Screening of an Inhibitor of the Tetracycline Efflux Pump in a Tetracycline-Resistant Clinical-Isolate of Staphylococcus aureus 743

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Clinically-isolated methicillin-resistant Staphylococcus aureus (MRSA) strain 743 exhibited resistance to tetracycline as judged from the active efflux of the drug. The efflux of tetracycline was inhibited by an uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), and minocycline. Inhibitors of the efflux pump were examined in this strain to determine the cellular accumulation of tetracycline. Out of seven compounds examined, three caused a significant increase in the cellular concentration of tetracycline by inhibiting the efflux pump. Two of them seem to be energy inhibitors. Ro 07-3149 inhibited the efflux pump without affecting the energy state, and exhibited very low antibacterial activity but showed weak synergy with tetracycline.

Key words S. aureus; tetracycline; drug resistance; inhibitor; drug efflux

Methicillin-resistant Staphylococcus aureus (MRSA) frequently shows antibiotic resistance against a wide range of antibiotics including tetracycline. The resistance of MRSA to tetracycline is based largely on two resistance determinants, tet(M) and tet(K). The resistance mechanisms of tet(M) and tet(K) are based on ribosomal protection and active drug efflux, respectively. If an efficient inhibitor of the tetracycline efflux pump is found, it should be very useful for the eradication of MRSA.

Tetracycline enters bacterial cells via simple diffusion through the lipid bilayer region of the cytoplasmic membrane as a protonated neutral form and then is accumulated as a deprotonated negatively-charged form or a monocationic chelation complex with a magnesium ion, because the pH of the cell interior is higher than that of the exterior in energized cells. Tetracycline efflux pumps, such as Tet(K) and Tet(B), actively export a tetracycline-divalent cation chelation complex through an antiport with a proton. Therefore, the tetracycline accumulation level of tetracycline-sensitive cells is high and deenergization by an uncoupler reduces the intracellular tetracycline concentration, whereas that of resistant cells is low and an uncoupler increases the intracellular concentration until there is a concentration equilibrium across the cell membrane. On the other hand, an efficient inhibitor of the tetracycline efflux pump, if one exists, is expected to greatly increase the tetracycline accumulation level of resistance cells to that of energized sensitive cells.

S. aureus strain 743 is a typical MRSA strain, which was clinically isolated and showed tetracycline resistance. In this study, we found that S. aureus 743 has an active tetracycline efflux pump and that minocycline efficiently inhibits it. We used this strain to examine efflux pump inhibitors.

MATERIALS AND METHODS

Strain S. aureus 743 was clinically isolated in Nutley, New Jersey, U.S.A. in 1983, and is resistant to tetracycline as well as to many other kinds of antibacterial agents, including methicillin, erythromycin, trimethoprim and sulfmethoxazole.

Reagents Muller-Hinton broth was obtained from Difco. [14C]Tetracycline was purchased from DuPont-New England Nuclear. Ro 07-3149 (Fig. 3) was synthesized by Roche Nutley (NJ, U.S.A.), and its purity was more than 95%, as determined by thin layer chromatography. Other compounds were obtained from our in-house compound library. Other materials were all of reagent grade.

Assaying of [14C]Tetracycline Uptake by Intact Cells S. aureus 743 cells were precultured with shaking overnight in Muller-Hinton broth supplemented with 20 μg/ml of tetracycline. One percent of the overnight culture was inoculated into the same medium and then the cells were grown to exponential phase (approximately 1.7×10⁵ CFU/ml). The cells were harvested and washed once with the storage buffer (100 mM potassium phosphate (pH 7.2) and 7.5 mM ammonium sulfate), and then resuspended and concentrated twenty-times in the same buffer containing 200 μg of chloramphenicol/ml. The cells were stored on ice until use.

For the uptake assay, the cells were diluted three times with the assay buffer (100 mM potassium phosphate (pH 6.6), 100 mM KCl, 2 mM MgSO₄, and 0.4% glucose). Uptake was initiated by adding 20 μl of 25 μM [14C]tetracycline (75 Ci/mole) to a mixture of 60 μl of the cell suspension and 20 μl of the inhibitor solution. After incubation at 37 °C for 80 min or the indicated times, the cells were filtered out on a glass filter and then immediately washed with the assay buffer. The radioactivity on the filter was counted with a Beta-Plate Counting System (Pharmacia). The intracellular concentration of tetracycline was calculated, with a counting efficiency of 56%, a cell volume of 1.3 μl/10⁶ cells being used.

Antimicrobial Activity Antimicrobial activity was measured by the broth dilution method with Muller Hinton broth. Approximately 5×10⁵ cells were inoculated into 100 μl of the broth in the presence of a series of 2-fold dilutions of the indicated drugs. The minimum inhibitory concentration was
determined after cells had grown for 18 h at 37 °C.

RESULT

**Active Efflux of Tetracycline from S. aureus 743 Cells**

*S. aureus* 743 showed antibiotic resistance to a wide variety of chemotherapeutic agents including tetracycline. The accumulation of tetracycline in *S. aureus* 743 cells was very low when intact cells were incubated with [3H]tetracycline (Fig. 1). The addition of an uncoupler, carbonyl cyanide m-chlorophenylhydrazone (CCCP), increased the accumulation level by a factor of about 2. Minocycline, which is a hydrophobic derivative of tetracycline, greatly increased the cellular accumulation of tetracycline by a factor of about 30 when 400 μM minocycline was added (Fig. 1). These observations indicated that the intracellular concentration of tetracycline in *S. aureus* 743 was lowered by an active efflux pump and that minocycline is an efficient inhibitor of the efflux pump. The accumulation level of tetracycline was dependent on the minocycline concentration (Fig. 2). The max-

![Fig. 1. Effects of Minocycline and CCCP on the Time Course of Tetracycline Accumulation in S. aureus 743 Cells](image)

\[ \triangle, \text{no drug added; } \blacktriangle, 200 \mu M \text{ CCCP; } \bigcirc, 200 \mu M \text{ minocycline; } \bullet, 400 \mu M \text{ minocycline.} \]

![Fig. 2. Minocycline Concentration Dependence of the Intracellular Tetracycline Concentration in S. aureus 743 Cells](image)

The tetracycline concentration was measured after 90 min incubation as described in Materials and Methods.

![Fig. 3. Chemical Structures of Ro 07-3149 and Their Derivatives](image)

<table>
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<th>R4</th>
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(1), Ro 07-3149: 1,1-dimethyl-3-(1-hydroxypropyl)-4,6,7-trimethylindan; (2), 4-isopropyl-6-methoxy-1,1,7-trimethylindan; (3), 5-hydroxy-3-isopropyl-1,1,4,7-tetramethylindan; (4), 6-hydroxy-4-isopropyl-1,1,7-trimethylindan; (5), 6-methoxy-1,1,4,7-tetramethylindan; (6), 6-methoxy-1,1,4,5-tetramethylindan; (7), 6-hydroxy-7-isopropyl-1,1,4-tetramethylindan.
Table 1. Effect of Ro 07-3149 on the Antibacterial Activities of Tetracycline toward Methicillin-resistant S. aureus 743 and a Drug Sensitive Wild-type S. aureus ATCC25923

<table>
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<tr>
<th>Strain</th>
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<td>Ro 07-3149</td>
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<tr>
<td>S. aureus 743 (MRSA)</td>
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The tetracycline concentration was measured after 75 min incubation as described in Materials and Methods. Ro 07-3149 (1); compound (2); compound (3); compound (4); compound (5); compound (6); ×, compound (7).

The intracellular level was reached with 400 µM minocycline, and at higher minocycline concentrations the level inclined to decrease, probably due to minocycline-induced de-energization.

Effects of Various Compounds on the Accumulation Level of Tetracycline in S. aureus 743 Cells The effects of a novel compound, 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylandan, Ro 07-3149, and six derivatives of it on the intracellular accumulation level of tetracycline in S. aureus 743 were investigated (Fig. 4). Three of these compounds did not affect the accumulation of tetracycline. Compounds (3) and (4) increased the intracellular tetracycline concentration at 25 µg/ml, whereas at higher concentrations, the intracellular concentration of tetracycline decreased to the level without compounds (Fig. 4), probably due to de-energization. These results indicate that these two compounds are certainly efflux pump inhibitors but that they also act as uncouplers at high concentrations. Compound (7) moderately increased the intracellular tetracycline concentration at 100 µg/ml, indicating this compound is a weak inhibitor. Only Ro 07-3149 greatly increased the intracellular tetracycline concentration at 25 µg/ml, and at higher concentrations did not affect the level of this drug (Fig. 4). Ro 07-3149 seems to be an efficient efflux pump inhibitor without uncoupling activity because (1) significant accumulation of tetracycline in cells was observed when Ro 07-3149 was added whereas only a low concentration of tetracycline in cells was observed in the presence of the uncoupler, CCCP and (2) the level of accumulation was constant over the range of concentrations tested.

Effect of Ro 07-3149 on the Antibacterial Activity of Tetracycline As shown in Table 1, the antibacterial activity of Ro 07-3149 is weak. The minimum inhibitory concentration (MIC) for S. aureus 743 cells is only 32 µg/ml. On the other hand, the MIC of tetracycline for these cells is 64 µg/ml (Table 1), which is much higher than that for tetracycline-sensitive S. aureus ATCC25923 cells (0.25 µg/ml). In the presence of 8 µg/ml (1/4 MIC) of Ro 07-3149, the MIC value of tetracycline decreased two-fold, whereas that of chloramphenicol was unchanged (Table 1).

DISCUSSION

We showed in this study, that S. aureus 743 has an active tetracycline efflux pump and that Ro 07-3149 is an efficient inhibitor of this pump. The presence of the tet(K) gene was detected by the PCR method in DNA isolated from S. aureus 743 cells (data not shown). The combined effect of Ro 07-3149 with the antibacterial activity of tetracycline was observed but the tetracycline resistance of this strain was not lost in the presence of Ro 07-3149. Therefore, the ribosomal protection mechanism may also contribute to the tetracycline resistance of the strain.

We investigated the mechanism of the Ro 07-3149 action on the Tet(K) efflux pump expressed in Escherichia coli in detail in our previous study.10 It was confirmed to inhibit the Tet(K) pump in everted membrane vesicles of E. coli without affecting the energy state of the membrane.

The screening method described here is based on the fact that efflux pump inhibitors greatly increase the intracellular drug concentration. The level is far higher than that reached on de-energization by an uncoupler. This is a simple and very specific method for the screening of efflux pump inhibitors without an uncoupling action. This is a very important factor because usual methods based on the synergetic effects of inhibitors with the antibiotic actions of drugs tend to reveal energy inhibitors, most of which are not suitable for chemotherapy due to their toxicity. The combination of synergy and drug accumulation methods is the most preferable for the screening of efflux pump inhibitors.

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REFERENCES