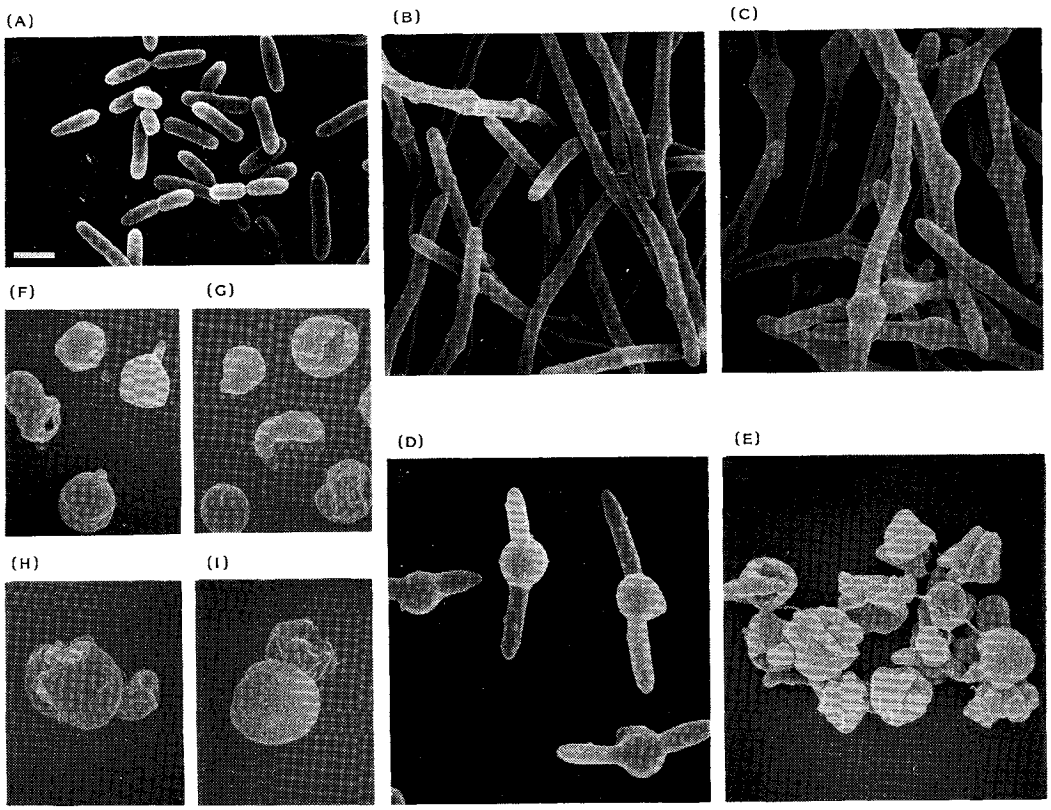
However, although primary morphological response of *P. aeruginosa* is due to PBP-3, this is somewhat curious when one considers that ID_{50}'s are almost the same but the relative amount of PBP-3 is greater than that of PBP-2. It is also possible that location of the two at the membrane makes PBP-3 more accessible even though the two PBPs have equivalent affinities.

On the other hand, imipenem induced the formation of round cells at each concentration. Imipenem appears to bind to PBP-2 principally (Fig. 2).

HASHIZUME *et al.* have already reported affinities of imipenem against *E.* coli and *P. aeruginosa*[10].

Fig. 2. Scanning electron micrographs of *Pseudomonas aeruginosa* NCTC10490 exposed for 2 hours at 37°C SM-7338, and imipenem at $\frac{1}{2}$MIC, MIC, 2MIC, and 4MIC, respectively.

Nothing (A), SM-7338 (B~E), imipenem (F~I).



The MICs (μg/ml) of SM-7338 and imipenem were 0.05 and 0.78, respectively, as measured by the broth dilution method. Bar in frame A indicates 1 μm. All micrographs are in the identical scale.

There was a strong correspondence between these results and ours except for the affinity for PBP-3 of *P. aeruginosa*; they reported an affinity about ten times lower than ours. However, we reproduced our results of higher affinities of imipenem for PBP-3 in three other strains of *P. aeruginosa* (data not shown). The *P. aeruginosa* used by HASHIZUME might be a little unusual strain compared with ours.

Against *E. coli*, the MIC of imipenem was eight times higher than that of SM-7338, although both antibiotics had about the same affinity for PBP-2 of this organism, thought to be a lethal target. These results may be due to the large difference affinity of both carbapenems for PBP-3. That is, high activity of SM-7338 may be explained by some type of synergistic effect caused by the inhibition of both PBP-2 and -3. It is possible that neither PBP alone would have to be completely inhibited for the cells to exhibit distress, especially if both were not fully functional. It is also possible that there may be a difference of permeability of the bacterial outer membrane between SM-7338 and imipenem.

These results seem to indicate that there are some differences of the mode of action between these two carbapenems, and further studies are necessary.

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