BINDING OF 3-O-ACETYL-4′"-O-ISOVALERYLTYLOSIN TO RIBOSOMES FROM A MACROLIDE-RESISTANT STRAIN OF STAPHYLOCOCCUS AUREUS

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The resistance of Staphylococcus aureus MS-9610 to tylosin and 3-O-acetyltylosin was due to the decreased affinity of its ribosome system to these macrolides. However, 3-O-acetyl-4″-O-isovaleryltylosin was found to bind to ribosomes of the strain about three times more than 3-O-acetyltylosin. This binding was not interfered by tylosin and 3-O-acetyltylosin. The 4″-O-acyl group and the mycinose moiety were suggested to have an important role in the binding of tylosin derivatives to ribosomes of resistant strains.

3-O-Acetyl-4″-O-isovaleryltylosin has an improved antibacterial activity against macrolide-resistant strains of Staphylococcus aureus clinically isolated. As reported previously1), the macrolide resistance of multiple-drug resistant strains of S. aureus can be divided into two types, the decreased sensitivity of ribosomes (Type I) and the decreased uptake (Type II). Among derivatives of macrolide antibiotics tested, 3-O-acetyl-4″-O-isovaleryltylosin inhibited the growth of both types of resistant strains. In this paper, we will report on the binding of the compound to 70S-ribosomes of Type I strain and the structural requirements for this binding.

Materials and Methods

Strains and Ribosomes

Staphylococcus aureus MS-9610 was isolated from a clinical specimen and its macrolide resistance was not inducible. The degree of the resistance was over 800 μg per ml of erythromycin, josaacyn, spiramycin, angolamycin, midacamycin, tylosin and 3-O-acetyltylosin and was 100 μg per ml to penicillin G. Staphylococcus aureus FDA 209P was used for comparison as a macrolide-sensitive strain. The culture condition was the same as described previously1).

70S-Ribosomes were prepared from S. aureus MS-9610 and FDA 209P by repeated suspension and centrifugation of ribosomes in a solution containing 1 M NH₄Cl, 10 mm tris-buffer, pH 7.8, 10 mm Mg-(OAc)₂, 60 mm KCl and 6 mm 2-mercaptoethanol as described in a previous paper1).

Chemicals

[1-¹⁴C]Acetyl group was introduced into the C-3 hydroxyl group of tylosin and 4″-O-isovaleryltylosin by an enzymatic method using [1-¹⁴C]acetyl-coenzyme A as described previously2). These labeled derivatives are described as 3-O-[1-¹⁴C]acetyltylosin and 3-O-[1-¹⁴C]acetyl-4″-O-isovaleryltylosin. Their specific activities were 7.63 and 8.34 (Lot 1) or 10.30 mCi/mmol (Lot 2), respectively.

L-[U-¹⁴C]Phenylalanine (424 mCi/mmol) and L-[U-¹⁴C]leucine (351 mCi/mmol) were purchased from Radiochemical Centre, Amersham, England; demycarosyl tylosin (desmycosin)3) and mycamino-
syl tylosinolide\(^4\)) were prepared according to the method reported previously; 3-O-acetyl-4'-O-isovaleryl-
demycinosyl tylosin was prepared from mycaminosyl tylosinolide by microbial transformation or from
demycinosyl tylosin\(^5\)) by chemical modification (the details for the procedure will be reported else-
where). All other chemicals were of commercial sources and of the highest grade available.

Determination of \(^{14}\text{C}\)Leucine Incorporation into Cellular Macromolecules

Inhibition of \(^{14}\text{C}\)leucine incorporation into cellular macromolecules was determined according to
the method of BYFILED et al.\(^6\)). The culture of \(S.\ aureus\) MS-9610 at an exponential growth phase (90 \(\mu l;\)
\(\text{OD}_{660}=0.2\)) containing 0.1 rim of deoxyadenosine was mixed with 10 \(\mu l\) of 5\% aqueous methanol con-
taining a test sample. The final concentration of the sample was 25 \(\mu M\) (Fig. 1) and 12.5 \(\mu M\) (Table 2).
It was incubated at 37°C for 10 minutes and 10 \(\mu l\) of \(L-[^{14}\text{C}]\)leucine (5 \(\mu Ci/ml\)) was added to the cell
suspension. After incubation at 37°C for 30 minutes, 100 \(\mu l\) 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\% trichloroacetic
acid (TCA) (5 ml/disc), and processed as reported previously\(^1\)). Radioactivity in cold TCA insoluble
materials was determined by a toluene scintillation method.

In Vitro Protein Synthesis on 70S-Ribosomes

In vitro protein synthesis in a system containing washed 70S-ribosomes of \(S.\ aureus\) and S-100 from
\(E.\ coli\) Q-13 (RNase I\(^-\)) was conducted as reported by MAO\(^7\)) with minor modifications and the condi-
tion as reported in a previous paper\(^1\)).

Determination of Tylosin Derivative-Ribosome Complexes

The formation of \(^{14}\text{C}\)-labeled tylosin derivative-ribosome complexes in staphylococcal strains was
determined by a gel-filtration method\(^9\)) and membrane filtration method as reported by PESTKA\(^8\)) and
MAO & PUTTERMAN\(^10\)).

1) Sephadex G-200 Gel Column Chromatography: Ribosomes (20 \(\mu l\), about 5.2 \(\text{OD}_{260}\) units) were mixed with 10 \(\mu l\) of 3-\(O\)-(1\(^-\)\(^{14}\text{C}\))acetyltylosin (0.61 \(\mu g\), \(6.4 \times 10^{-10}\) mole, 7.63 mCi/m mole) or 3-\(O\)-(1\(^-\)\(^{14}\text{C}\))-
acetyl-4'-O-isovaleryltylosin (0.46 \(\mu g\), \(4.4 \times 10^{-10}\) mole, 10.30 mCi/m mole) solution in a buffer (total
volume 100 \(\mu l\); 50 mm tris-buffer, pH 7.8, 50 mm NH\(_4\)Cl, 16 mm Mg(OAc)\(_2\) and 100 \(\mu M\) dithiothreitol
which was described below as the standard buffer and the mixtures were incubated at 37°C for 20
minutes. \(^{14}\text{C}\)-Labeled tylosin derivative-ribosome complexes were subjected to Sephadex G-200 gel
column chromatography (column size; 0.6 \(\times\) 20 cm, 0.25 ml/fraction) preequilibrated with the standard
buffer, using the same buffer for elution.

2) Millipore Membrane Filtration: Ribosomes (20 \(\mu l\), 5.2 \(\text{OD}_{260}\) units for MS-9610 and 7.6 \(\text{OD}_{260}\)
units for FDA 209P) were mixed with 5 \(\mu l\) of 3-\(O\)-(1\(^-\)\(^{14}\text{C}\))acetyltylosin (7.63 mCi/mole, 3.05 \(\mu g\)) or 10 \(\mu l\)
of 3-\(O\)-(1\(^-\)\(^{14}\text{C}\))acetyl-4'-O-isovaleryltylosin (8.34 mCi/m mole, 2.50 \(\mu g\)) solution in the standard buffer
(total volume 100 \(\mu l\)) and the mixture was incubated at 37°C for 20 minutes. The mixture was diluted
with 3 ml of a cold standard buffer containing the corresponding non-radioactive tylosin derivative at
50 \(\mu l/ml\), filtered by gentle suction through a Millipore membrane filter (HA, 0.45 \(\mu m\) pore size, 2.4 cm
diameter). The membrane filter was presoaked with the cold standard buffer described above. This
pretreatment of the filter with a non-labeled derivative decreased the background value. The value with-
out ribosomes was taken as the background value. The \(^{14}\text{C}\)-labeled tylosin derivative-ribosome com-
plexes on the membrane filter were washed twice with 3 ml of the above cold standard buffer and the
radioactivity remaining on the filter disc was measured by a liquid-scintillation method.

Interference of the Binding of Labeled Tylosin Derivatives to Ribosomes by Unlabeled Antibiotics

The inhibition of the binding of labeled tylosin derivatives to MS-9610 ribosomes by unlabeled anti-
biotics structurally related and unrelated was examined by the abilities of the latter to replace the former
in the formation of the ribosome complexes. Ribosomes (40 \(\mu l\), 6.9 \(\text{OD}_{260}\) units) were mixed with an
indicated amount of an unlabeled compound (20 \(\mu l\)) in the standard buffer (120 \(\mu l\), Fig. 3, Table 2).
After preincubation at 37°C for 10 minutes, 20 \(\mu l\) of 3-\(O\)-(1\(^-\)\(^{14}\text{C}\))acetyltylosin (1.22 \(\mu g\), \(1.27 \times 10^{-9}\) mole,
7.63 mCi/mmole) or 3-\(O\)-(1\(^-\)\(^{14}\text{C}\))acetyl-4'-O-isovaleryltylosin (0.92 \(\mu g\), \(8.8 \times 10^{-10}\) mole, 10.30 mCi/
mmole) was added to the reaction mixture. After incubation at 37°C for 20 minutes, the reaction mix-
ture was diluted, filtered and washed as described in a previous paragraph.
Results and Discussion

Inhibition of Protein Synthesis by Tylosin Derivatives

The effect of tylosin derivatives on in vivo protein synthesis in Type I macrolide-resistant Staphylococcus strain, MS-9610, is shown in Fig. 1. 3-O-Acetyl-4''-O-isovaleryltylosin and 4''-O-isovaleryltylosin showed a strong inhibition. This was in a good agreement with the data on growth inhibitory activities of tylosin derivatives. The dose-dependent effect of tylosin derivatives on protein synthesis on ribosomes1 prepared from macrolide-resistant (MS-9610) or -sensitive (FDA 209P) strains was examined. Fifty percent inhibition concentrations (ID$_{50}$) of tylosin, 3-O-acetytylosin, 4''-O-isovaleryltylosin and 3-O-acetyl-4''-O-isovaleryltylosin for the protein synthesis on MS-9610 ribosomes were 9.20, > 20, 0.90 and 0.80 µg/ml, respectively, and for the protein synthesis on ribosomes of FDA 209P were 0.19, 0.39, 0.78 and 0.65 µg/ml, respectively. Thus, MS-9610 ribosomes were found to be resistant to tylosin and 3-O-acetyltylosin but sensitive to 3-O-acetyl-4''-O-isovaleryltylosin or 4''-O-isovaleryltylosin.

Formation of Tylosin Derivative-Ribosome Complexes

The amounts of binding of tylosin derivatives to ribosomes of MS-9610 and FDA 209P determined by a membrane filter method are shown in Table 1. Thirteen pmole of 3-O-[1-$^{14}$C]acetyltylosin bound to 1 OD$_{260}$ unit of MS-9610 ribosomes and this amount was one third of the amount of 3-O-[1-$^{14}$C]acetyltylosin bound to FDA 209P ribosomes. The amount of 3-O-[1-$^{14}$C]acetyl-4''-O-isovaleryltylosin bound to MS-9610 ribosomes was three times more than that of 3-O-[1-$^{14}$C]acetyltylosin. This suggests that a stronger inhibition of protein synthesis by 3-O-acetyl-4''-O-isovaleryltylosin is due to the greater ability

Table 1. The formation of tylosin derivative-ribosome complexes in a macrolide-sensitive strain (FDA 209P) and -resistant strain (MS-9610) of Staphylococcus aureus.

<table>
<thead>
<tr>
<th>Source of ribosome*1</th>
<th>Tylosin derivative</th>
<th>The amount bound to ribosomes*2 (pmole/OD$_{260}$)</th>
<th>MIC (µg/ml)</th>
<th>ID$_{50}$*3 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA 209P</td>
<td>3-O-Acetyltylosin</td>
<td>38</td>
<td>0.78</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>3-O-Acetyl-4''-O-isovaleryltylosin</td>
<td>185</td>
<td>1.56</td>
<td>0.7</td>
</tr>
<tr>
<td>MS-9610</td>
<td>3-O-Acetyltylosin</td>
<td>13</td>
<td>&gt;1,600</td>
<td>&gt;20</td>
</tr>
<tr>
<td></td>
<td>3-O-Acetyl-4''-O-isovaleryltylosin</td>
<td>39</td>
<td>100</td>
<td>0.80</td>
</tr>
</tbody>
</table>

*1 FDA 209P (7.6 OD$_{260}$ units), MS-9610 (5.2 OD$_{260}$ units)

*2 The amount of a compound bound to ribosomes was determined using 3-O-[1-$^{14}$C]acetyltylosin (7.63 mCi/mmole, 5 µl, 3.05 µg) and 3-O-[1-$^{14}$C]acetyl-4''-O-isovaleryltylosin (8.34 mCi/mmole, 10 µl, 2.50 µg) as described in the Methods.

*3 50% Inhibition concentration against poly (U)-directed poly Phe synthesis in a cell-free system.
of this derivative to bind to MS-9610 ribosomes. The same result was also observed by the gel filtration method (Fig. 2).

While, in spite of having almost the same antibacterial activities or inhibitory activities in a cell-free protein system, the amount of 3-O-[1-14C]acetyl-4"-O-isovaleryltylosin bound to FDA 209P ribosomes was 4.9 times more than that of 3-O-[1-14C]acetyltylosin (Table 1). This indicates that the increase of binding affinity to ribosomes from a macrolide-sensitive strain does not necessarily correspond to high antibacterial activity.
To compare the affinity of 3-O-acetyl-4′′-O-isovaleryltylosin to MS-9610 ribosomes with that of 3-O-acetyltylosin, the inhibition between them in ribosome binding was examined at various concentrations, as shown in Fig. 3. The binding of 3-O-[1-14C]acetyltylosin was almost completely inhibited by 3-O-acetyl-4′′-O-isovaleryltylosin at one half concentration of the former, while the binding of 3-O-[1-14C]acetyl-4′′-O-isovaleryltylosin was inhibited only 25% by the addition of two fold concentration of tylosin or 3-O-acetyltylosin. This indicates that the affinity of 3-O-acetyl-4′′-O-isovaleryltylosin is much higher than that of tylosin. The binding sites of tylosin derivatives on MS-9610 ribosomes and the nature of this ribosomes will be reported elsewhere. The large difference between the MIC (100 μg/ml) and ID₅₀ (0.8 μg/ml) values of 3-O-acetyl-4′′-O-isovaleryltylosin on MS-9610 strain may indicate that there is an internal inhibition of the passage of the derivative caused by its high binding ability to cellular components.

### Structural Relationships among Tylosin Derivatives in Binding to Ribosomes

Structural relationships in binding to ribosomes were examined by interference between compounds in binding to ribosomes. Compounds used in this experiment are shown in Fig. 4. The results are listed in Table 2 together with the results of inhibitory activities of the compounds against protein synthesis in intact cells and a cell-free system of MS-9610 cells. In case of the compounds lacking an acyl group at the C-4′′ position of tylosin analogues, such as 3-O-acetyltylosin, tylosin, demycarosyl tylosin and mycaminosyl tylonolide, the ability to bind to ribosomes was markedly lower compared with tylosin derivatives having an acyl group at 4′′-position. In spite of having an acyl group at the 4′′-position, 3-O-acetyl-4′′-O-isovaleryl-demycinosyl tylosin also had low binding ability, indicating that the mycinose moiety is necessary for binding to ribosomes of MS-9610 strain. The requirement of mycinose was also suggested...
by a high binding ability of angolamycin compared with josamycin and spiramycin. Angolamycin has mycinose at the same position of the aglycone as tylosin, while josamycin and spiramycin have not. Compared with 3-O-acetyl-4'-O-isovaleryltylosin, the binding ability of angolamycin was significantly low. Introduction of acyl group into the C-3 hydroxyl group of the aglycone did not enhance the binding ability to ribosomes.

Other antibiotics known as bacterial protein synthesis inhibitors such as, chloramphenicol, streptomycin, tetracycline and kanamycin, did not inhibit with the binding of 3-O-[1-14C]acetyl-4'-O-isovaleryltylosin to ribosomes.

Table 2. The influence of various antibiotics on the binding of 3-O-acetyl-4'-O-isovaleryltylosin to ribosomes of MS-9610 cells in comparison with their effects on protein synthesis in vivo and in vitro and growth inhibition.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition by a compound (12.5 µM) of Binding of [14C]-3Ac4'-iVTS (4.4 µM) to 70S-ribosomes*1</th>
<th>ID50*2 (µg/ml)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-O-Acetyl-4'-O-isovaleryltylosin</td>
<td>79</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>4-O-Isovaleryltylosin</td>
<td>84</td>
<td>0.9</td>
<td>100</td>
</tr>
<tr>
<td>3-O-Acetyl-4'-O-isovaleryl-demycinosyltylosin</td>
<td>19</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>3-O-Acetytylosin</td>
<td>24</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Tylosin</td>
<td>30</td>
<td>9.3</td>
<td>1600</td>
</tr>
<tr>
<td>Demycarosyl tylosin</td>
<td>24</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Mycaminosyl tylonolide</td>
<td>13</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Josamycin</td>
<td>8</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>0</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Angolamycin</td>
<td>40</td>
<td>&gt;20</td>
<td>&gt;1600</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>-6</td>
<td>12.3</td>
<td>1600</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>-10</td>
<td>-</td>
<td>6.25</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>-3</td>
<td>-</td>
<td>1.56</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5</td>
<td>-</td>
<td>6.25</td>
</tr>
</tbody>
</table>

*1 70S-ribosomes (6.9 OD260 units)  
[14C]-3Ac4'-iVTS: 3-O-[1-14C]acetyl-4'-O-isovaleryltylosin.  
*2 50% Inhibition concentration in in vitro protein synthesis with 70S ribosomes from S. aureus MS-9610.

References


