STUDIES ON THE EFFECTS OF 3-ACETYL-4''-ISOVALERYLTYLOSIN AGAINST MULTIPLE-DRUG RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

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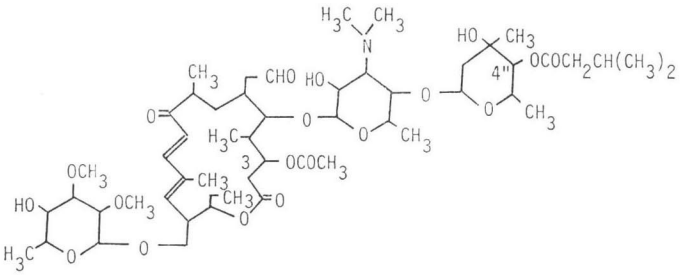
The macrolide-resistance of multiple-drug resistant strains of *Staphylococcus aureus* was divided into two types; the decreased sensitivity of ribosomes (type I) and the decreased uptake (type II). Both types were resistant to erythromycin, tylosin and 3-acetyltylosin, and their resistance was not inducible. 3-Acetyl-4''-isovaleryltylosin inhibited the growth of both types. Protein synthesis on ribosomes of type I *in vitro* (*S. aureus* MS-9610) was inhibited by 3-acetyl-4''-isovaleryltylosin, but little or no inhibition was seen with either tylosin or 3-acetyltylosin. Ribosomes of type II *in vitro* (*S. aureus* MS-8710) were sensitive to all macrolides. 3-Acetyl-4''-isovaleryltylosin accumulated about twice as much as 3-acetyltylosin in intact cells of type II.

Macrolide-resistant staphylococci have increased among clinical isolates, and we have endeavored to find derivatives which inhibit the growth of these resistant strains. We found that tylosin, one of the 16-membered macrolide antibiotics, inhibited resistant strains at about twice lower concentration than erythromycin, acetylspiramycin, josamycin and midecamycin. Therefore, we chose tylosin as the starting material to prepare derivatives with an improved activity.

It is well known that C-20 aldehyde group of tylosin is important for its activity and can not be modified without the reduction of the antibacterial activity. The hydroxy groups at C-3, C-2', C-3'', C-4'' and C-4''' are the candidates for possible modification. As previously reported[1,2], the introduction of an acyl group at 4''-OH resulted in moderately improved antimicrobial activity against resistant strains but not the acylation of 3-OH, 2'-OH or 4'''-OH. Among 4''-acyl derivatives, 4''-isovaleryltylosin had the strongest activity. Furthermore, 3-acetyl-4''-isovaleryltylosin (Fig. 1), which was easily obtained by microbial acylation of tylosin, showed a strong activity against macrolide-resistant strains as well as 4''-isovaleryltylosin. We studied the mechanism of the effect of these tylosin derivatives against resistant strains, that is, we examined whether these derivatives are taken up into resistant cells more efficiently than the parent compound and whether these derivatives inhibit protein synthesis on ribosomes of resistant strains. We also examined whether tylosin and its derivatives are inactivated by cell-homogenates of resistant strains.

Fig. 1. Chemical structure of 3-acetyl-4″-isovaleryltylosin.



Materials and Methods

Materials

L-[U-^{14}C]Phenylalanine (424 mCi/mmole), [2-^{14}C]thymidine (56.6 mCi/mmole), [2-^{14}C]uridine (57 mCi/mmole), L-[U-^{14}C]leucine (351 mCi/mmole) and [1-^{14}C]acetyl-coenzyme A (54 mCi/mmole) were the products of Radiochemical Centre, Amersham, England; polyuridylic acid (poly U) potassium salt, tRNA from *Escherichia coli* MRE600 (RNase I$^-$) and deoxyribonuclease I were the products of Boehringer Mannheim GmbH; ATP, GTP, phosphoenolpyruvate (PEP), dithiothreitol (DTT) and pyruvate kinase were the products of Sigma Chemical Co., Ltd. All other chemicals were of commerical sources and of the highest grade available.

3-^{14}C-Acetyltylosin and 3-^{14}C-acetyl-4″-isovaleryltylosin were prepared by enzymatic acetylation of tylosin and 4″-isovaleryltylosin with [(1-^{14}C)acetyl]-coenzyme A as described previously[3]. The specific activities of 3-[(1-^{14}C)-acetyl]-tylosin and 3-[(1-^{14}C)-acetyl]-4″-isovaleryltylosin were 7.63 and 8.34 mCi/mmole, respectively. They are abbreviated as 3-^{14}C-acetyltylosin and 3-^{14}C-acetyl-4″-isovaleryltylosin.

Bacterial strains and culture conditions

The multiple-drug resistant strains of *Staphylococcus aureus* clinically isolated were supplied by Prof. S. MITSUHASHI, Gunma University, Maebashi, Japan. Brain heart infusion broth (Difco) was used as the culture medium. A 2-ml static culture, overnight at 37°C, was inoculated to a flask containing 100 ml fresh medium and shaken at 37°C until the optical density at 660 nm reached 0.2~0.3. These cells were used for the experiments.

Preparation of sub-cellular fractions and washed 70S-ribosomes

Cells of *S. aureus* or *E. coli* Q-13 in the exponential phase (0.2 OD_{660}) were collected by pouring on crushed ice and washed twice by centrifugation and suspension in a cold Medium A (0.01 M Tris-HCl, pH 7.8, containing 0.01 M $Mg(OAc)_2$, 0.06 M KCl and 0.006 M 2-mercaptoethanol). The cells (10 g in wet weight obtained from 5 liters culture) thus obtained were disrupted by hand-grinding with 15 g of alumina or sea sand for 5 minutes under the ice-chilled condition. To this mixture, Medium A, 20 ml, was added and centrifuged at 12,000×g for 30 minutes and the supernatant, 19 ml, was obtained. Deoxyribonuclease I was added to the supernatant at a final concentration of 5 μg/ml and the solution was kept standing for 30 minutes in an ice bath. After centrifugation at 12,000×g for 30 minutes, the supernatant (18 ml) was taken, incubated at 34°C for 30 minutes to reduce endogeneous protein synthesis and centrifuged at 30,000×g for 30 minutes. The supernatant fluid (S-30 fraction) was dialyzed overnight against 2 liters of cold Medium A and centrifuged at 100,000×g for 2 hours to collect ribosomes. The supernatant fluid (S-100 fraction) was removed from the precipitate (ribosomes) by aspiration. The ribosomes were washed twice with 10 ml of cold Medium A supplemented with 1 M NH_4Cl by suspension and centrifugation at 100,000×g for 2 hours. The washed ribosomes were suspended in 0.01 M Tris-HCl, pH 7.8 supplemented with 0.03 M NH_4Cl, 0.01 M $Mg(OAc)_2$ and 0.006 M 2-mercaptoethanol (washed 70S-ribosomes, 250 OD_{260}, 8.5 ml). These fractions (S-30, S-100, washed 70S-ribosomes) were stored in small portions at −80°C until use.

In vitro protein synthesis

In vitro protein synthesis in a system containing washed 70S-ribosomes from *S. aureus* and S-100 from *E. coli* Q-13 (RNase I$^-$) was conducted as reported by MAO[4] with minor modifications as follows. A reaction mixture (0.1 ml) contained 50 mM Tris-HCl, pH 7.8, 16 mM $Mg(OAc)_2$, 160 mM NH_4Cl, 3 mM ATP, 0.2 mM GTP, 2 mM PEP, 5 μg of pyruvate kinase, 2 mM DTT, 0.08 μCi of L-[U-^{14}C]-phenylalanine (424 mCi/mmole), 0.01 mM each of 19 amino acids, 50 μg of tRNA from *E. coli* MRE600, 70 μg protein of the S-100 fraction from *E. coli* Q-13, 2 OD_{260} units of washed 70S-ribosomes from *S. aureus* and a desired amount of an antibiotic. The reaction was started by adding 10 μg of poly(U) and proceeded at 37°C for 30 minutes. After incubation, 90-μl portions of the assay mixture were withdrawn and placed on 2.4 cm filter paper discs of Whatman 3 MM paper. The discs were immersed in 10% trichloroacetic acid (TCA) (5 ml/disc), and processed as reported[4] and radioactivities in hot TCA insoluble materials were measured by a toluene-scintillation method.

Determination of cellular macromolecule synthesis

Incorporation of ^{14}C-labeled precursors into cellular macromolecules was determined according to the method of BYFIELD *et al.*[5] In brief, an experiment was made up of 3 sets of 2 tubes, each containing 0.9 ml of the culture of *S. aureus* at an exponential growth phase (0.2 OD_{660}) and 0.1 mM of deoxyadenosine. Of each set, the first tube was mixed with 50 μl of methanol solution of 3-acetyl-4''-isovaleryltylosin of either 25 or 50 μg/ml at a final concentration, and the second tube simply methanol (control). They were incubated at 37°C for three minutes and mixed with any of the following radioactive precursors; 0.5 μCi of [2-^{14}C]thymidine (56.6 mCi/mmole) for Set 1, 0.5 μCi of [2-^{14}C]uridine (57 mCi/mmole) for Set 2, and 0.5 μCi of L-[U-^{14}C]leucine (351 mCi/mmole) for Set 3. Incubation was resumed and at indicated time, 0.1 ml sample was taken and placed onto a Whatman 3 MM filter paper disc (2.4 cm diameter). The wet discs were immersed in ice-cold 10% TCA and processed[5]. Radioactivity in cold TCA insoluble materials was determined by a toluene scintillation method.

Uptake of ^{14}C-labeled tylosin derivatives

Uptake of ^{14}C-labeled tylosin derivatives by intact cells from the medium was measured by filtering a 0.25-ml sample of a bacterial suspension in the medium through a membrane filter (HA, 0.45 μm; Millipore Corp.) according to the method of MCMURRY *et al.*[6] with minor modifications as follows. Cells of *S. aureus* at the growth stage were harvested by centrifugation and suspended in 0.05 M phosphate buffer, pH 7.0, to give a cell density of about 3 OD_{660}. After preincubation at 37°C for 10 minutes, either 3.05 μg of 3-^{14}C-acetyltylosin (7.63 mCi/mmole) or 2.50 μg of 3-^{14}C-acetyl-4''-isovaleryltylosin (8.34 mCi/mmole) was added to 1.5 ml of the cell suspension. After incubation at the indicated time, 0.25-ml portion of the cell suspension was withdrawn, diluted with 5 ml of ice-cold 0.05 M phosphate buffer, pH 7.0, containing the corresponding non-radioactive tylosin derivative at 50 μg/ml. The cells were filtered through a membrane filter by gentle suction, washed twice with 5 ml of a cold phosphate buffer containing a non-radioactive tylosin derivative described above. The radioactivity of the membrane filter containing the cells was determined by a liquid-scintillation method.

The ability of unlabeled tylosin and tylosin derivatives to inhibit the uptake of 3-^{14}C-acetyltylosin by intact cells was determined as follows. Prior to the addition of 3-^{14}C-acetyltylosin to 0.4 ml of the cell suspension of *S. aureus*, 0.1 ml of aqueous solution containing an unlabeled test compound was added to the cell suspension. After preincubation at 37°C for 10 minutes, 2.50 μg of 3-^{14}C-acetyltylosin was added to the cell suspension, incubated for 20 minutes and processed as described above.

Results

1. Antibacterial Activity of 3-Acetyl-4''-isovaleryltylosin

The minimum inhibitory concentrations (MIC, μg/ml) of 3-acetyl-4''-isovaleryltylosin against various clinical isolates of multiple-drug resistant *S. aureus* are shown in Table 1 in comparison with those of other macrolide antibiotics. All resistant strains were strongly resistant to tylosin, josamycin,

Table 1. Antibacterial activity of macrolide antibiotics to inhibit the growth of *Staphylococcus aureus* clinically isolated.

S. aureus	MIC (μg/ml)							Resistance to other drugs
	EM	JM	MM	Ac-SPM	Tylosin	3-Acetyl-tylosin[2]	3-Acetyl-4''-isovaleryl-tylosin[2]	
MS-8710[1]	>1,600	>1,600	>400	>400	800	800	25	Pc-G, TC, SM, CP
MS-9610[1]	>1,600	>1,600	>400	>400	1,600	>1,600	100	Pc-G
MS-9937[1]	>1,600	800	>400	>400	400	400	12.5	TC, CP
MS-11612[1]	>1,600	>1,600	>400	>400	800	1,600	25	
MS-11615[1]	>1,600	1,600	>400	>400	800	800	25	
MS-11620[1]	>1,600	>1,600	>400	>400	1,600	>1,600	100	Pc-G, CP
MS-11630[1]	>1,600	>1,600	>400	>400	1,600	>1,600	50	Pc-G, CP
MS-11636[1]	>1,600	1,600	>400	>400	1,600	>1,600	25	KM, TC, SM, CP
FDA 209P	0.09	0.39	0.39	1.56	0.78	0.78	1.56	

The minimum inhibitory concentration (MIC, μg/ml) was determined by the broth dilution method.

The inoculum size of each tester microorganisms was at 10^6 cells per ml.

1) Constitutive resistant to macrolides.

2) New tylosin derivatives by microbial acylation.

EM; erythromycin, JM; josamycin, MM; midecamycin, Ac-SPM; acetylspiramycin, Pc-G; benzylpenicillin, TC; tetracycline, SM; streptomycin, KM; kanamycin, CP; chloramphenicol.

erythromycin and other commercial macrolide antibiotics; the MIC was more than 400 μg/ml. In contrast, these strains were fairly sensitive to 3-acetyl-4''-isovaleryltylosin; the MIC was 25 μg/ml or less.

MS-9610 and MS-8710 strains were chosen for the study of the mechanism of action of 3-acetyl-4''-isovaleryltylosin, because the former showed the highest resistance against all macrolide antibiotics and the latter showed the widest range of resistance covering drugs other than macrolides.

Inhibition of growth of these resistant strains by 3-acetyl-4''-isovaleryltylosin was determined in more detail; the turbidity of the culture was followed with incubation time after the addition of the drug. As shown in Fig. 2, marked inhibition of the growth of MS-8710 and MS-9610 was observed at concentrations not less than 25 μg/ml and 50 μg/ml, respectively, so far tested for 6 hours. Therefore, these concentrations were suggested to be suitable for further biochemical studies of this derivative on these resistant strains. In comparison with this derivative, tylosin and 3-acetyltylosin showed only weak and temporary inhibition of the growth of the resistant strains. However, there was no difference among these drugs, in the effect on FDA 209P, a macrolide-sensitive strain, which was inhibited at 0.78 μg/ml of any drugs.

2. Effect on Cellular Macromolecule Synthesis

To determine the effect of 3-acetyl-4''-isovaleryltylosin on synthesis of cellular DNA, RNA and protein, cells were labeled with radioactive thymidine, uridine and leucine, respectively, in the presence or absence of 3-acetyl-4''-isovaleryltylosin.

As shown in Fig. 3, the incorporation of leucine into TCA insoluble fraction of MS-8710 and MS-9610 was strongly inhibited by the addition of 3-acetyl-4''-isovaleryltylosin at 25 μg/ml or 50 μg/ml respectively. Thymidine and uridine incorporations were not inhibited (data not shown).

Fig. 2. Inhibition of the growth of *Staphylococcus aureus* FDA 209P (a macrolide sensitive strain) and MS-8710, MS-9610 (resistant strains) by tylosin and tylosin derivatives.

Microorganisms were grown in four L-tubes containing 5 ml of brain heart infusion broth at 37°C with shaking. When the cultures reached a cell density of about 0.1 OD_{660}, drug was added to each L-tube to give a final concentration of 0.78 μg/ml for FDA 209P, 25 μg/ml for MS-8710 and 50 μg/ml for MS-9610, respectively. Incubation proceeded for additional hours and turbidity at OD_{660nm} was determined at indicated times.

—— control (no antibiotic), – – – tylosin, ------ 3-acetyltylosin, ----- 3-acetyl-4″-isovaleryltylosin

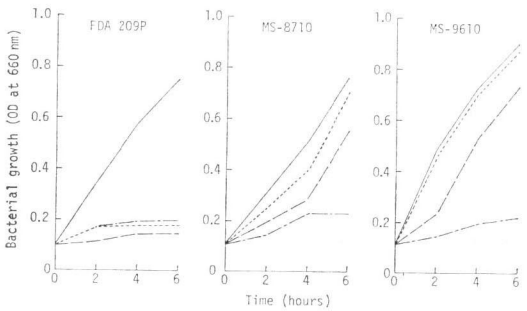
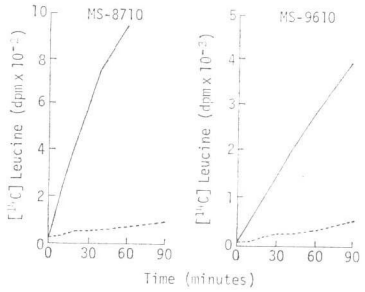


Fig. 3. Inhibition of protein synthesis in intact cells of the resistant strains MS-8710 and MS-9610.

Experimental conditions were given under Materials and Methods. 3-Acetyl-4″-isovaleryltylosin was added to give a final concentration of 25 μg/ml for MS-8710 and 50 μg/ml for MS-9610, respectively.

—— control (none), ------ 3-acetyl-4″-isovaleryltylosin



3. Uptake of 3-Acetyl-4″-isovaleryltylosin by Intact Cells

SHIMIZU *et al.*[7] reported that the decrease in accumulation of macrolide antibiotics in cells of macrolide-resistant strains of *S. aureus* was found.

The improved antibacterial activity of 3-acetyl-4″-isovaleryltylosin against macrolide-resistant strains suggested that this derivative could overcome the increased permeability barrier and/or could bind efficiently to the ribosomes of the resistant cells. For comparison, a parallel experiment was conducted with the strain FDA 209P, which was sensitive to macrolides. Uptake of 3-^{14}C-acetyl-4″-isovaleryltylosin into cells of these strains were determined by the membrane filter method and the results were compared with those of 3-^{14}C-acetyltylosin, a radioactive derivative of tylosin which did not inhibit the growth of the resistant strains as well as tylosin.

The results shown in Fig. 4 indicate, first of all, that decreased permeability was at least partly responsible for the resistance of *S. aureus* strains MS-8710 and MS-9610; the uptake of 3-^{14}C-acetyltylosin in resistant cells was significantly less than that of the sensitive strain(s). The degree of resistance of strain MS-9610 was stronger than the strain MS-8710 and the uptake by the former was less than that by the latter. 3-^{14}C-Acetyl-4″-isovaleryltylosin, compared with 3-^{14}C-acetyltylosin, showed better uptake especially into cells of the resistant strains; about 4 times as much uptake was observed with MS-9610. Specificity of the uptake was confirmed by a competition experiment between 3-^{14}C-acetyltylosin and unlabeled members of the family including 3-acetyl-4″-isovaleryltylosin. As shown in Table 2, 3-acetyl-4″-isovaleryltylosin showed the strongest inhibition of the uptake of 3-^{14}C-acetyltylosin. The result indicates that 3-acetyl-4″-isovaleryltylosin can penetrate more easily into cells of both resistant and sensitive strains than that of other tylosin derivatives.

4. Effect on *In Vitro* Protein Synthesis

We determined the effect of 3-acetyl-4″-isovaleryltylosin on *in vitro* protein synthesis on washed

Fig. 4. Uptake of ^{14}C-tylosin derivatives into intact cells of FDA 209P (a sensitive strain) and MS-8710, MS-9610 (resistant strains) of *Staphylococcus aureus*.

Details for preparation of the cell suspension and the procedures for the measurement of uptake of ^{14}C-labeled tylosin derivatives by intact cells from the medium are given under Materials and Methods. Either 3.05 μg of 3-^{14}C-acetyltylosin or 2.50 μg of 3-^{14}C-acetyl-4''-isovaleryltylosin was added to 1.5 ml of the cell suspension.

------ 3-^{14}C-acetyl-4''-isovaleryltylosin (8.34 mCi/mmole)

—— 3-^{14}C-acetyltylosin (7.63 mCi/mmole)

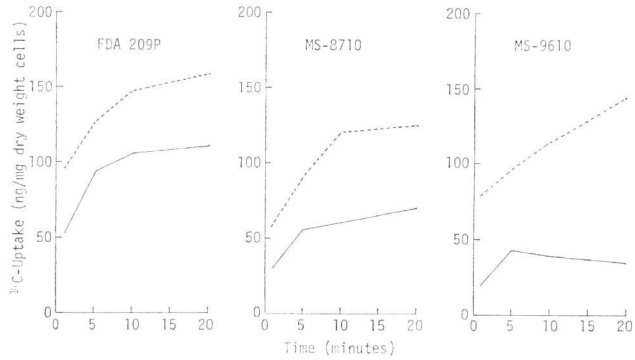


Table 2. Inhibitory activity of unlabeled 3-acetyl-4''-isovaleryltylosin on uptake of 3-^{14}C-acetyltylosin into intact cells of *Staphylococcus aureus* in comparison with those of other compounds.

Concentration of unlabeled compound (μg/ml)		Inhibition % compared with the control		
		FDA 209P	MS-8710	MS-9610
Tylosin	20	41	40	35
	200	59	63	46
3-Acetyltylosin	20	39	49	29
	200	72	70	58
3-Acetyl-4''-isovaleryltylosin	20	66	68	54
	200	90	88	85

Prior to the addition of 3-^{14}C-acetyltylosin to 0.4 ml of the cell suspension of *S. aureus*, 0.1 ml of aqueous solution of an unlabeled test compound was added to the cell suspension. After preincubation at 37°C for 10 minutes, 3.05 μg of 3-^{14}C-acetyltylosin (7.63 mCi/mmole) was added to the cell suspension and incubated for 20 minutes. ^{14}C-Uptake of control (without unlabeled compound) was 116 ng for FDA 209P, 82 ng for MS-8710 and 52 ng for MS-9610 per mg dry weight cells, respectively. Inhibition was expressed in percent of control without inhibitor.

70S-ribosomes of macrolide-sensitive and -resistant strains, in comparison with tylosin and 3-acetyltylosin.

As shown in Fig. 5, the protein synthesis on ribosomes of MS-9610 cells was partially inhibited by 3-acetyl-4''-isovaleryltylosin, but little or no inhibition was demonstrated by tylosin or 3-acetyltylosin. On the other hand, ribosomes of MS-8710 were sensitive to tylosin and 3-acetyltylosin as well as to 3-acetyl-4''-isovaleryltylosin, although intact cells of MS-8710 were resistant to tylosin and 3-acetyltylosin. These results indicated that strains MS-9610 and MS-8710 differed in the mechanism of resistance. The MIC of tylosin and josamycin and the inhibition of protein synthesis *in vitro* were

Fig. 5. Effect of 3-acetyl-4″-isovaleryltylosin, 3-acetyltylosin and tylosin on poly(U)-directed polyphenylalanine synthesis with 70S-ribosomes from MS-8710, MS-9610 (resistant strains) and FDA 209P (a sensitive strain) of *Staphylococcus aureus*.

A reaction mixture for determination of protein synthesis directed by poly(U) contained in 0.1 ml, 50 mM Tris-HCl, pH 7.8, 16 mM $Mg(OAc)_2$, 160 mM NH_4Cl, 3 mM ATP, 0.2 mM GTP, 2 mM PEP, 5 μg of pyruvate kinase, 2 mM DTT, 0.08 μCi of L-[U-^{14}C]phenylalanine (424 mCi/mmole), 0.01 mM each of 19 amino acids, 50 μg of tRNA from *E. coli* MRE 600, 70 μg protein of the S-100 fraction from *E. coli* Q-13, 2 OD_{280} units of washed 70S-ribosomes from *S. aureus* and a desired amount of an antibiotic. The reaction was started by adding 10 μg of poly(U) and proceeded at 37°C for 30 minutes. Under these conditions, the incorporation of ^{14}C-phenylalanine was almost a linear function of time up to 30 minutes of incubation, while the minus poly(U) system showed negligible incorporation. —— tylosin, – – – 3-acetyltylosin, ------ 3-acety-4″-isovaleryltylosin

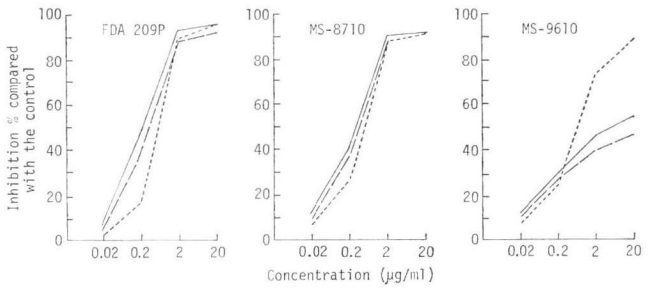


Table 3. Effects of josamycin (JM) and tylosin (TS) on the growth of intact cells (MIC) of *Staphylococcus* and the cell-free protein synthesis systems (ID_{50}).

Group of resistant* strains	*S. aureus* strain	MIC (μg/ml)		ID_{50}** (μg/ml)	
		JM	TS	JM	TS
Group I	MS-9610	>1,600	1,600	>20	9.33
Group II	MS-8710	>1,600	800	0.43	0.32
	MS-9937	800	400	0.35	0.26
	MS-11612	>1,600	800	0.18	0.32
	MS-11636	1,600	1,600	0.13	0.28
Group III	MS-11615	1,600	800	0.72	0.70
	MS-11620	>1,600	1,600	0.72	0.55
	MS-11630	>1,600	1,600	0.78	1.15
A sensitive strain	FDA 209P	0.39	0.78	0.43	0.19

* All resistant strains were classified into Groups I, II and III by their sensitivity to the cell free protein synthesis (ID_{50}).

** ID_{50}=50 % inhibition concentration against poly(U)-directed poly phe synthesis on 70S-ribosomes.

examined for all MS strains shown in Table 1. As shown in Table 3, the strains MS-9937, MS-11612 and MS-11636 were the same type of resistant strains as MS-8710: their ribosome system was sensitive to macrolides and their resistance was mainly due to the decreased permeability, and ribosomes of MS-11615, MS-11620 and MS-11630 were resistant, but the degree of resistance was lower than that of MS-9610. It is worthy to note that 3-acetyl-4″-isovaleryltylosin inhibits the growth of all strains including MS-9610.

Discussion

The antibacterial activity of 3-acetyl-4″-isovaleryltylosin against clinical isolates of multiple drug resistant *S. aureus* was found to be based on the inhibition of protein synthesis, as is the parent compound.

To exert antibacterial activity, macrolide antibiotics must penetrate the cell wall and plasma membrane and reach their ribosome targets. Introduction of an isovaleryl group at 4″-OH position of tylosin increased the efficiency of penetration into both sensitive and resistant strains of cells. The effect of the isovalerylester at $C_{4''}$ may be due to alteration in the physicochemical properties, *i.e.*, hydrophobicity and/or an alteration of the steric conformation.

In general, macrolide antibiotics are more hydrophobic than other protein synthesis inhibitors, such as aminoglycosides, tetracyclines, *etc.*, and their interaction with the phospholipid bilayer of the plasma membrane seems important for their penetration, probably by passive diffusion.

Hydrophobicities of these drugs were examined by thin-layer chromatography (TLC) using silica gel plates and by high performance liquid chromatography (HPLC) using C_{18} reversed phase column. These results indicate that 3-acetyl-4″-isovaleryltylosin is more lipophilic than tylosin and 3-acetyltylosin.

Although MS-8710 was highly resistant to tylosin, its ribosomes were susceptible to the action of macrolides just as the sensitive strain (FDA 209P). Therefore, genetic modification of ribosomes was ruled out as the mechanism of resistance of this strain. Either alteration in the membrane transport system or induction of drug-inactivating enzymes is a possible mechanism. The latter possibility was shown to be unlikely because tylosin was stable in the cell homogenate of the drug-resistant strains.

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