Specific Action Mechanisms of YM133 against Macrolide-Lincosamide-Streptogramin Type B Constitutive Resistant *Staphylococcus aureus*

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Abstract

4”-*O*-(4-methoxyphenyl)acetyltyosin (YM133) is an acyl derivative of tylosin (TS), and shows results of antibacterial activity against macrolide-lincosamide-streptogramin type B (MLS_B) constitutive resistant *Staphylococcus aureus*. The minimum inhibitory concentrations (MICs) for MLS_B constitutive resistant strain ISP217 were 12.5μg/ml for YM133 and puromycin (PM), whereas the MICs were more than 800μg/ml for erythromycin (EM), josamycin (JM), TS, lincomycin (LCM), and mikamycin type B. The MICs for PM were 12.5μg/ml for MLS_B sensitive strain NCTC8325, MLS_B inducible resistant strain ISP447, and MLS_B constitutive resistant strain ISP217. The 50% growth inhibition doses (ID_{50}) of YM133 and TS against MLS_B sensitive strain and MLS_B inducible resistant stain were both approximately 0.12μg/ml, indicating that these antibiotics have the same effect towards these strains. The ID_{50} of YM133 against MLS_B constitutive resistant strain was 29.9μg/ml, which was approximately 36 times larger in its effect compared to TS. The results from the Drug-probit relationship showed that the degrees of the slopes concerning YM133 against ISP217 and PM against ISP217 were identical, suggesting YM133 and PM having the same action mechanism against strain ISP217. The slopes concerning TS against NCTC8325 and TS against ISP217 share almost identical degrees in their slopes, indicating that the action mechanisms for TS against those two strains are the same.

1. Introduction

It is commonly known that multiple drug resistant bacteria are reproduced as a result of overuse and/or repeated use of antibiotics. Clinically isolated *S. aureus* resistance strains are one example of multiple drug resistant bacteria and are found to exhibit inducible-resistance and constitutive- resistance to not only macrolide (Mac) antibiotics, but also to lincosamide and streptogramin type B antibiotic (MLS_B) (1,2,3,4). Therefore, it should be considered as a social priority to rationally develop effective antimicrobial drugs which could overcome the resistant mechanism of bacteria, as well as search for new antibiotics.

Since 1968, Nakajima et al. have been contributing to the research of macrolide’s mode of action and drug resistance mechanism using Mac resistant *S. aureus*, for the purpose of improving the effectiveness of Mac antibiotics. In recent years, several new semisynthetic derivatives of Mac have been developed by chemical modification and it has been reported to be effective against MLS_B resistance *S. aureus* in both clinical treatments and in vitro. In 1987, Takeuchi et al.(5) reported the findings which showed that the various derivatives of tylosin (TS) in 16-membered ring Mac had an antibacterial activity against MLS_B resistance *S. aureus.*

The present study aimed to examine the properties of action mechanism of YM133, that is, the derivatives of TS. The following *S. aureus* were used: Mac susceptibility strain, MLS_B inducible resistance strain, and MLS_B constitutive resistance strain. The current study also aimed to describe the new antimicrobial activity against MLS_B constitutive resistance strain, by addressing the findings of the MICs of YM133, and the effect of YM133 on the growth of MLS_B constitutive resistance *S. aureus.*

2. Materials and Methods

2.1 Strain

S. aureus NCTC8325 was used as a Mac sensitive strain. MLS_B inducible-resistant *S. aureus* ISP447 and MLS_B constitutive-resistant *S. aureus* ISP217 kindly donated by Dr. P. A. Pattee, were also used (1,6).

2.2 Media and reagents

As media for MIC measurements, trypticase soy broth (TSB) and Muller Hinton Ⅱ Agar (MH Ⅱ) obtained from Beckton Dickinson Company (BBL) were used. As media for resistance induction ability

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measurements, the following media were used; Heart Infusion Agar (HIA) obtained from BBL; Inducible Base (Ind. B) agar medium containing polypeptone 5 g, yeast extract 5 g, monopotassium phosphate 1 g, glucose 2 g, and agar 20 g per 1 L of purified water; and Inducible Soft (Ind. S) agar medium which had the same composition to Ind. B except that 5 g of agar was used instead of 20 g (7). For drug susceptibility measurements by liquid culture method, TSB and medium H (pH 7.6) containing pancreatic digest of gelatin 5 g, beef extract 3 g, yeast extract 2 g, and HEPES 11.8 g per 1 l of purified water, were used (8). The reagent of HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid) from Nacalai Tesque Ltd. was used. All other reagents were purchased.

2.3 Antibiotics

Antibiotics used in this study were; erythromycin (EM) and lincomycin (LCM) obtained from Japan Upjohn; tylosin (TS) and puromycin (PM) obtained from SIGMA; josamycin (JM) from Meiji Seika Ltd.; 4"-*O*-(4-methoxyphenyl) acetyltylosin (YM133) obtained from Mercian Central Institute Ltd.; and mikamycin-B (MKM-B) was purified from a mikamycin mixture (obtained from Banyu Pharmaceutical Ltd.) using the HPLC method.

2.4 Minimum Inhibitory Concentrations (MICs)

Determination of MICs was achieved by a serial two-hold dilution of antibiotics in MH Ⅱ agar. Each plate containing the drug was seeded by Micro Inoculater (MI type 1; Sakuma factory) with an inoculum size of about 10^4 cfu/ml of overnight-cultured strains. The MIC was defined as being the lowest concentration of drug that would inhibit visible growth after incubation at 37℃ for 18 to 24 hr (9).

2.5 Effect of resistance induction of erythromycin

The inducibility of resistance to Mac antibiotics by EM was assayed by the agar disc diffusion test: Ind. S (8 ml) including bacteria (10^6 cells) was poured onto the previous Ind. B (40 ml). After the Ind. S layer was hardened, several paper discs of 8 mm in diameter with antibiotics were separately placed on the agar surface. The plates were then incubated at 37℃ for 18 to 24 hours (7,9,10,11).

2.6 Growth inhibition An overnight culture of *S. aureus* in BHI broth was diluted 1:9 with a fresh culture medium H (pH 7.6). After 2-hour incubation at 37℃, the culture was further diluted to an optical density (OD) of about 0.1 at 610 nm in an L-shaped tube (L-tube) containing medium H plus antibiotic, (total volume of 10 ml) incubated at 37℃ for one hour with shaking, and chilled rapidly to 0℃. The chilled cells were harvested by centrifugation (Hitachi, HIMAC Centrifuge CR5B), at 4,000×g for 10 minutes at 4℃ and washed twice with two 5-ml portions of cold 0.9% NaCl solution. The washed cells were suspended in 10 ml of antibiotic- free medium H, and adjusted to an OD of about 0.1 at 610 nm. The cell suspension in an L- tube was further incubated at 37℃ with shaking, and the growth of the suspension was determined by monitoring OD at 610 nm (12).

The 50% inhibitory dose (ID_{50}) was determined as follows: overnight cultures of *S. aureus* were added to a fresh medium H and incubated to log phase. After cells were collected by centrifugation and were suspended in fresh medium H, mixed with a serial two-third dilution of antibiotics, and incubated at 37℃ with gentle shaking. The ID_{50} of antibiotics was determined by probit analysis (13).

3. Results

3.1 Antimicrobial susceptibility of *Staphylococcus aureus*

The MICs for a susceptible strain NCTC8325 and MLS_B inducible resistant strain ISP447 (prior to resistance induction) were 6.25μg/ml or less for EM, JM, TS, YM133, LCM and MKM-B (Table 1). The MICs for MLS_B constitutive resistant strain ISP217 were more than 800μg/ml for all antibiotics tested, except for YM133 and PM (12.5μg/ml, 12.5μg/ml respectively) (Table 1). Moreover, the MICs for PM were 12.5μg/ml for all three strains (Table 1).

Table 1 MICs of MLS_B and PM antibiotics against *Staphylococcus aureus*

Strain	MIC (μg/ml)						
	EM	JM	TS	YM133	LCM	MKM-B	PM
NCTC8325	0.20	0.39	0.78	1.56	0.39	3.13	12.5
ISP447	6.25	0.78	0.39	3.13	3.13	6.25	12.5
ISP217	>800	>800	>800	12.5	>800	>800	12.5

Abbreviations: EM, erythromycin; JM, josamycin; TS, tylosin; YM133, 4"-*O*-(4-methoxyphenyl) acetyltylosin; LCM, lincomycin; MKM-B, mikamycin B

3.2 The effect of resistance induction with erythromycin

As EM diffused into agar medium, strain ISP447 formed 'D-shaped' inhibition zones due to the induction of resistance to JM, YM133, and TS (Fig. 1). In contrast, for strain ISP217, no zone was formed due to the induction of resistance to JM and TS, whereas 'round-shaped' inhibition zone was formed for YM133.

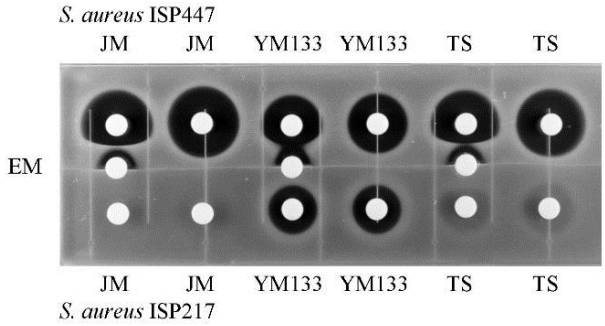

Fig. 1 The effect of erythromycin on the *S. aureus* ISP447 and *S. aureus* ISP217 against macrolide antibiotics

EM, 10 μ g/disc; antibiotics, 25 μ g/disc
Abbreviations: JM, josamysin; YM133, 4"-*O*-(4-methoxyphenyl) acetyltylosin; TS, tylosin

3.3 The effects of YM133, tylosin, and puromycin on the growth of *Staphylococcus aureus* NCTC 8325

Figure 2 (A) shows that the growth rate of NCTC 8325 did not differ between control (0.0μg/ml) and 0.23μg/ml of YM133 up to two hours. After two hours, OD stopped rising at the point of 0.5, showing inhibition of growth for YM133 concentration of 0.23μg/ml. In contrast, when TS was used, the growth inhibition occurred in proportion to its concentration (Fig. 2B). When PM was used with a concentration of 2.70μg/ml, the growth inhibition curb resembled the curb for YM133 with the concentration of 0.23μg/ml, showing growth inhibition after 2 hours (Fig. 2C).

3.4 The concentration-probit relationship and 50% growth inhibitory doses (ID_{50}) of antibiotics against *Staphylococcus aureus*.

The concentration-probit (10) relationship was analyzed using the least squares method.

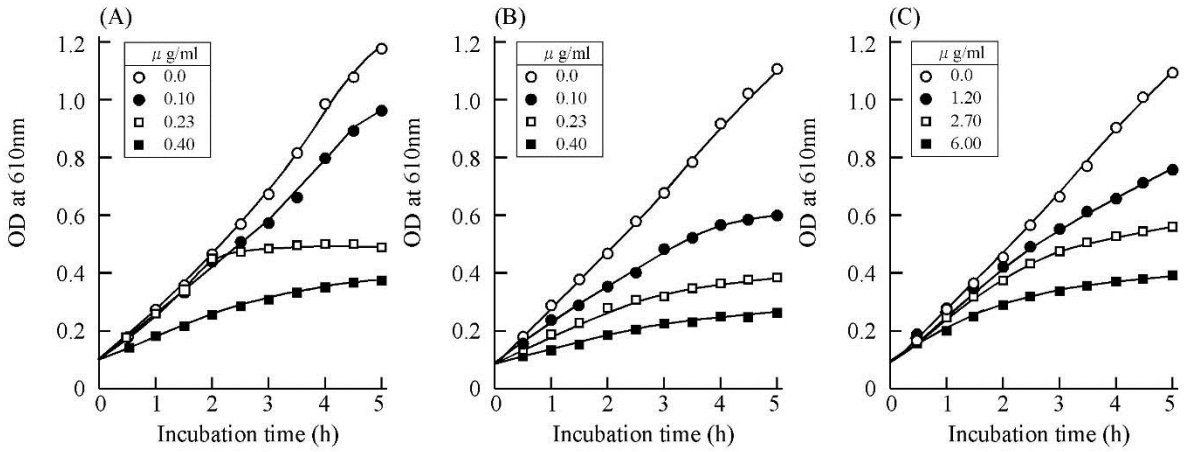

Fig. 2 The effect of YM133 (A), tylosin (B), and puromycin (C) on the growth of *S. aureus* NCTC8325

The relationship shows that the degrees of the slope for YM133 against strain ISP217, PM against strain ISP217, and PM against NCTC8325 were identical (Fig. 3). The results also show that the degrees of the slopes for TS against strain ISP217 and TS against NCTC8325 were almost identical (Fig. 3). The degree of the slope was found to be sharper for YM133 against NCTC8325 compared to other slopes (Fig. 3).

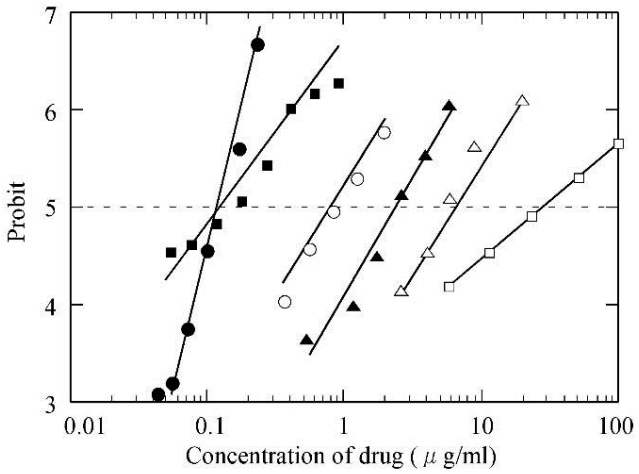

Fig. 3 The effects of YM133, tylosin, and puromycin on the growth of *S. aureus*.

Symbols: *S. aureus* NCTC8325 run with YM133 (●), TS (■),and PM (▲). *S. aureus* ISP217 run with YM133 (○), PM (△), and TS (□).

To compare susceptibility of YM133, TS, and PM quantitatively, 50% growth inhibitory doses were calculated by liquid culture method. The ID_{50} values of YM133 and TS did not show a great difference between strain NCTC8325 and ISP447 (ranging from 0.12 to 0.13μg/ml). In contrast, the ID_{50} values of YM133 and TS for ISP217 were 0.82μg/ml and 29.9μg/ml respectively (Table 2). The ID_{50} values of YM133 and TS showed that these antibiotics had the same effects to MLS_B sensitive-, resistant strain and MLS_B inducible-resistant strain, whereas YM133 had the effect to MLS_B resistant strain which was an approximately 36 times larger effect to MLS_B-constitutive resistant strain compared to TS.

Table 2 The fifty per cent growth inhibitory doses (ID_{50}) of YM133, tylosin, and puromycin against *S. aureus*

Strain	ID_{50} (μ g/ml)		
	YM133	TS	PM
NCTC8325	0.12	0.12	2.51
ISP447	0.13	0.12	2.67
ISP217	0.82	29.90	5.71

Abbreviations: YM133, 4"-*O*-(4-methoxyphenyl) acetyl-tylosin; TS, tylosin; PM, puromycin

4. Discussion

Mac is known to be bound to ribosome 50S subunit, bringing along the change in conformation of ribosome. As a result of this, protein synthesis is inhibited. From the process of cell-free protein synthesis and its inhibitory mechanism, it can be said that the translocation of peptidyl-transfer RNA and peptidyltransferase are inhibited (14,15,16,17). In contrast, the resistance mechanism of MLS_B inducible resistant strain ISP447 and MLS_B constitutive resistant strain IPS217 produce ribosome methylase. As a result of this, the mechanism that is due to N^6, N^6-dimethylation of a specific adenine residue in 23S ribosomal RNA, renders ribosomes unable to bind to EM, as the resistance was due to alteration of ribosome (4,18,19).

However, the current research indicated that unlike new and old Mac, YM133 showed an antimicrobial activity in not only MLS_B-sensitive *S. aureus* NCTC8325 but also in Mac constitutive MLS_B-resistant *S. aureus* ISP217. Being consistent with the findings of Takeuchi et al. (5) and Terasawa et al. (20), the MICs for both YM133 and PM against ISP217 strain were 12.5 mg/ml (Table 1), indicating susceptibility. Furthermore, in examining the induction of resistance using EM against inducible MLS_B-resistant *S. aureus* ISP447, YM133 formed 'D-shaped' growth inhibitory zone, showing the same effect to the already-known Mac (Fig. 1). On the other hand, a completely different effect to the already-known Mac was found for the experiment using *S. aureus* ISP217, illustrating that YM133 formed 'round-shaped' growth inhibitory zone with a presence of EM, instead of 'D-shaped'. The results

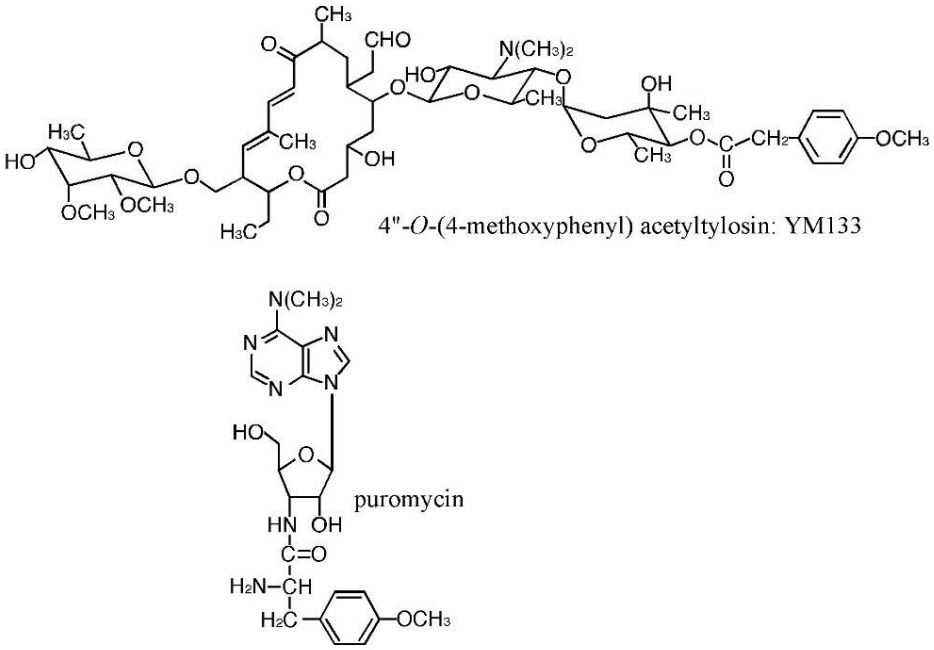

Fig. 4 Chemical structures of 4"-*O*-(4-methoxyphenyl) acetyltylosin and puromycin

strongly suggest that YM133 has a resistance against the resistance mechanism of Mac which occurred as a result of dimethylation of ribosomes.

In the experiment of the effects of YM133 against the growth of *S. aureus* NCTC8325, the growth inhibition was only observed after 2 hours and forward. Generally, it is known that Mac has the ability to inhibit the growth of bacteria in proportion to its drug concentration. The authors noticed, by observing YM133 inhibiting the growth of MLS_B constitutive resistant strain ISP217, that YM133 and PM had great similarity in their chemical structures, both having methoxyphenyl (Fig. 4).

The results from Drug-Probit relationship illustrated that the degrees of the slopes concerning YM133 against strain ISP217 and PM against strain ISP217 were identical (Fig. 3). Therefore, it can be suggested that YM133 and PM have the same action mechanism against strain ISP217 (Fig. 3). Moreover, TS against strain ISP217 and TS against strain NCTC8325 share almost identical degrees in their slopes, indicating that the action mechanisms for TS against those two strains are the same (Fig. 3). The finding also suggests that the effect of YM133 against strain NCTC8325 is the additive action of TS and PM.

5. Acknowledgements

We express our gratitude to Mercian central institute Ltd. and Dr. Takeo Yoshioka who distributes YM133. We thank Sayaka Endo (who was belonging to the University of Otago in New Zealand) for her helpful assistance.

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