A novel compound, 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylindan, is an effective inhibitor of the *tet*(K) gene-encoded metal-tetracycline/H^+ antiporter of *Staphylococcus aureus*

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Abstract A novel indan derivative, 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylindan (Ro 07-3149), was found to be a strong inhibitor of the *tet*(K) gene-encoded tetracycline/H^+ antiporter of *Staphylococcus aureus*. One micromole of this compound per mg membrane protein was enough for complete inhibition of the Tet(K)-mediated tetracycline transport and tetracycline-coupled proton transport, without the energy state of the membrane being affected. The mode of inhibition was non-competitive. Although this compound caused membrane de-energization at a high concentration, the IC_{50} value for de-energization (7.3 μmol/mg membrane protein) was about 17 times and 33 times higher than the values for Tet(K)-mediated proton/tetracycline antiport and [^{3}H]tetracycline transport, respectively, indicating that the inhibitory action of Ro 07-3149 is not due to the uncoupling effect of the inhibitor.

Key words: Ro 07-3149; Tetracycline; Antiporter; TetK; *Staphylococcus aureus*

1. Introduction

Tetracyclines are useful antibiotics and widely used because of their broad antimicrobial activity [1]. However, the recent increase in microorganisms resistant to tetracyclines has become a problem regarding the use of tetracyclines [1,2]. One of the major tetracycline-resistance mechanisms is known to be the pumping out of tetracyclines from bacterial cells through a specific Tet transporter [1,2].

Two classes of efflux proteins are found in Gram-positive bacteria, Tet(K) in *Staphylococcus aureus* [3,4], and Tet(L) in *Bacillus, Staphylococcus* and *Streptcoccus* species [5,6], whereas several other classes, such as Tet(A), (B) and (C), are found in Gram-negative organisms including *Enterobacteriaceae* [1]. The former and latter types of Tet proteins are greatly different from each other not only in molecular size and sequence but also in putative membrane topology [7]. However, the two types of Tet proteins mediate the efflux of tetracyclines in a similar manner, that is, electrically neutral antiport of a metal–tetracycline chelation complex with a proton [8–10].

Inhibitors of these tetracycline efflux systems are expected to be useful for overcoming the bacterial resistance in combination with existing tetracyclines, especially in the case of methicillin-resistant *S. aureus* (MRSA), because Tet(K) confers the tetracycline resistance on *S. aureus* as well as Tet(M) based on ribosomal protection [11]. Recently, we screened for inhibitors of the tetracycline efflux system in *S. aureus* by measuring the intracellular concentration of [^{3}H]tetracycline and found that 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylindan (Ro 07-3149, Fig. 1) showed the ability to increase the cellular concentration of tetracycline even in the presence of a drug efflux system [12]. However, with such indirect measurements it is impossible to determine whether inhibition is due to the uncoupling effect of the inhibitor on the energized state of the cell membrane. Since the in vitro inside-out (ISO) membrane vesicles prepared from *S. aureus* did not work, we transferred the *tet*(K) gene into *E. coli* in order to perform in vitro experiment.

In this study, we analyzed the mode of inhibition by Ro 07-3149 of a tetracycline efflux system using ISO membrane vesicles prepared from *E. coli* cells in which the *tet*(K) gene of *S. aureus* was constitutively expressed [13].

2. Materials and methods

2.1. Materials

Ro 07-3149 (Fig. 1) was synthesized at Roche (Nutley, NJ). Tetracycline was obtained from Wako Pure Chemical Industries, Ltd., and Sigma. 7-[^{3}H]Tetracycline and [^{14}C]methylamine hydrochloride was obtained from DuPont New England Nuclear and Amersham, respectively. Other chemicals were of reagent grade and were from commercial sources.

2.2. Bacterial strains and plasmids

E. coli W3104 [14] was used as the host strain for expression of the *tet*(K) gene and the preparation of ISO membrane vesicles. pTZ1252 [13] is a plasmid carrying the *tet*(K) gene for expression in *E. coli*.

2.3. Preparation of ISO membrane vesicles

The preparation of membrane vesicles from *E. coli* W3104/pTZ1252 was carried out as described previously [15]. Briefly, cells were grown in 1 l of minimal medium supplemented with 0.2% glucose and 0.1% casamino acids until OD_{530} reached 0.6. ISO vesicles were prepared by disruption of cells with a French press in 50 mM MOPS-KOH buffer (pH 6.6) containing 0.1 M KCl and 10 mM EDTA. Then the vesicles were washed once with 50 mM MOPS-KOH (pH 7.0) containing 0.1 M KCl.

2.4. Measurement of proton translocation as the fluorescence change of quinacrine

Proton translocation across ISO vesicles was measured as the change in the fluorescence of quinacrine [16]. A suspension (10 μl)

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Abbreviations: Ro 07-3149, 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethylindan; DMSO, dimethyl sulfoxide

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of ISO vesicles (5 mg protein/ml) was diluted with 1 ml of 50 mM MOPS-KOH (pH 7.0) containing 0.1 M KCl and 10 mM $MgSO_4$. After the addition of 5 μl of 160 μM quinacrine, 0.5–5 μl of 40.6 mM Ro 07-3149 in DMSO was added. The membrane vesicles were energized by adding 2.5 μl of 250 mM β-NADH (final, 625 μM), the emission (500 nm)/excitation (440 nm) ratio being monitored. Then, 10 μl of a 2 mM tetracycline solution (final, 20 μM) was added when the ΔpH formation was completed. Finally, 10 μl of 1 M NH_4Cl was added to disrupt the ΔpH.

2.5. Measurement of [^{3}H]tetracycline and [^{14}C]methylamine uptake by ISO vesicles

A mixture of 10 μl of the vesicle suspension (3.5 mg/ml) and 0.5 μl of 250 mM NADH was pre-incubated at 30°C for 1 min. The uptake was started by the addition of [^{3}H]tetracycline (final conc., 10 μM) or [^{14}C]methylamine (final conc., 2 mM) in 40 μl of 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl and 62.5 μM $CoCl_2$ (final, 50 μM), unless otherwise stated. For the inhibition study, 0.5 μl aliquots of various concentrations of the inhibitor dissolved in DMSO were added simultaneously with tetracycline. After incubation at 30°C for the indicated periods, the uptake was stopped by adding 2 ml of 5 mM MOPS-KOH (pH 7.0) containing 0.15 M LiCl, and then the mixture was immediately passed through a Millipore filter (pore size, 0.45 μm), and washed twice with the same buffer. The radioactivity on the filter was counted.

For the kinetic study, the tetracycline concentration was varied from 10 to 80 μM in the presence of 800 μM $CoCl_2$.

3. Results

3.1. Inhibition of Tet(K)-mediated [^{3}H]tetracycline uptake by ISO vesicles with Ro 07-3149

We examined the inhibitory activity of Ro 07-3149 toward Tet(K)-mediated tetracycline uptake by ISO membrane vesicles prepared from *E. coli* W3104/pTZ1252. Fig. 2A shows the time course of [^{3}H]tetracycline uptake by Tet(K)-containing ISO membrane vesicles in the presence or absence of Ro 07-3149. This experiment is a representative one of three independent experiments. Tetracycline uptake was almost completely inhibited in the presence of 324 μM Ro 07-3149, while in the presence of 162 μM Ro 07-3149, about one-third of the uptake activity remained (Fig. 2A). The degree of inhibition was dependent on the concentration of Ro 07-3149 relative to the total amount of membrane protein, as shown in Fig. 2C. The IC_{50} value was 0.25 μmol/mg membrane protein. The relative concentration of Ro 07-3149 as to total membrane protein reflects the concentration of this compound inside the membrane because of the high hydrophobicity of Ro 07-3149.

3.2. Effects of Ro 07-3149 on ΔpH-dependent [^{14}C]methylamine accumulation by ISO vesicles

Tet(K) proteins mediate the electrically neutral metal-tetracycline/H^+ antiport similar to Tet(B) [8]. The Tet(K)-mediated tetracycline uptake by ISO vesicles was more than 80% inhibited by the presence of 3.5 μg/ml nigericin, whereas it was hardly affected by valinomycin (unpublished observation), indicating that the driving force for the Tet(K)-mediated tetracycline transport is ΔpH instead of Δψ. Therefore, in order to determine whether the inhibition of the Tet(K)-mediated tetracycline uptake by ISO vesicles with Ro 07-3149 is due to disruption of ΔpH, we examined the effect of Ro 07-3149 on ΔpH-dependent [^{14}C]methylamine accumulation by ISO vesicles under the same conditions used for measurement of tetracycline uptake. Since a neutral form of weak bases such as methylamine can penetrate the lipid bilayer and the charged form will accumulate on the acidic side of membrane vesicles, the respiration-driven accumulation of methylamine by ISO vesicles is usually used for an indicator of ΔpH [17]. In contrast to tetracycline uptake, ΔpH-dependent [^{14}C]methylamine uptake was not inhibited by either 165 μM or 325 μM Ro 07-3149 (Fig. 2B). Dose dependence of [^{14}C]methylamine accumulation on Ro 07-3149 showed that the accumulation was not significantly affected by Ro 07-3149 up to 650 μM of the inhibitor (Fig. 2C), clearly indicating that the inhibitory effect of Ro 07-3149 is not due to the uncoupling effect of the inhibitor.

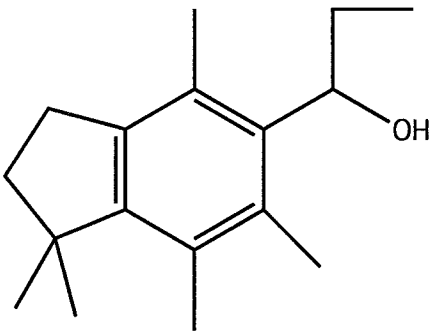

Fig. 1. Chemical structure of 1,1-dimethyl-5-(1-hydroxypropyl)-4,6,7-trimethyl-indan (Ro 07-3149).

3.3. Effect of Ro 07-3149 on proton translocation across ISO vesicles

In order to confirm that the inhibitory effect of Ro 07-3149 on Tet(K) function was not due to the uncoupling effect, we next examined the effect of Ro 07-3149 on respiration-dependent proton translocation and Tet(K)-mediated tetracycline-dependent proton translocation using quinacrine fluorescence. When NADH was added, quinacrine fluorescence was quenched due to the respiration-dependent proton influx into the vesicles (Fig. 3A). When tetracycline was added to the energized vesicles, Tet(K)-mediated tetracycline/H^+ antiport partially collapsed the ΔpH across the membrane resulting in an increase in quinacrine fluorescence (Fig. 3A). When 41 μM (0.81 μmol/mg membrane protein) Ro 07-3149 was added prior to the addition of NADH, the Tet(K)-mediated tetracycline-dependent fluorescence increase was almost completely inhibited, without the NADH-induced fluorescence quenching being affected (Fig. 3B), clearly confirming that the inhibitory action of Ro 07-3149 on the Tet(K) function is not due to the uncoupling effect. The inhibition of the tetracycline-dependent proton translocation was also dependent on the concentration of Ro 07-3149 (Fig. 4). The IC_{50} value was 0.43 μmol/mg membrane protein, being similar to that of [^{3}H]tetracycline uptake.

When a high concentration (8.1 μmol/mg membrane protein) of Ro 07-3149 was added before the membrane vesicles were energized with NADH, the fluorescence quenching was significantly decreased to about 46% of that in the absence of Ro 07-3149, reflecting the de-energization caused by Ro 07-3149 at a high concentration. The dose dependence of NADH-dependent ΔpH formation on Ro 07-3149 in a high concentration range is shown in Fig. 4 (inset). The IC_{50} value for NADH-dependent ΔpH formation was 7.3 μmol/mg membrane protein, which was 17 and 33 times higher than those for Tet(K)-mediated proton translocation and tetracycline uptake, respectively. F_0F_1-ATPase-mediated ATP-dependent

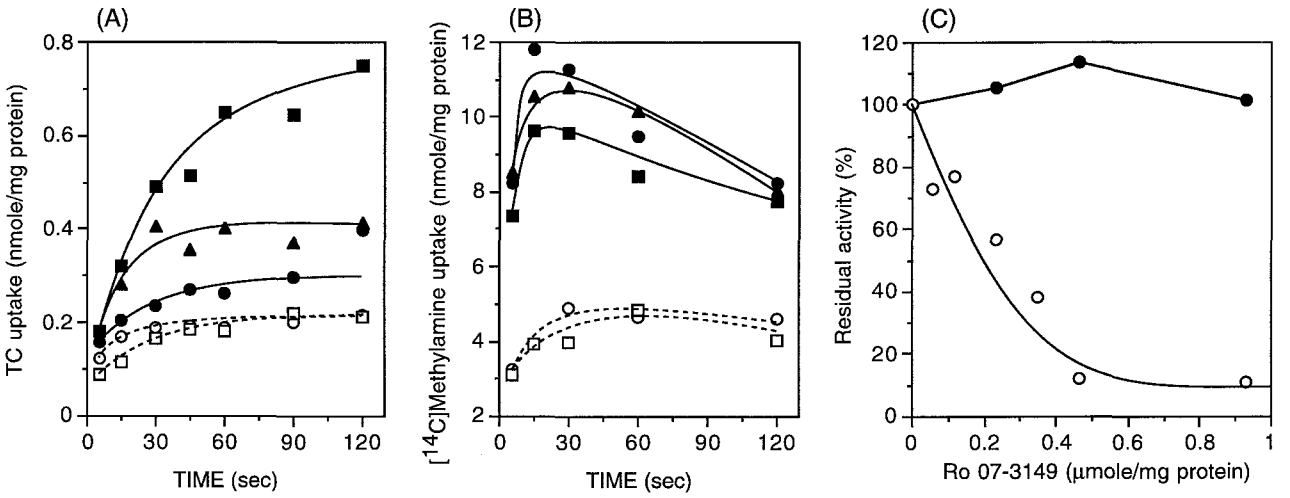

Fig. 2. Effects of Ro 07-3149 on the Tet(K)-mediated [^{3}H]tetracycline uptake and the ΔpH-dependent accumulation of [^{14}C]methylamine by ISO vesicles prepared from *E. coli* W3104/pTZ1252 cells. A: Time course of [^{3}H]tetracycline uptake by ISO vesicles in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$. B: Time course of [^{14}C]methylamine accumulation by ISO vesicles in the presence of 2 mM [^{14}C]methylamine. Symbols in (A) and (B): ■, no Ro 07-3149 with 2.5 mM NADH; ▲, 162 μM Ro 07-3149 with 2.5 mM NADH; ●, 325 μM Ro 07-3149 with 2.5 mM NADH; □, no Ro 07-3149 without NADH; ○, 325 μM Ro 07-3149 without NADH. C: Dose dependence of the residual Tet(K)-mediated [^{3}H]tetracycline uptake (○) and the residual ΔpH-dependent [^{14}C]methylamine accumulation (●) on Ro 07-3149. The net residual tetracycline uptake and methylamine accumulation were measured at 30 s after the addition of labeled compounds and indicated as a percentage relative to that in the absence of Ro 07-3149. The concentration of Ro 07-3149 was normalized as to the total membrane protein concentration (0.7 mg/ml).

ΔpH formation was also inhibited by Ro 07-3149 (data not shown), indicating that Ro 07-3149 acts as a weak uncoupler at a high concentration. However, the uncoupling activity of Ro 07-3149 was too weak to be detected prior to the inhibition of Tet(K)-mediated tetracycline transport. Therefore, Ro 07-3149 was concluded to be a Tet(K)-specific inhibitor.

3.4. *Kinetics of the inhibition of tetracycline uptake by Ro 07-3149*

The initial rate of [^{3}H]tetracycline uptake by Tet(K)-containing ISO vesicles was measured in the presence of various concentrations of [^{3}H]tetracycline and Ro 07-3149. In the absence of Ro 07-3149, the apparent K_m value for tetracycline uptake was 28 μM, which is in good agreement with the previously reported value [8]. As shown by Lineweaver-Burk plots (Fig. 5), Ro 07-3149 reduced the V_{max} value without affecting the K_m value, indicating that it acts on a non-competitive inhibitor. The K_i value was calculated to be 230 μM (0.32 μmol/mg protein) by non-linear regression, which was similar to the IC_{50} value for the inhibition of [^{3}H]tetracycline uptake shown in Fig. 2.

4. Discussion

Two different kinds of tetracycline efflux pump inhibitors have hitherto been reported [18,19]. One of them comprises a group of tetracycline derivatives [18] which inhibit the efflux pump-mediated tetracycline uptake by ISO vesicles, probably in a competitive manner, although a kinetic experiment was not performed. The other kind is nocardamine which is not a tetracycline derivative. The latter was identified as an antagonist of the tetracycline efflux pump in intact cells. However, none of them was examined as to whether they indirectly act through their uncoupling action.

Tet(K) mediates metal-tetracycline/H^+ antiport similar to Tet(B) [8]. The main driving force for Tet(K) is ΔpH because nigericin inhibited the Tet(K)-mediated tetracycline transport but valinomycin did not. This behavior of Tet(K) is similar to that of Tet(B) [20] and different from the electrogenic nature of chromosomally encoded Tet(L) in *Bacillus subtilis* reported by Guffanti and Krulwich [21]. It is clear that Tet(K) and the chromosomally encoded Tet(L) have different characteristics because unlike Tet(L), Tet(K) does not mediate Na^+/H^+ antiport (unpublished observation). In this manuscript, ΔpH was monitored as a driving force for Tet(K)-mediated tetracycline transport. Ro 07-3149 completely inhibited the Tet(K)-mediated [^{3}H]tetracycline uptake by ISO vesicles at the concentration in which ΔpH was not affected when monitored with [^{14}C]methylamine accumulation. In addition, the IC_{50} value of Ro 07-3149 for ΔpH disruption monitored with quinacrine

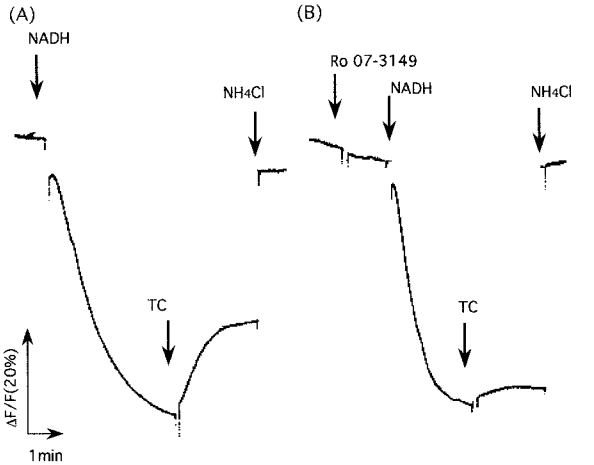

Fig. 3. Proton translocation across ISO membrane vesicles prepared from *E. coli* W3104/pTZ1252. Proton translocation was measured by monitoring the fluorescence quenching of quinacrine. Arrows indicate the times of the addition of the indicated reagents. The final concentrations of Ro 07-3149, NADH, tetracycline (TC), and NH_4Cl were 41, 625, 20, and 10 μM, respectively.

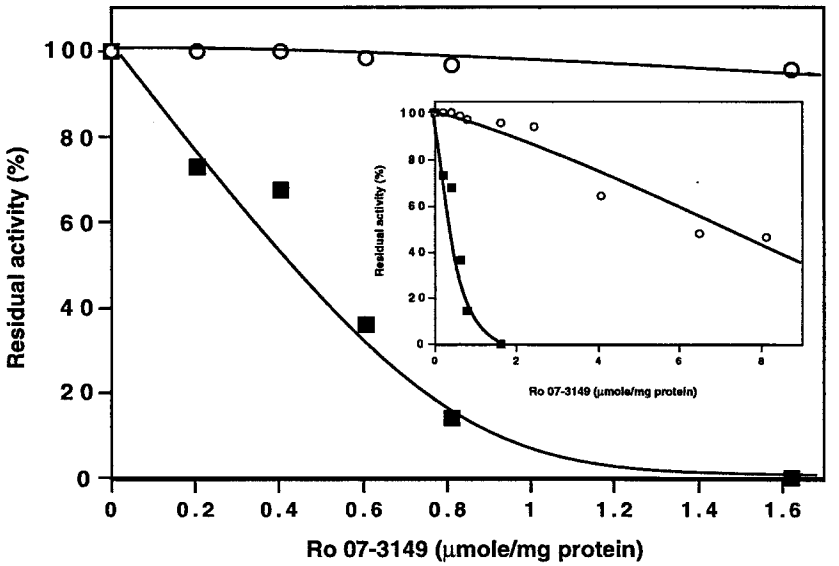

Fig. 4. The effect of Ro 07-3149 on the *tet*(K)-mediated proton translocation activity (■), and NADH-dependent ΔpH formation activity (○) of Tet(K)-containing ISO vesicles, as measured as the fluorescence quenching of quinacrine. The concentration of Ro 07-3149 was normalized as to the total membrane protein concentration (0.05 mg/ml). Inset: results in the presence of a high concentration of Ro 07-3149.

fluorescence change was at least 17 times higher than that for inhibition of tetracycline-dependent proton translocation mediated by Tet(K). Therefore, it can be concluded that the inhibitory action of Ro 07-3149 on Tet(K) function is not due to the uncoupling effect.

Minimum inhibitory concentration (MIC) of tetracycline and Ro 07-3149 against *S. aureus* cells carrying *tet*(K) gene was 64 and 32 μg/ml, respectively. In the presence of 8 μg/ml of Ro 07-3149, the MIC value of tetracycline was reduced to 32 μg/ml. In contrast, Ro 07-3149 showed neither antibacterial activity nor synergistic effect for tetracycline action against *E. coli* cells carrying pTZ1252, probably due to the low permeability of Ro 07-3149 across the outer membrane. Anyway, Ro 07-3149 is a first compound established as a non-competitive inhibitor of Tet(K) clearly not due to the uncoupling action.

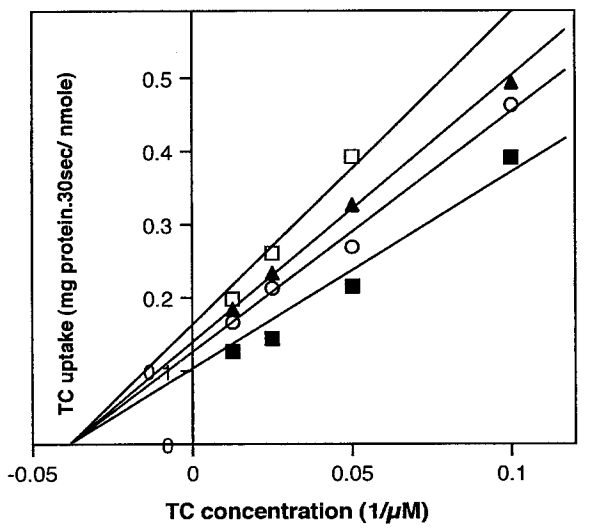

Fig. 5. Lineweaver-Burk plots of the initial rate (15 s) of [^{3}H]tetracycline uptake by Tet(K)-containing ISO vesicles against tetracycline concentration. ■, no Ro 07-3149; ○, 81 μM (0.12 μmol/mg protein); ▲, 162 μM (0.23 μmol/mg protein); □, 244 μM (0.35 μmol/mg protein) of Ro 07-3149.

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