SCE-963, a New Potent Cephalosporin with High Affinity for Penicillin-Binding Proteins 1 and 3 of *Escherichia coli*

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A few biochemical activities of SCE-963, a new cephalosporin with potent antibacterial activities against gram-negative bacteria, were compared with those of several currently available cephalosporins against strains of *Escherichia coli* K-12. The minimum inhibitory concentrations of SCE-963, cefazolin, cephaloridine, cephalothin, and cephalaxin were 0.2, 1.56, 3.13, 12.5, and 25 μg/ml, respectively. Affinities of these cephalosporins for the penicillin-binding protein (PBP) 1B of *E. coli* correlated well with their antibacterial activities; among tested cephalosporins, SCE-963 showed the highest affinity for PBP 1B. SCE-963 inhibited cross-linking of peptidoglycan in a cell-free system the most strongly suggesting that this inhibition results from its high affinity for PBP 1B. SCE-963 also showed the highest affinity for PBP 3; it caused filamentation of cells over a wide range of relatively lower concentrations. Thus its superior antibacterial activity is believed to be manifested through its high affinity for the PBPs.

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### MATERIALS AND METHODS

**Bacterial strains.** The following *E. coli* K-12 strains were used. *E. coli* Y10 (thr-1 leu-6 thi-1 supE44) (4), obtained from T. Kamiryo of Kyoto University, was the source of particulate enzyme for peptidoglycan synthesis in a cell-free system. *E. coli* LD-2 is a lysine and meso-diaminopimelic acid (DAP) auxotroph derived from *E. coli* Y10 in our laboratories by treatment with N-methyl-N'-nitro-N-nitrosoguanidine (1) followed by replica plating. This mutant incorporates radioactive DAP specifically into the peptidoglycan of cell walls. Envelope fraction containing PBPs was prepared from *E. coli* KN126 [F′ trpE9829 (am) Tyr (am) ilv sup-126] (8) obtained from T. Nagata of Kyoto University.

**Measurement of MICs.** The MICs were determined by the twofold agar dilution method (16) on YAB (for strain KN126) or DYAB (for strain LD-2) agar plates (5). The YAB medium contained 17.5 g of Difco antibiotic medium 3 and 5 g of Difco yeast extract per liter. The DYAB medium contained 20 mg of DAP per liter of YAB medium (5).

**Measurement of inhibition of peptidoglycan synthesis.** *E. coli* LD-2 was grown in DYAB medium at 37°C for 2 h without shaking to exponential growth phase. Cells were harvested, washed twice with YAB medium, and suspended in 5/4×-strength YAB me-
Assay of PBPs. The procedure reported by Spratt (12) was followed with some modifications. PBPs of E. coli KN126 were fractionated by a gel system which enables the separation of PBP 1 into 1A and 1B (14). The PBPs were quantitated by densitometry of X-ray films with a TLC scanner (Shimadzu CS 910). The binding affinities of cephalosporins for each PBP are expressed in terms of IC₅₀, which are the concentrations (micrograms per milliliter) required to prevent [³⁵C] benzylpenicillin binding by 50%.

Antibiotics, labeled compounds, and other chemicals. SCE-963 and [³⁵C]SCE-963 were synthesized in our division. CEZ, CER, CET, CEX, and benzylpenicillin were obtained commercially. [⁵⁷C]-benzylpenicillin, [³⁵C]DAP, and UDP-[³⁵C]GlcNAc were purchased from the Radiochemical Centre, Amersham, England. UDP-MurNAc-pentapeptide was prepared according to the method of Izaki et al. (4). All other chemicals were of reagent grade.

RESULTS

Inhibitory activities of cephalosporins for peptidoglycan synthesis. Preliminary experiments showed that SCE-963, as well as CEZ, CER, CET, and CEX, specifically inhibited the biosynthesis of peptidoglycan without affecting other macromolecular synthesis. As shown in Fig. 2, SCE-963 inhibited peptidoglycan synthesis to almost the same degree as CEZ and CER,

![Fig. 1. Chemical structure of SCE-963.](image1)

![Fig. 2. Comparison of cephalosporins in their activities to inhibit peptidoglycan synthesis in E. coli LD-2. The amount of DAP incorporated in the control incubation mixture was 0.35 nmol. MICs were (µg/ml): SCE-963, 0.2; CEZ, 1.56; CER, 6.25; CET, 12.5; CEX, 25. They are indicated by arrows in this figure.](image2)
whereas CET and CEX showed less activity. The inhibition by CEZ, CER, and CET increased linearly in proportion to their logarithmic concentrations starting with their MIC levels, and, in contrast, the inhibition by SCE-963 and CEX increased only at the 10 \times MIC levels. These results indicate that the antibacterial activity of cephalosporins, expressed in terms of MIC, does not necessarily result from the inhibition of peptidoglycan synthesis. Morphological examinations of cells during this isotope incorporation (data not shown) indicated that the inhibition of peptidoglycan synthesis paralleled the lytic action of cephalosporins and did not occur during filamentation of cells.

**Effect of cephalosporins on growth and cell morphology.** SCE-963, CEZ, and CER exhibited lytic action against *E. coli* KN126 at 10 \mu g/ml, whereas CET did so only at 100 \mu g/ml (Fig. 3). CEX had no effect even at 100 \mu g/ml. The lytic action of SCE-963 was less than expected from its antibacterial activity, suggesting that the mode of action of SCE-963 is somewhat different from that of CEZ or CER. The capacity of SCE-963 to induce filamentous cells was greater than that of the other cephalosporins; cells were elongated up to 10 to 20 times and 40 times the length of normal cells at 0.1 and 1 \mu g/ml, respectively; after a 3-h incubation, CEX induced the formation of filamentous cells of up to 10 times and 15 times the length of normal cells at 10 and 100 \mu g/ml, respectively. CEZ and CET changed rods to elongated forms at 1 and 10 \mu g/ml, respectively. Filamentation did not occur with CER at any tested concentrations. At concentrations where lysis occurred, there appeared the ghosts of rod-shaped cells. These results paralleled the inhibition of peptidoglycan synthesis (see Fig. 2).

**Inhibition of cross-linking of peptidoglycan in a cell-free system.** Particulate enzymes of *E. coli* Y10 synthesized highly cross-linked (52.4%) peptidoglycan under our assay conditions. Among the cephalosporins tested, SCE-963 was the most effective inhibitor of cross-linking (Fig. 4). The order of the inhibitory activities of the cephalosporins for cross-linking roughly paralleled that of their antibacterial activities, suggesting a correlation between these two activities.

**Affinities of cephalosporins for PBPs and their correlation with antibacterial activity.** Figure 5a shows the competition of increasing concentrations of SCE-963 for \[^{14}C\]penicillin binding to PBPs of *E. coli* KN126. Figure 5b shows the quantitated data obtained by densitometry. Affinities of other cephalosporins for PBPs were determined in the same way. Table 1 shows their *I_50* for PBPs 1A, 1B, 2, 3, 4, and 5/6. All the cephalosporins tested had highest affinities for PBP 1A. The function of PBP 1B, however, has been reported to compensate that of PBP 1A (14, 17, 18). Consequently, the concentration required to prevent the total function of PBP 1 would actually correspond to that of PBP 1B. Besides, PBP 4 and 5/6 have been pointed out to be nonessential for normal growth of *E. coli* (3, 7, 17). As a result, one or all of the other PBPs, i.e., PBP 1B, 2, and 3, seem likely to be the targets of the cephalosporins involved in their antibacterial activities. The kinetics of saturation of PBP 2 were biphasic with SCE-963, CER, and CET, and there was no feasible correlation between *I_50* for this PBP and MICs of these cephalosporins even with the higher or the lower *I_50*. Figure 6 illustrates the relationship between *I_50* for PBP 1B and 3 and MICs of these cephalosporins. There was a striking correlation between *I_50* for PBP 1B and MICs and some correlation between *I_50* for PBP 3 and MICs. SCE-963, the most potent cephalosporin, showed the lowest *I_50* for both PBP 1B and 3.

**DISCUSSION**

SCE-963 and all other tested cephalosporins specifically inhibited peptidoglycan synthesis. The capacities of cephalosporins to inhibit peptidoglycan synthesis did not completely parallel their antibacterial activities; SCE-963 was 8 and 16 times more active than CEZ and CER, respectively, when measured by MIC, but the inhibitory activities of peptidoglycan synthesis of these three cephalosporins were almost the same (Fig. 2). Generally, inhibition of peptidoglycan synthesis occurred at higher concentrations, when cephalosporins lysed *E. coli* cells, but not at lower concentrations, when they prevented only cell division. SCE-963 induced formation of extremely long filamentous cells over a wide range of lower concentrations. These morphological alterations of *E. coli* can be explained by the high affinity of SCE-963 for PBP 3, which is involved in cell division (11, 13).

Among tested cephalosporins, SCE-963 was the most effective inhibitor of cross-linking of the peptidoglycan in a cell-free system (Fig. 4). The order of the inhibitory activities of tested cephalosporins followed their affinities for PBP 1B. Tamaki et al. demonstrated that PBP 1B may be one of the essential enzymes involved in polymerization and cross-linking of the peptidoglycan in a cell-free system (18).
The minor components of PBPs, i.e., PBP 1 consisting of 1A and 1B, 2, and 3, have been shown to be essential for cell elongation, cell shape, and cell division, respectively, and to be possible targets of β-lactam antibiotics (11), while the major ones, i.e., PBP 4 and 5/6, are not indispensable for normal growth of *E. coli* (3, 7, 17). Furthermore, the functions of some PBPs have been reported to compensate each other (14, 17, 18). In this study, all of the cephalosporins tested showed the highest affinities for PBP 1A (Table 1). Their antibacterial activ-
ties, however, may not directly reflect their affinities for PBP 1A, since PBP 1B makes up for the defect of the function of PBP 1A (17, 18). Consequently the concentration required to prevent the total function of PBP 1 would correspond to that of PBP 1B. This presumption is supported by: (i) the existence of a striking correlation between $I_{50}$ for PBP 1B and MICs of the cephalosporins (Fig. 6), and (ii) the high sensitivity of mutants defective in PBP 1B to cephalosporins (17).

A correlation between $I_{50}$ for PBP 3 and MICs was suggested, although the evidence was not as strong as in the case of PBP 1B. The correlation between $I_{50}$ for PBP 2 and MICs was not feasible. These facts are consistent with the hypothesis (11) that typical $\beta$-lactams usually exert their antibacterial activity through inhibition of the functions of PBP 1 and 3. Based on the same hypothesis, the potent activity of SCE-963 can be explained by its extremely high affinity for these PBPs. The remarkable affinity for PBP 3 of SCE-963 is in good agreement with its high ability to induce filamentous cells within a wide range of lower concentrations. At a given concentration, a $\beta$-lactam antibiotic can inhibit functions of several PBPs at the same time, but it is not clear at present how such composite actions would result in antibacterial activity. We have found some discrepancies in the correlation between MICs and $I_{50}$s for PBP 1B and 3 among the cephalosporins studied. For instance, the antibacterial activity of CET was not as strong as expected from its affinity for PBP 3 (Fig. 6). This discrepancy may be due to the fact that the assay of PBPs was performed in the absence of permeability barriers, while the MIC was determined on intact cells (12). It has been pointed out that the permeability of CET is much poorer than that of several other cephalosporins in E. coli (19) and Proteus vulgaris (10). Therefore, an experimental system must be developed that

**TABLE 1.** Binding affinities of cephalosporins for each PBP of E. coli KN126 and their MICs against the same organism

<table>
<thead>
<tr>
<th>Cephalosporin</th>
<th>MIC (µg/ml)</th>
<th>$I_{50}$ for PBP:</th>
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<tr>
<td></td>
<td></td>
<td>1A</td>
</tr>
<tr>
<td>SCE-963</td>
<td>0.2</td>
<td>0.70</td>
</tr>
<tr>
<td>CEZ</td>
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<td>0.19</td>
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<td>CER</td>
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<td>CET</td>
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<tr>
<td>CEX</td>
<td>25</td>
<td>1.5</td>
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*Competition was biphasic.*

**FIG. 4.** Comparison of cephalosporins in their activities to inhibit cross-linking of peptidoglycan in a cell-free system.
SCE-963, A NEW CEPHALOSPORIN

Fig. 5. Competition of SCE-963 for [$^{14}$C]benzylpenicillin binding. Envelope samples (11.2 mg of protein per ml) were preincubated for 10 min at 30°C with water (A) or increasing concentrations of SCE-963, and the PBPs remaining accessible were labeled with a saturating concentration of [$^{14}$C]benzylpenicillin. PBPs were detected by exposure of the dried gel to X-ray films for 12 days at -80°C. (a) Fluorogram of SCE-963. The final concentrations of SCE-963 were (μg/ml): (B) 0.13, (C) 0.33, (D) 0.82, (E) 2.05, (F) 5.12, (G) 12.8, (H) 32, (I) 80, (J) 200, and (K) 500. (b) Quantitated data of (a). Symbols: (○) PBP 1A; (●) 1B; (×) 2; (Δ) 3; (□) 4. The data on PBP 5/6 are not shown in this figure because the competition was negligible.
can directly determine the binding of β-lactams to PBPs of intact cells. One successful example has been reported by Reynolds et al. (9) using whole cells of Bacillus megaterium.

The antibacterial activities of the cephalosporins tested in this study correlated with their affinities for PBP 1 and 3 of E. coli. The superior antibacterial activity of SCE-963 may be explained by this hypothesis.

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