The vanadyl ribonucleoside complex inhibits ribosomal subunit formation in Staphylococcus aureus

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Received 23 January 2012; returned 11 March 2012; revised 30 March 2012; accepted 17 April 2012

Objectives: The discovery of new antibiotic targets is important to stem the increase in antibiotic resistance to most currently used antimicrobials. The bacterial ribosome is a major target for a large number of antibiotics that inhibit different aspects of translation. Most of these antimicrobial agents also inhibit ribosomal subunit formation as a second cellular target. Precise subunit assembly requires the activity of several distinct RNases for proper rRNA processing. The present work shows that the vanadyl ribonucleoside complex (VRC) inhibited RNases in Staphylococcus aureus involved in ribosomal subunit formation without an effect on translation.

Methods: Methicillin-susceptible and -resistant strains of S. aureus were examined for the inhibitory effects of VRC on cell viability by colony counting. Protein synthesis rates were measured by isotopic methionine incorporation. Ribosome synthesis was measured by radiolabelled uridine incorporation into ribosomal subunits as displayed on sucrose gradients. Pulse and chase radiolabelling was used to measure subunit synthesis rates. RNA turnover was determined by a gel on a chip assay.

Results: The rates of subunit synthesis and the amounts of both subunits were significantly reduced in the presence of the compound. Ribosomal RNA was degraded and cell viability was reduced as a consequence. VRC also stimulated the inhibitory effects of a macrolide and an aminoglycoside antibiotic on ribosome formation.

Conclusions: Bacterial ribosomal subunit synthesis was specifically impaired in VRC-treated cells, with the rates and amounts of both subunits reduced. Cell viability was significantly reduced and rRNA turnover was stimulated.

Keywords: VRC, ribosome formation, protein synthesis, macrolides, aminoglycosides

Introduction

Antibiotics play an important role in global health. However, antibiotic resistance is becoming an increasing problem worldwide.1 The discovery of novel drug targets and new types of antimicrobial agents are necessary to control infections caused by resistant microorganisms.2 A major cellular target for various currently used antimicrobial agents is the bacterial ribosome. Many steps in the complex process of translation can be inhibited by different compounds. Information on the specific inhibitory effects of many translational inhibitors has been reviewed recently.3 The biogenesis of the large and small ribosomal subunit in bacteria is an additional target for many of these same antibiotics.4

Bacterial ribosomal subunit assembly begins with specific ribosomal proteins adding to the nascent 16S, 23S and 5S ribosomal RNA transcripts. For both 30S and 50S synthesis, an intermediate precursor particle is formed initially. These particles contain a subset of the total ribosomal proteins found in the mature subunit and precursor forms of the 16S and 23S rRNA.5 Endo- and exonucleolytic cleavage of the precursor rRNAs by ribonucleases is essential for generation of the mature subunits.6 Mutant strains of Escherichia coli deficient in specific RNases are hypersensitive to azithromycin and to aminoglycoside antibiotics (Ashley D. Frazier, unpublished results). Maturation of the precursors into mature subunits is delayed in these mutants and an increase in the degradation of rRNA can be observed.7 Antibiotics targeting subunit assembly inhibition have been investigated,8 however, the use of RNase inhibition to potentiate the effects of currently used antibiotics has not been examined.

The vanadyl ribonucleoside complex (VRC) is a low molecular weight inhibitor of RNases that has been used during the isolation of RNA from cells.9-11 It is an effective inhibitor of cellular RNases, although its target specificity is unknown. We reasoned

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that VRC could target and inhibit RNases involved in ribosomal subunit assembly and thus function as a novel antimicrobial agent. Staphylococcus aureus strains were selected because of the need to develop new antimicrobial agents against this important human pathogen.12 This work shows that VRC can specifically inhibit ribosomal subunit formation in both methicillin-susceptible S. aureus (MSSA) and methicillin-resistant S. aureus (MRSA) cells without an inhibitory effect on protein synthesis. VRC was found to enhance the inhibitory effects of paromomycin and azithromycin in these organisms. The results indicate that RNases may be a novel antibiotic target.

Material and methods

**Cellular growth and viability**

MSSA RN178617 and MRSA A102418 were grown at 37°C in tryptic soy broth (TSB). Strain A1024 was provided from the clinical isolate collection of M. J. Ferraro.12 Strain RN1786 was a gift from Richard Novick.14 The macroside and aminoglycoside IC50 values for these two strains of S. aureus have been previously documented.15,16 After 1 h of initial bacterial growth, 5 mM VRC (New England Biolabs) was added to the cells. For some experiments, paromomycin or azithromycin was added at a concentration of 1.5 or 5 mg/L, respectively. Growth rates were measured as an increase in cellular density over time using a Klett–Summerson colorimeter, as previously described.16

**Radioactivity in specific gradient regions was summed and expressed as a percentage of total amount of isotope in the gradient.**

**Protein synthesis assay**

Cells were grown as described above in the presence or absence of 5 mM VRC. After two cellular doublings, 1 mCi/L [35S]methionine (1175 Ci/mmole; MP Biomedicals) was added. Following the addition of the isotope, the samples were removed at 5 min intervals. Each sample was precipitated in 10% TCA with 100 mg/L BSA, collected and washed on Whatman GF/A glass fibre filters. The filters were placed into vials containing 3 mL of Scintisafe gel. [3H]Uridine radioactivity was measured by liquid scintillation counting.

**Uridine pulse and chase labelling**

Two 12 mL cultures of cells, one control and one with VRC at 5 mM, were grown to a Klett of 40. The cells were pulse labelled with 1 mCi/L [3H]uridine (30 Ci/mmole, American Radiolabeled Chemicals, St Louis, MO, USA) for 90 s and then chased with uridine at 25 mg/L. At intervals, 2 mL samples were removed, collected by centrifugation, washed and stored frozen before lysis for sucrose gradient centrifugation as described.4

**Radioactivity in specific gradient regions was summed and expressed as a percentage of the total amount of isotope in the gradient at each timepoint.**

**Ribosomal subunit assembly**

Bacterial cell cultures were grown in TSB as described. Following the addition of VRC at a Klett of 20, paromomycin or azithromycin was added to appropriate cultures. After 15 min of growth with the antibiotics, [3H]uridine at a concentration of 1 mCi/L and uridine at a concentration of 2 mg/L were added. The cells were allowed to grow for two cellular doublings. At that time, uridine was added to 50 mg/mL and the cells were incubated an additional 15 min. Cells were collected by centrifugation and stored frozen at −70°C.

Cellular lysates were prepared with lysostaphin, DNase I and Triton X-100, as previously described.15 The samples were centrifuged through 5%–20% sucrose gradients in S buffer (10 mM Tris-HCl, pH 8.0/0.5 mM magnesium acetate/50 mM NH4Cl/0.2 mM β-mercaptoethanol) in an SW41 rotor at 187,813 × g for 3.5 h. Following centrifugation, sample fractions were collected by pumping them through an ISCO Model UA-5 absorbance monitor set at 254 nm. The fractions were collected into vials and mixed with 3 mL of Scintisafe gel before measuring the [3H]uridine by liquid scintillation counting.

**Agilent bioanalysis of RNA**

Bacterial cells were grown as previously described above. At a density of 4 × 10^8 cells/mL, the cells were collected by centrifugation and RNA was extracted from the cell pellet. Total RNA was isolated using a spin column procedure with the RNA isolation kit from Norgen (Norgen Biotek Corp.). RNA was examined using an Agilent Bioanalyzer 2100 and the RNA 6000 lab-on-a-chip. Typically 0.5–1 μg of RNA was analysed via chip analysis conducted according to the manufacturer’s instructions for total RNA analysis. The amount of 23S, 16S and small RNA species was measured by integration and is expressed as a percentage of total RNA in a given gel lane.

**Eukaryotic cell growth**

Growth of eukaryotic cells in tissue culture was performed as follows: 20000 macrophage cells (RAW 264.7)15 or 10000 fibroblast cells (BJ cells; ATCC) were seeded into 96-well plates with 200 μL of supplemented RPMI 1640 or DMEM, respectively. Both macrophage and fibroblasts were grown at 37°C in a 5% CO2/95% air humidified atmosphere. After 2 or 12 h of initial growth, respectively, 0.5, 1 or 5 mM VRC was added to the appropriate wells. After an additional 2 h of growth for each cell type, 10 mg/L paromomycin or azithromycin was added to the wells. The cells were allowed to grow for ~32 additional hours. Cell viability was measured by performing a Cell Titer 96® AQueous one solution cell proliferation assay as per the manufacturer’s instructions (Promega).

**Statistical analysis**

Statistical differences between samples were determined by Student’s t-test. In each table, an asterisk indicates a statistical significance of P < 0.05. Each antibiotic or VRC sample was compared with the control cells grown without VRC or antibiotics.

**Results**

The VRC is known to be a non-specific inhibitor of ribonuclease activity.10 It was examined in S. aureus to see if the RNases needed for ribosomal subunit assembly could be targeted to impair cell growth. VRC was initially tested to determine whether it had an inhibitory effect on cell viability. In both MSSA and MRSA cells, the addition of VRC decreased the cfu (Table 1). When 5 mM VRC was used, cell numbers were decreased by 90% in both strains. VRC also enhanced the inhibitory effects of paromomycin and azithromycin. The addition of VRC led to an increase in the efficiency of the antibiotics as seen by a statistically significant decrease in cfus compared with the effects of the antibiotics alone (Table 1).
Ribonucleases are essential for both ribosomal subunit assembly and for turnover of mRNA after translation. The inhibitory effects of VRC on translation and subunit formation were examined separately. The incorporation of $[^35]S\text{methionine}$ into total cellular proteins was examined in both strains in the absence and presence of VRC. As Figure 1 shows, the residual ribosomes in cells treated with VRC were not inhibited in their ability to incorporate amino acids into proteins as determined by the rate of protein synthesis. Comparable rates of translation were observed in each case.

Ribosomal subunit assembly was examined in both strains by $[^3]H\text{uridine}$ labelling during growth in the absence and presence of VRC. The subunits were separated by sucrose density gradient centrifugation to reveal the distribution of $[^3]H\text{uridine}$-labelled RNA in the ribosomal particles. Figure 2(a and b) shows the sucrose gradient profiles for the labelled subunits. In both strains there was an overall 90% reduction in the formation of both subunits in the VRC-treated samples when compared with the untreated control cells. This is apparent in the 10-fold difference in isotope amounts as shown by the left and right $y$-axes in Figure 2. The relative amounts of 50S subunits were reduced by about 15% after VRC treatment (Table 2). The gradient profiles also show a 15%–25% increase in $[^3]H\text{uridine}$-labelled RNA in the slowly sedimenting top gradient fractions after VRC treatment (Figure 2 and Table 2). This result is indicative of rRNA degradation.7

The influence of VRC on the rate of ribosomal subunit formation was examined by a $[^3]H\text{uridine}$ pulse and chase labelling procedure. The rates of subunit formation in the absence of the inhibitor were equivalent to those observed previously in these S. aureus strains (Figure 3).15,19 Synthesis of both subunits was completed in 60 min in the absence of VRC. Compared with control cultures, VRC reduced the rate of formation of both subunits in these organisms. The 50S subunit synthesis rate was reduced by 4-fold under these conditions (Figure 3).

The status of rRNA in control and VRC-treated cells was examined by the Agilent Bioanalyzer lab-on-a-chip procedure. Decreases in the relative amounts of both 16S and 23S rRNA were observed in VRC-treated cells of both strains (Figure 4). Precursor forms of both rRNA species could be seen. In both strains, the addition of VRC alone led to about a 5% increase in RNA fragmentation. The addition of paromomycin with and without VRC led to a further significant increase in smaller RNA molecules when compared with the control cells. Growth with both VRC and azithromycin led to a 22% increase in fragmented small RNA in MRSA cells. This increase in smaller RNA species was the greatest stimulation of rRNA degradation observed. The results in Figure 4 reveal the enhancing effects of VRC on inhibition by both antibiotics. This RNA analysis substantiates the observations suggested by the sucrose gradient centrifugation analysis.

In order to determine whether VRC could be used as an antimicrobial agent in eukaryotic cells, the compound and antibiotics were applied to fibroblasts and to macrophage cells in culture. When antibiotics were added to the eukaryotic cells, there was no significant change in the overall amount of viable cells. However, when 1 or 5 mM VRC was added to the eukaryotic cell cultures, there was an approximately 90%–98% reduction in the number of viable eukaryotic cells. This led to the conclusion that VRC was toxic to these cells and could not be used to stimulate antibiotic effectiveness.

Discussion

The bacterial ribosome is an important target for current antibiotic treatments.5 These antimicrobial agents target both translation and ribosomal subunit formation.6 For ribosomal subunit biogenesis, a number of different endonucleases and exoribonucleases are necessary for precursor rRNA processing.20 These include RNases III, E, G, T and PH.6 This processed RNA is used to form the subunit precursors, and without an inhibitor present, the precursors mature to generate the 30S and 50S ribosomal subunits, respectively.5 When an inhibitor, such as an antibiotic, is present, the precursor RNA is broken
down. Ribonucleases are used by the cell to degrade the subunit precursors and their rRNA into nucleotides to be recycled.\textsuperscript{4,5,21} RNase activity is therefore an important mechanism for both rRNA processing and rRNA turnover.

We have previously shown that \textit{E. coli} strains deficient in RNase E, RNase II or polynucleotide phosphorylase (PNPase) are hypersensitive to azithromycin.\textsuperscript{7} These mutants showed an enhanced accumulation of 23S rRNA, a stimulation of rRNA breakdown and a reduced recovery rate of 50S ribosomal synthesis after azithromycin removal. These results suggested that the use of an RNase inhibitor in bacterial cells could enhance the efficiency of current antibiotics.

The present results can be interpreted to suggest that the antibiotics alone stall subunit formation, leading to precursor particle accumulation.\textsuperscript{22,23} VRC alone may inhibit the activity of critical rRNA processing RNases. Either antibiotic with VRC causes the accumulation of a subunit precursor and the inhibition of rRNA processing, leading to an enhanced inhibitory effect on subunit formation and a further reduction in cell viability.

The relatively high concentration of VRC used here (5 mM) and its inhibitory effects on eukaryotic cells would preclude its use as a human antimicrobial agent. Its effects on ribosome synthesis indicate its use as a model compound and suggest that inhibition of bacterial RNases can be a novel target for drug development.

It is important to note that VRC specifically inhibited subunit formation without an effect on translation. This reinforces the established idea that translation and subunit assembly are separate and equivalent targets for ribosomal antibiotics.\textsuperscript{5,24–26} Either process can be a target for specific antimicrobials. The cellular RNase target for VRC is unknown, but RNase III is a likely target since the formation of both subunits was affected to the same extent. Inhibition of subunit assembly-specific RNases by VRC suggests that these may be a target for other small molecule inhibitors or for RNA interference approaches. This research further indicates the importance of RNases as a novel target in antibiotic research.

**Acknowledgements**

We appreciate the technical help of Ward Rodgers. We would like to thank Courtney Netherland and Jaime Parman-Ryans for the generous donation of macrophage and fibroblast cells.
Figure 3. Kinetics of ribosomal subunit formation in cells growing with and without 5 mM VRC. (a) Rate of formation of 30S subunits in MSSA cells. (b) Rate of formation of 50S subunits in MSSA cells. (c) Rate of formation of 30S subunits in MRSA cells. (d) Rate of formation of 50S subunits in MRSA cells. Results are the mean ± standard error of two independent experiments.

Figure 4. RNA samples analysed by the Agilent Bioanalyzer lab-on-a-chip. Total RNA was isolated from MSSA and MRSA cells grown without and with VRC and antibiotics. (a) Agilent chip analysis of RNA from MSSA cells. The RNA samples are: lane 1, control cells; lane 2, cells grown with paromomycin; lane 3, cells grown with azithromycin; lane 4, cells grown with 5 mM VRC; lane 5, cells grown with paromomycin and 5 mM VRC; and lane 6, cells grown with azithromycin and 5 mM VRC. (b) Agilent chip analysis of RNA from MRSA cells. Samples are the same as in (a).
Vanadyl ribonucleoside complex and ribosome assembly

Funding
This project was supported by award number 1R15GM086783 from the National Institute of General Medical Sciences.

Transparency declarations
None to declare.

References