Evernimicin (SCH27899) Inhibits both Translation and 50S Ribosomal Subunit Formation in Staphylococcus aureus Cells

W. SCOTT CHAMPNEY* AND CRAIG L. TOBER
Department of Biochemistry and Molecular Biology, J. H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee 37614

Received 29 June 1999/Returned for modification 24 December 1999/Accepted 17 February 2000

The effects of the evernimicin antibiotic evernimicin (SCH27899) on growing Staphylococcus aureus cells were investigated. Cellular growth rates and viable cell numbers decreased with increasing antibiotic concentrations. The rate of protein synthesis, measured as 35S-amino acid incorporation, declined in parallel with the growth rate. Significantly, the formation of the 50S ribosomal subunit was inhibited in a dose-dependent fashion as well. 30S ribosomal subunit synthesis was not affected over the same concentration range. Evernimicin did not stimulate the breakdown of mature ribosomal subunits. Pulse-chase labeling experiments revealed a reduced rate of 50S subunit formation in drug-treated cells. Two erythromycin-resistant strains of S. aureus that carried the ermC gene were as sensitive as wild-type cells to antibiotic inhibition. In addition, two methicillin-resistant S. aureus organisms, one sensitive to erythromycin and one resistant to the macrolide, showed similar sensitivities to evernimicin. These results suggest a use for this novel antimicrobial agent against antibiotic-resistant bacterial infections.

In the struggle to keep up with the current increase in the number of antibiotic-resistant infectious organisms, both new antimicrobial agents and new cellular targets must be found (10). A compound identified 35 years ago has recently been reinvestigated as a new and potentially effective antibiotic. The evernimomicins are a group of complex, sugar-derived antibiotics isolated from Micromonospora carbonacea (25, 26). They were described and characterized many years ago, but very few studies have been conducted to examine their modes of action (12, 21; A. K. Ganguly and A. K. Saksena, Communications to the editor, J. Antibiot. (Tokyo) 28:707–709, 1975). Avilamycin, a poly saccharide antibiotic with similarities to the evernimomicins, was shown to affect protein synthesis by interacting with the 30S ribosomal subunit (27). This compound has been used as an antimicrobial agent in animal feed (1).


We have identified a novel target for macrolide antibiotics in bacterial cells, the assembly of 50S ribosomal subunits (3–5). Macrolide and ketolide antibiotics have equivalent inhibitory effects on both translation and 50S subunit formation in S. aureus (7, 8). Since it has been suggested that evernimicin inhibits protein synthesis by interacting with the 50S subunit (Adrian and Klugman, 38th ICAAC), we decided to investigate its inhibitory effects on translation and subunit assembly in a systematic fashion. We found that both translation and 50S subunit formation were targets for inhibition in wild-type S. aureus cells and in both MRSA and erythromycin-resistant mutant strains. The significance of these findings is discussed in terms of the effects both on 50S subunit formation and on the potential clinical utility of this antimicrobial agent.

MATERIALS AND METHODS

Measurements of cell growth, subunit assembly, and translation rates. Studies were conducted with S. aureus strains RN1786, RN4220  ermC (inducible macrolide-lincosamide-streptogramin B [MLSb] resistance), and SK983  ermC (constitutive MLSb resistance), MRSA A1018  ermA (constitutive MLSb resistance), and MRSA A1024, all of which were provided by J. Sutcliffe of Pfizer Central Research. Evernimicin (SCH27899) and its placebo were generously provided by T. Black of Schering-Plough Corp. Evernimicin was made as a stock solution at 50 mg/ml in placebo and was diluted in placebo as needed. Cells were grown at 37°C in tryptic soy broth (TSB) in the presence and absence of evernimicin as described previously (7, 8). The erythromycin-resistant strains were grown with erythromycin at 50 mg/ml, and the MRSA organisms were grown with ampicillin at 50 mg/ml. Growth rates were measured by following the increase in cell density in a Klett-Summerson colorimeter. To examine ribosomal subunit assembly, the cells were labeled with [3H]uridine (1 uCi/ml; 2 uCi/ml) and were allowed to grow for two doublings in the presence of the drug. Isotope incorporation was followed by adding uridine to a final concentration of 50 uCi/ml, followed by a 30-min chase period. The rate of protein synthesis was determined in each culture during the chase period by adding [35S]methionine and cysteine (Tran 35S-label; ICN Pharmaceuticals) to 1 uCi/ml. Three samples of 0.2 ml were collected at 5-min intervals and were precipitated with 10% trichloroacetic acid, and the 35S-methionine and cysteine in the proteins were measured by liquid scintillation counting. At the end of the chase period, viable cell counts were measured by serial dilution of cells in A salts (18) followed by plating of 10 ml on square TSB agar plates by the method of Jett et al. (15). The colonies were counted after 48 h at 37°C.

Cell lysis and sucrose gradient sedimentation of ribosomal subunits were performed as described previously (7, 8). The absorbance at 254 nm for each...
TABLE 1. Effect of evernimicin (SCH27899) on doubling time, total cell number, protein synthesis rate, and 30S and 50S subunit amounts in *S. aureus* cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>Evernimicin conc (µg/ml)</th>
<th>Doubling time (h)</th>
<th>No. of cells (10³/ml)</th>
<th>Protein synthesis: 30S (% of control)</th>
<th>Protein synthesis: 50S (% of control)</th>
<th>30S Total cpm</th>
<th>50S Total cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK1786</td>
<td>0.0</td>
<td>0.8</td>
<td>22</td>
<td>100</td>
<td>20</td>
<td>38 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>1.3</td>
<td>21</td>
<td>59</td>
<td>21</td>
<td>34 (69)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>2.5</td>
<td>18</td>
<td>35</td>
<td>23</td>
<td>33 (87)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>2.9</td>
<td>18</td>
<td>24</td>
<td>22</td>
<td>31 (82)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>3.5</td>
<td>13</td>
<td>19</td>
<td>22</td>
<td>30 (79)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>4.7</td>
<td>6.3</td>
<td>17</td>
<td>22</td>
<td>24 (63)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.64</td>
<td>7.8</td>
<td>3.9</td>
<td>14</td>
<td>18</td>
<td>16 (42)</td>
<td></td>
</tr>
<tr>
<td>RN4220 (ermC)</td>
<td>0.0</td>
<td>0.6</td>
<td>19</td>
<td>100</td>
<td>23</td>
<td>33 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>3.3</td>
<td>6.3</td>
<td>17</td>
<td>22</td>
<td>25 (76)</td>
<td></td>
</tr>
<tr>
<td>SK903 (ermC)</td>
<td>0.0</td>
<td>0.6</td>
<td>10</td>
<td>100</td>
<td>23</td>
<td>35 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>4.3</td>
<td>7.2</td>
<td>16</td>
<td>22</td>
<td>19 (54)</td>
<td></td>
</tr>
<tr>
<td>A1018 (MRSA  ermA)</td>
<td>0.0</td>
<td>0.6</td>
<td>44</td>
<td>100</td>
<td>19</td>
<td>37 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>1.4</td>
<td>20</td>
<td>33</td>
<td>33</td>
<td>33 (89)</td>
<td></td>
</tr>
<tr>
<td>A1024 (MRSA)</td>
<td>0.0</td>
<td>0.75</td>
<td>27</td>
<td>100</td>
<td>21</td>
<td>35 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>1.9</td>
<td>15</td>
<td>23</td>
<td>22</td>
<td>26 (74)</td>
<td></td>
</tr>
</tbody>
</table>

* The results for doubling times, cell numbers, and protein synthesis rates are the averages of three or more experiments, with the standard error of the mean being ±3.5% for control samples (n = 7). Protein synthesis rates for the different strains are based on the data in Fig. 1. The percentage of the total radioactivity in the 30S and 50S subunit regions of each sucrose gradient is indicated. The results for the ribosomal subunit synthesis experiments are the averages of at least three experiments, with the standard error of the mean being ±2.0% for controls (n = 6).

* The percentage of the total gradient radioactivity in the 50S subunit fractions is given in parentheses.

Results

The effects of evernimicin on the growth of *S. aureus* cells were examined. An MIC of 0.8 µg/ml was found for the wild-type strain and for two erythromycin-resistant strains that carried the *ermC* gene. The two MRSA strains examined were slightly more sensitive to evernimicin (MIC, 0.4 µg/ml). The growth rates of the five strains examined were all reduced in liquid culture with evernimicin (Table 1). The decline in the cellular growth rate was paralleled by a decline in viable cell number for each organism. In addition, the growth rate of the wild-type cells in TSB was reduced with increasing concentrations of evernimicin. These results are presented in Table 1.

Evernimicin and avilamycin have both been shown to inhibit protein synthesis in cells (27; Black et al., 38th ICAAC). For each of the five strains studied, the rates of 35S-amino acid incorporation into cellular proteins were examined in the presence and the absence of the antibiotic. A dose-dependent decline in the rate of protein synthesis was observed for wild-type cells growing in the presence of evernimicin, as Fig. 1A shows. The growth rate and cell number declined in parallel with the reduction in protein synthesis rate (Fig. 2A), with a 50% inhibitory dose of 0.03 µg/ml. The two *ermC* strains showed similar sensitivities to the inhibitory effects of the drug (Fig. 1B). Protein synthesis in the two MRSA strains was also affected in a similar fashion, as Fig. 1C indicates. These effects of evernimicin on cellular protein synthesis are summarized in Table 1.

The assembly of the large 50S ribosomal subunit in *S. aureus* cells is a novel target for macrolide and ketolide antibiotics (2, 7). Evernimicin was tested to see if this drug would have a similar effect in these cells. 50S ribosomal subunit formation in each strain was susceptible to evernimicin inhibition, as Table 1 indicates. Compared with the untreated controls, evernimicin specifically reduced the percentage of the total [3H]uridine radioactivity found in the 50S subunit region of sucrose gradients, without affecting 30S subunit amounts. Table 1 also shows the effects of increasing antibiotic concentrations on 50S formation in wild-type cells. A dose-dependent inhibition in the formation of the larger subunit was found. This dose dependency for 50S assembly inhibition is displayed in Fig. 2B. A 50% inhibitory dose of 0.4 µg/ml was found, which is 13 times more than the value found for the effect on translation. 30S subunit formation was unaffected by evernimicin, except at the highest dose examined.

The macrolide antibiotics specifically prevent the formation of the 50S subunit and do not stimulate the breakdown of mature 50S particles (3, 9). Evernimicin was tested for a stimulatory effect on 50S subunit breakdown. Ribosomal subunits labeled with [3H]uridine in the absence of evernimicin were completely stable in the presence of the antibiotic, as Fig. 3A shows. There was no difference in the amount of [3H]uridine in 50S particles from cells grown with or without evernimicin. By contrast, the UV absorbance profile of the ribosomal subunits from evernimicin-treated cells showed a decline in the amount of 50S particles present (Fig. 3B).

Pulse-and-chase labeling procedures have been used to examine the rates of ribosomal subunit formation in bacterial cells (22). The kinetics of ribosomal subunit formation were examined by measuring the level of incorporation of [3H]uridine into ribosomal particles after a 90-s pulse labeling. In the absence of antibiotic, synthesis of both subunits was complete in 20 min, with the characteristic 2:1 ratio of subunits found at this time (Fig. 4A). In evernimicin-treated cells, the kinetics of 30S formation were comparable to those for cells in the control culture. By contrast, 50S formation was substantially retarded, with a gradual, linear increase in subunits observed for up to 1 h following the uridine chase (Fig. 4B).

Discussion

The results from this work indicate that evernimicin is a potent inhibitor of the growth and viability of *S. aureus* cells. It is effective at low concentrations and has an MIC for *S. aureus* which is equal to or lower than those of most macrolide antibiotics that we have examined (7, 8). The MICs of evernimicin...
for the strains that we have tested are very comparable to the
MICs found by others for methicillin-susceptible S. aureus
and MRSA organisms (19, 23). Viable cell numbers were reduced
in parallel with the reduction in growth rate at increased ever-
nimicin concentrations, indicating a bacteriocidal activity of
the drug. Protein synthesis and specifically the 50S subunit
have been indicated as the inhibitory targets for this compound
by other work (Adrian and Klugman, 38th ICAAC; Black et

FIG. 1. Effect of evernimicin on cellular protein synthesis. (A) The rates of incorporation of 35S-amino acids into proteins were determined as described in Materials
and Methods for wild-type S. aureus cells growing without evernimicin (C) and with evernimicin at 0.02 μg/ml (○), 0.04 μg/ml (□), 0.08 μg/ml (△), and 0.16 μg/ml (□). (B) Protein synthesis rates for wild-type cells growing without evernimicin (C) and with evernimicin at 0.32 μg/ml (○) and for ermC strains RN4220 (□) and SK983 (△) growing with evernimicin at 0.32 μg/ml. (C) Protein synthesis rates for wild-type cells growing without evernimicin (C) and with evernimicin at 0.04 μg/ml (△) and for MRSA strains A1018 (□) and A1024 (○) growing with evernimicin at 0.04 μg/ml.

FIG. 2. Effect of increasing evernimicin (Ziracin) concentrations on protein synthesis, growth rate, cell number, and ribosomal subunit synthesis in growing S. aureus
cells. (A) Percentage of control protein synthesis rate (C) and control growth rate (C). △, viable cell number. (B) Percentage of total gradient radioactivity (in counts
per minute) in 50S subunit (□) and 30S subunit (○) sucrose gradient fractions.
We have confirmed and extended these observations.

Inhibition of translation was found to be the preferential target for evernimicin in these cells, unlike our findings with macrolide antibiotics (4, 5). The inhibition of growth rate was directly related to the inhibition of cellular protein synthesis. The antibiotic’s effect on 50S formation was less significant, with about 13 times as much drug needed to give an equivalent degree of inhibition. This suggests that the interaction of the antibiotic with the mature subunit is stronger than the interaction with the particle(s) in the assembly pathway. By contrast, three different macrolide antibiotics were shown to have equivalent inhibitory effects on translation and 50S subunit formation (4, 5). The difference in structure between the macrolide compounds and evernimicin suggests a different interaction with the macromolecules in the 50S particle. Evernimicin-resistant mutants of S. aureus with alterations in ribosomal protein L16 bind to the antibiotic with a six- to eightfold lower affinity (P. M. McNicholas, P. A. Mann, D. J. Najarian, L. Miesel, T. A. Black, R. S. Hare, and K. J. Shaw, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-846, p. 117, 1999). In S. aureus, evernimicin could interact weakly with 23S rRNA and proteins in the precursor particle to affect assembly and more strongly only when L16 is assembled into the mature 50S subunit. It is important in this regard that ribosomal protein L16 is not found in either 50S precursor particle in growing Escherichia coli cells (20).

It is significant that four antibiotic-resistant strains of S. aureus were as sensitive as wild-type cells to the inhibitory effects of this drug. The inducible and constitutive ermC strains (MLSB organisms) were as susceptible to evernimicin as an antibiotic-susceptible organism was. The MRSA strains also showed comparable sensitivities to evernimicin. The MRSA strains have been tested previously for their sensitivities to erythromycin and other macrolides (6). Their clear susceptibility to evernimicin suggests a potentially important clinical role for this evernimicin in the treatment of infectious diseases caused by antibiotic-resistant organisms.

Significantly, this work is the first to describe a nonmacrolide antibiotic which functions by inhibition of 50S subunit formation in cells (3, 9). Evernimicin specifically prevents the complete formation of the large ribosomal subunit, apparently inhibiting both translation and assembly by 50S particle binding. Like the macrolides and ketolides, this antibiotic does not prevent 30S subunit formation except at very high concent-

FIG. 3. Effect of evernimicin on ribosomal subunit breakdown. Cells were grown in the presence of [3H]uridine, washed, diluted, and regrown with and without evernimicin at 0.32 μg/ml. (A) [3H]uridine radioactivity profiles of sucrose gradients of cell lysates from cultures grown without (C) and with (●) evernimicin. (B) UV absorbance profiles of sucrose gradients of cell lysates from cultures grown without (□) and with (●) evernimicin.

FIG. 4. Pulse-chase labeling kinetics of ribosomal subunit formation in cells growing with and without evernimicin at 0.32 μg/ml. (A) Kinetics of 30S (□) and 50S (●) subunit formation in control cells. (B) Kinetics of 30S (□) and 50S (●) subunit formation in evernimicin-treated cells.
trations and does not stimulate the breakdown of mature particles.

The effect on 50S subunit formation is clearly seen in pulse-chase analysis of subunit formation. Control cells assembled subunits with kinetics very similar to those observed previously in E. coli cells, with 10 min required for 30S subunit formation and 20 min required for 50S subunit synthesis (2, 17, 22). Evernimicin had no effect on 30S subunit synthesis but substantially reduced the rate of 50S subunit formation in treated cells. About 16% of the expected amount of 50S particles were assembled after 1 h in the presence of the drug.

These observations help to substantiate the model that we have proposed that 50S subunit translational inhibitors can also interact with a precursor stage of the nascent 50S particle and prevent its further maturation (2). It will be interesting to compare the rRNA and protein compositions of the precursor targets for evernimicin and the macrolides in cells. This work has identified two cellular targets for this novel antimicrobial agent. The inhibition of these two vital cellular activities is responsible for its killing effect on bacterial cells. Its effectiveness against two types of antibiotic-resistant S. aureus strains suggests that it will be a welcome addition to the diminishing list of compounds effective against antibiotic-resistant microorganisms.

ACKNOWLEDGMENTS

We are pleased to acknowledge Joyce Sutcliffe at Pfizer Central Research for strains and Todd Black at Schering-Plough Corp. for evernimicin. This work was funded in part by a grant from Schering-Plough Corp.

REFERENCES


