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Lack of Effect of Carbonyl Cyanide *m*-Chlorophenylhydrazone on KB-5246 Accumulation by *Staphylococcus aureus*

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The accumulation of KB-5246 in a quinolone-susceptible strain of *Staphylococcus aureus* was about 70 times that of norfloxacin. Carbonyl cyanide *m*-chlorophenylhydrazone increased the accumulation of norfloxacin about eightfold, but it did not influence that of KB-5246. The low efflux of KB-5246 from *S. aureus* may contribute to its potent antibacterial activity.

KB-5246, a new quinolone with a tetracyclic structure (9), has been demonstrated to have a broad spectrum of antibacterial activity. We have reported that the strong antibacterial activity of KB-5246 against gram-negative bacteria can be explained by its inhibition of DNA gyrase and its accumulation by both porin and non-porin pathways (8). However, the reason for the potent antibacterial activity of KB-5246 against gram-positive bacteria is still unknown.

In this study, we examined the activity of KB-5246 against *Staphylococcus aureus*, which was used as a representative gram-positive bacterium. The antibacterial activities of quinolones have been explained by their inhibitions of DNA gyrase and their permeation (1–7, 10, 11). We were unable to examine the inhibition of *S. aureus* DNA gyrase by KB-5246 because of difficulties in purifying DNA gyrase from clinical isolates of *S. aureus* (12). Therefore, to determine the reason for the potent antibacterial activity of KB-5246 against gram-positive bacteria, we compared the accumulation of KB-5246 and norfloxacin by a quinolone-susceptible strain of *S. aureus*.

KB-5246 and norfloxacin were synthesized in the Department of Chemistry of our laboratories. Methyltriphenylphosphonium ($TPMP^+$) was purchased from Tokyo Kasei Co. The quinolone-susceptible clinical isolate used in this study, *S. aureus* INK-1, which was isolated during 1988 to 1989 in Japan, was donated by K. Deguchi.

MICs were determined by the twofold agar dilution method described previously (9).

The accumulation of KB-5246 and norfloxacin and the binding of $TPMP^+$ were measured by the method of Hirai et al. (6, 7), with the following modifications. Bacterial cells were grown to an optical density at 660 nm of 0.6. KB-5246, norfloxacin, or $TPMP^+$ was added to the bacterial suspension to a concentration of 10 μg/ml (final concentrations, KB-5246, 26 μM; norfloxacin, 31 μM; $TPMP^+$, 28 μM), and the suspensions were incubated for 10 min at 37°C. In tests of the dose-response of accumulation, KB-5246 was added at concentrations of 0.05, 0.10, 0.50, 1.0, 5.0, and 10.0 μg/ml. After incubation, the cells were washed with 1 ml of saline by centrifugation, and the precipitated cells were suspended in 5% acetic acid, allowed to stand in boiling water for 5 min, and then centrifuged. The amount of KB-5246, norfloxacin, or $TPMP^+$ in the resulting supernatant was defined as that amount accumulated by the cells. For evaluation of the energy-dependent efflux of quinolones, an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), was added 5 min before the addition of KB-5246, norfloxacin, or $TPMP^+$. The concentration in the supernatant was determined by high-pressure liquid chromatography by using an Inertsil ODS-2 column (Gasukuro Kogyo Co.), a mobile phase composed of 5% acetic acid-methanol (85:15), a flow rate of 1.5 ml/min, and a detection wavelength of 270 nm at a column temperature of 40°C. All data on accumulation were means of two points.

Energy-dependent efflux has been reported to influence the accumulation of quinolones (2, 4). We examined the effect of CCCP on the binding of $TPMP^+$ or the accumulation of norfloxacin by *S. aureus* INK-1, because binding of $TPMP^+$ is known to be an indicator of the energized state in oxidative phosphorylation (5). $TPMP^+$ or norfloxacin was added at a concentration of 10 μg/ml. CCCP was added at a concentration of 200 μM 5 min before the addition of $TPMP^+$ or norfloxacin. The accumulation was measured after 10 min of incubation. Without CCCP, the accumulations of $TPMP^+$ and norfloxacin were 20.90 and 0.07 μg/mg of dry cell, respectively. With CCCP, $TPMP^+$ accumulation was not detected, and the accumulation of norfloxacin was 0.54 μg/mg of dry cell. Because the binding of $TPMP^+$ was completely inhibited by 200 μM CCCP, we used a CCCP concentration of 200 μM. The accumulation of norfloxacin was increased by the addition of 200 μM CCCP, as reported previously (13).

The dose-response curve for the accumulation of KB-5246 by the quinolone-susceptible strain (INK-1) of *S. aureus* is shown in Fig. 1. The accumulation increased linearly with an increase in the extracellular KB-5246 concentration over a range of extracellular concentrations of KB-5246 of 0.05 to 10.0 μg/ml. Moreover, the accumulation was not influenced by 200 μM CCCP over a range of extracellular KB-5246 concentrations of 0.10 to 10 μg/ml.

The accumulations of KB-5246 and norfloxacin by *S. aureus* INK-1 were compared (Fig. 2). The accumulation of KB-5246 by the clinical isolate (INK-1) which was susceptible to quinolones (norfloxacin MIC, 3.13 μg/ml; KB-5246 MIC, 0.10 μg/ml) was approximately 70-fold that of norfloxacin. Moreover, 200 μM CCCP increased the accumulation of norfloxacin in this quinolone-susceptible strain approximately eightfold but did not influence its accumulation of KB-5246. These results suggest that the ineffective efflux of

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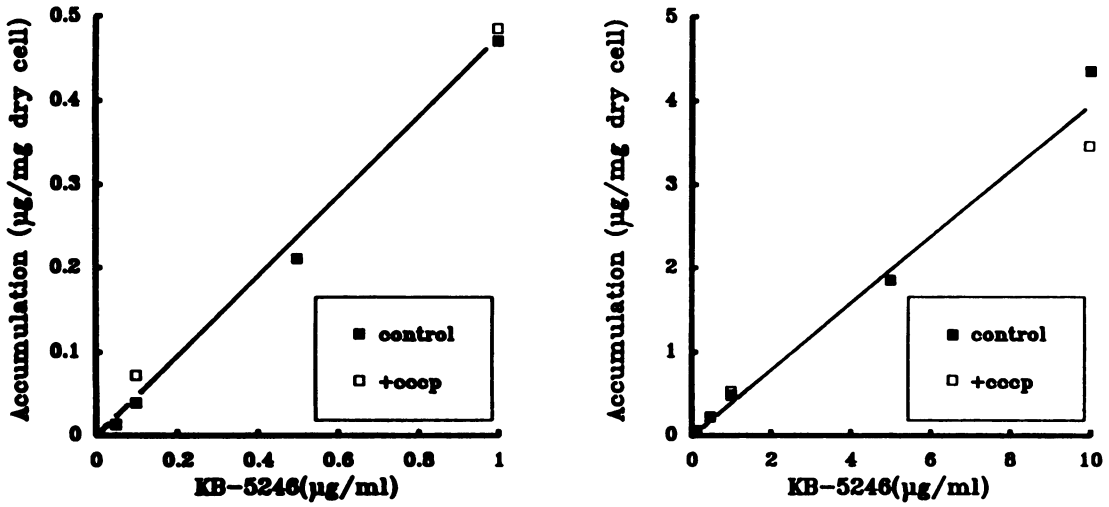

FIG. 1. Dose-response curve of the accumulation of KB-5246 by a quinolone-susceptible strain (INK-1) of *S. aureus*. KB-5246 was added at concentrations of 0.05, 0.10, 0.50, 1.0, 5.0, and 10 μg/ml. CCCP was added at a concentration of 200 μM 5 min before the addition of KB-5246. The accumulation was measured after 10 min of incubation.

KB-5246 from the quinolone-susceptible strain of *S. aureus* may be one factor responsible for the potent antibacterial activity of KB-5246. Norfloxacin might be effectively ejected by an energy-dependent efflux system.

In this study, we measured the accumulation of KB-5246 in *S. aureus* and the effect of CCCP on its accumulation to determine the reason for its potent antibacterial activity against gram-positive bacteria. We found that the accumulation of KB-5246 in the quinolone-susceptible strain was 70 times that of norfloxacin. One reason for its high accumulation was its ineffective efflux.

Recently, H. Yoshida et al. (13) established the sequence of a *norA* gene that encoded a membrane-associated active efflux pump. They reported that this efflux pump effectively excretes a hydrophilic agent but that it does not excrete a hydrophobic agent. Thus, the poor efflux of KB-5246 from *S. aureus* cells might be due to its hydrophobicity (8). S. Yoshida et al. (14) reported that one mechanism of resis-

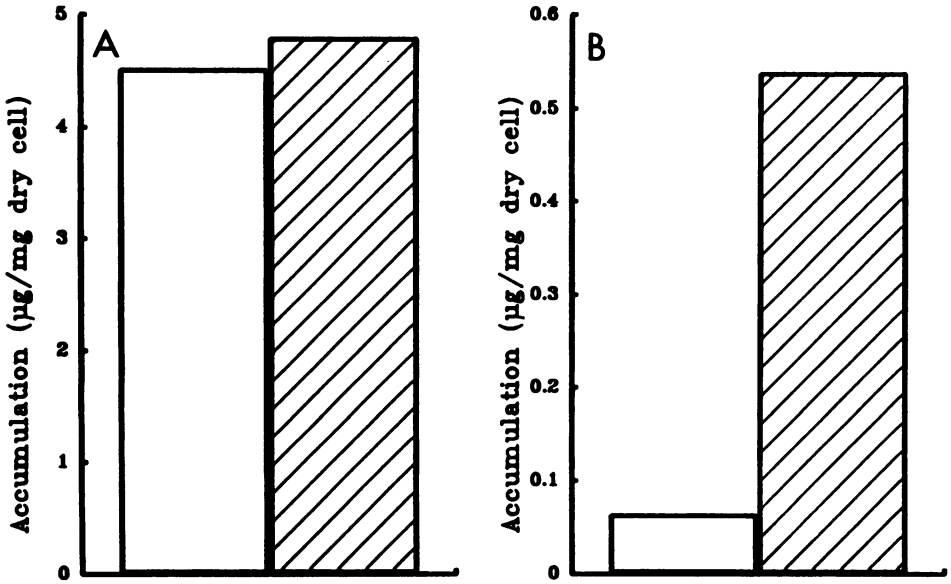

FIG. 2. Accumulation of KB-5246 (A) and norfloxacin (B) by a quinolone-susceptible strain (INK-1) of *S. aureus*. KB-5246 or norfloxacin was added at a concentration of 10 μg/ml. CCCP was added at a concentration of 200 μM 5 min before the addition of KB-5246 or norfloxacin. The accumulation was measured after 10 min of incubation. Open columns, without CCCP; hatched columns, with 200 μM CCCP.

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tance of *S. aureus* to quinolones is energy-dependent efflux. KB-5246 should be a useful tool for investigating the mechanism of resistance in these strains.

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