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Inhibition of Ribosomal Subunit Synthesis in *Escherichia coli* by the Vanadyl Ribonucleoside Complex

Ashley D. Frazier and W. Scott Champney*

Department of Biomedical Sciences, J.H. Quillen College of Medicine, East Tennessee State University, Johnson City, TN 37614 USA

Abstract

The increase in antibiotic resistant microorganisms has driven a search for new antibiotic targets and novel antimicrobial agents. A large number of different antibiotics target bacterial ribosomal subunit formation. Several specific ribonucleases are important in the processing of rRNA during subunit biogenesis. This work demonstrates that the ribonuclease inhibitor, vanadyl ribonucleoside complex (VRC), can inhibit RNases involved in ribosomal subunit formation. The ribosomal subunit synthesis rate was significantly decreased and ribosomal RNA from the subunit precursors was degraded. VRC had no inhibitory effect on translation. VRC also potentiated the inhibitory effects of an aminoglycoside and a macrolide antibiotic.

Keywords

VRC; ribosome formation; protein synthesis; paromomycin; azithromycin

Introduction

The increase in antibiotic resistance is a global threat which worries both physicians and research scientists [28]. An important area of investigation is the discovery of novel antimicrobial agents to which resistance has not yet been developed. In addition, a significant effort is being made to identify new cellular targets for drug discovery [1, 4, 19].

The bacterial ribosome is a target for a diverse collection of different antibiotics [8, 34]. Drug binding sites and molecular mechanisms of action have been described recently for many of these compounds based on refined crystal structures [6, 8, 13, 15, 24, 32]. Most of these antibiotics bind to the 30S or 50S ribosomal subunit and inhibit translation of mRNA. Importantly, biogenesis of the subunits is an additional cellular target for many of these agents [7]. When synthesis of either subunit is inhibited, specific ribonucleases (RNases) proceed to degrade the rRNA in the ribosomal subunit assembly intermediates [8]. In particular, RNase E is important in the turnover of a 50S subunit precursor particle present in erythromycin-inhibited cells [32] and the enzymes RNase II and PNPase are also involved in rRNA degradation in cells inhibited by azithromycin [29].

In *Escherichia coli* cells, at least five different RNases are needed to generate and process the 5' and 3' ends of precursors to 16S, 23S and 5S rRNA [18, 22, 30, 33]. Subunit assembly intermediates are the substrates for these activities. Mutant organisms missing any of these proteins show an enhanced sensitivity to both paromomycin and neomycin [16]. These observations suggest that RNases in *E. coli* cells could be novel targets for

*Corresponding author. Phone: (423) 439-2022. Fax: (423) 439-2030. Champney@etsu.edu.

antimicrobial agents. Inhibition of certain RNases is predicted to enhance the inhibitory effects of antibiotics targeting subunit formation [29, 32].

The vanadyl ribonucleoside complex (VRC) is a low molecular weight inhibitor of RNases that is commonly used during the isolation of RNA from cells [2, 26, 27]. Although its target specificity is unknown, the compound is thought to target endoribonucleases [2]. VRC has been shown to reduce the synthesis of both ribosomal subunits in *Staphylococcus aureus* [17]. Cell viability was reduced in this organism and rRNA degradation was stimulated. The compound also enhanced the inhibitory effects of both paromomycin and azithromycin.

We hypothesized that VRC could therefore function as a novel antimicrobial agent in a gram-negative organism, like *E. coli*. This work shows that VRC caused an inhibitory effect on ribosomal subunit formation in *E. coli* cells without affecting bacterial protein synthesis rates. VRC also enhanced the growth inhibitory effects of paromomycin and azithromycin in these cells. This work indicates that inhibition of RNases is a potential drug target.

Materials and Methods

Cellular Growth and Viability Measurements

Escherichia coli strain SK901 [21] cells were grown in a 37°C water bath in 5 ml of tryptic soy broth (TSB). After bacterial growth was evident, vanadyl ribonucleoside complex (VRC, New England Biolabs) was added to the cells at a concentration of 5 mM. Paromomycin or azithromycin were added to some cultures at 5µg/mL. The cells were grown for two cellular doublings to approximately 4×10^8 colony forming units (CFU)/mL before measurements were made. Cell numbers were measured after serial dilution and plating on TSB agar plates as previously described [20]. VRC caused a color change to the TSB media used here which made an estimate of the growth rate by turbidity changes inaccurate.

Uridine Pulse and Chase Labeling

Cell cultures of 12ml were grown to a Klett of 20 at 27°C and then VRC was added to 5mM. Following one cellular doubling, the cells were pulse labeled with ^3H -uridine (1µCi/ml) for 90 sec and then chased with uridine at a concentration of 25µg/ml. At six time intervals, 2ml samples were removed, collected by centrifugation, and stored frozen before analysis by sucrose gradient centrifugation as previously described [11, 29].

Rates of Protein Synthesis

Bacterial cultures were grown in the presence or absence of 0.5, 1, or 5mM VRC. After two cellular doublings, 1µCi/mL of ^{35}S -methionine (1175 Ci/mmol, MP Biomedicals) was added. Following the addition of the radioisotope, three 0.2mL samples were removed at 5 min intervals. Samples were precipitated in 10% TCA with 100µg of BSA, collected and washed on Whatman GF/A glass fiber filters. The filters were placed into vials containing 3mL Scintisafe gel. Radioactivity was measured by liquid scintillation counting.

Ribosomal Subunit Assembly

Cells were grown as described above. At a Klett of 20, cultures received VRC at 5mM and/or paromomycin or azithromycin as appropriate. After 15 minutes of additional growth, ^3H -uridine at 1µCi/mL (30Ci/mmol, Am. Radiochemicals) and uridine at 2µg/mL were added. After two cell doublings, 50µg/mL of uridine was added and the cells were incubated an additional 15 min. Cells were collected by centrifugation and stored frozen at -70°C.

Cellular lysates were prepared by a lysozyme-freeze thaw method as previously described [13]. The lysates were centrifuged through 5–20% sucrose gradients in S buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM Mg acetate, 50 mM NH₄Cl) in an SW 41 rotor at $187813 \times g$ for 3.5 hours [29]. Following centrifugation fractions were collected after pumping the gradient through an ISCO Model UA-5 absorbance monitor set at 254nm. The fractions were collected into vials and mixed with 3mL Scintisafe gel before measuring the radioactivity by liquid scintillation counting. The distribution of the total radioactivity in the top, 30S, or 50S regions was determined by summing the amount of isotope in each peak and four surrounding fractions.

Agilent Bioanalysis of RNA

Bacterial cells were grown as described with 5mM VRC and antibiotics. Antibiotics were added at 10µg/mL in order to augment the sensitivity of RNA analysis following isolation. At a density of 4×10^8 cells/mL, the cells were collected by centrifugation and RNA was extracted from the cell pellet using a chloroform/phenol extraction procedure as detailed [27]. Total RNA was examined using an Agilent Bioanalyzer 2100 and the RNA 6000 chip. The sample preparation, loading procedure and run was carried out according to manufacturer's instructions for total RNA analysis. Generally 0.5 to 1µg of RNA was examined.

Northern Blot Hybridization

Biotinylated 16S and 23S specific probes were constructed by PCR as previously described [29]. The 16S (241 bp) and 23S (146 bp) DNA probes were amplified from plasmid pKK3535 DNA [3] using the polymerase chain reaction with primers from Life Technologies. The 23S primers used were (23S F) TAG GGG AGC GTT CTG TAA G and (23S R) CCC ATT AAC GTT GGA C (nt. nos. 1188–1334). The 16S primers used were (16S F) GGA GGA AGG TGG GGA TGA CG and (16S R) ATG GTG ACG GGC GGT GTG (nt. nos. 1173–1414). PCR products were purified by extraction with phenol and chloroform and precipitation with 2 volumes of ethanol. The pellets were resuspended in 30µL of sterile water. The purified DNA probes were labeled with biotin using the Label-IT biotin labeling kit (Mirus) [29].

Six micrograms of total RNA was denatured by heating at 55°C for 10 minutes and separated on a 5% TAE PAGE gel as previously described [27]. An estimation of RNA fragment sizes were provided by a biotinylated RNA standard that was run in each gel. Intact 16S and 23S RNA was evident by ethidium bromide staining prior to removing this region of the gel for Northern hybridization. This removal allowed for a more sensitive detection of the RNA fragments. RNA was transferred from the gel onto Nytran nylon membranes using a Turbo blot apparatus (S&S). The membranes were pre-hybridized in 15mL of 1X pre-hybridization solution at 42°C for 30 minutes. The membranes were then hybridized overnight at 42°C with 6mL hybridization buffer, 1X background quencher, and 4pmol of the denatured 16S or 23S specific probe as previously described [29].

After hybridization, the membranes were washed and the probe detected via the North2South chemiluminescent hybridization kit (Pierce Chemical Co.) with streptavidin-conjugated horseradish peroxidase. Quantitative analysis of the rRNA fragmentation was determined by the G Box image analysis system (SynGene).

Results

The RNase inhibitor VRC has been shown to reduce the synthesis rates and amounts of ribosomal subunits in *Staphylococcus aureus* cells without an inhibitory effect on protein

biosynthesis [17]. The compound was examined in *Escherichia coli* cells to see if similar inhibitory effects could be found in a Gram-negative organism. The effect of VRC on ribosomal subunit synthesis rates was measured by a ³H-uridine pulse and chase labeling procedure. Figure 1 shows the rates of 30S (Figure 1a) and 50S (Figure 1b) subunit formation in the absence of VRC. Assembly was complete in 60 min at 27°C (Figure 1). Growth with 5mM VRC lengthened the time for the rate of subunit assembly by about 4-fold, with 50% of net subunit formation completed in 120 min. The synthesis of both subunits was reduced to the same extent by VRC treatment.

Compounds which inhibit ribosomal subunit formation also affect translation [31]. Protein synthesis rates in bacterial cells were measured in the presence of 3 different concentrations of VRC. The data in Figure 2 demonstrate that the rate of protein synthesis was increased slightly with increasing VRC concentrations but no inhibitory effects were seen. Combined with the decrease in 30S and 50S subunits, the protein synthesis results indicate that VRC slows the assembly of ribosomal subunits without affecting either subunit's function and suggests that VRC inhibits rRNA processing ribonucleases.

We have shown previously that 5mM VRC reduced the viability of *S. aureus* cells by over 90% and the compound also enhanced the effectiveness of two antibiotics in this microorganism. The effects of VRC and the same antibiotics on the viability of *E. coli* cells were determined by colony counting of cell samples. Cells grown with 5mM VRC alone showed a decrease in colony forming units (Table 1). Paromomycin by itself also lead to a significant decrease in the viable cell numbers (7.7% of control); while the addition of VRC with paromomycin had a similar effect on cell numbers (Table 1). Azithromycin alone gave an 83% reduction in cell numbers and the inhibitory activity of azithromycin was enhanced in the presence of 5mM VRC (Table 1).

Based on the prior work in *S. aureus* [17], we tested whether the addition of VRC alone or with either antibiotic would lead to a decrease in the amounts of ribosomal subunits in *E. coli* cells. Cells were radio-labeled with ³H-uridine for two doublings with and without VRC and the two antibiotics. The distribution of ³H-uridine labeled rRNA after sucrose gradient centrifugation was used to examine 30S and 50S subunit levels (Figure 3). VRC treatment led to substantial reductions in the amounts of both particles (Figure 3a). The addition of 5mM VRC gave a 26% reduction in 30S subunits and a 45% reduction in 50S subunit levels (Table 2). In addition, a 26% increase in radio-labeled RNA was seen in the top gradient fractions, indicative of rRNA degradation [29]. The increase was proportional to the loss of rRNA from both subunits (24%).

When paromomycin or azithromycin was added to the bacterial cells, 30S and 50S subunit assembly was impaired, as seen previously [8] (Fig. 3b,c). 50S subunit formation was reduced by more than 40%. Increases in the amounts of RNA in the top gradient region indicate that degradation of rRNA was stimulated by both antibiotics (Table 2). The addition of VRC in combination with the antibiotics led to a further reduction in 50S subunit amounts and an apparent enhancement of rRNA degradation (Table 2). The proportional increase in RNA degradation was seen in each instance (Table 2). The increased levels of radiolabeled RNA in the 30S subunit region is likely due to the accumulation of 50S subunit precursor particles here [23, 25, 29, 32].

The effect of VRC and the two antibiotics on rRNA turnover was examined in more detail. The Agilent chip methodology was used to examine rRNA status in the cells. Figure 4 shows the gel pattern and Table 3 gives a quantitative analysis of the rRNA distribution. The amounts of both 16S and 23S rRNAs in *E. coli* were reduced by each experimental treatment compared with untreated control samples (Table 3). Cell growth with antibiotics

alone increased the amounts of small RNA fragments while decreasing 16S and 23S rRNA. VRC alone by itself reduced the 16S and 23S rRNA amounts by about 20%, and increased the RNA degradation observed when either antibiotic was added. The fragmentation was also apparent in the reduced RIN seen with the antibiotic samples (Table 3). An RNA band of the approximate size of the precursor form of 16S rRNA was apparent in each case where antibiotics were added (Figure 4).

Northern hybridization analysis was used to examine the rRNA fragmentation with more specificity. Probes specific for 16S and 23S rRNA sequences were used to test for the degradation of each type of rRNA. The probe hybridization results are shown in Figure 5 and indicate that VRC alone stimulated some fragmentation of 16S (Figure 5a). Each antibiotic enhanced 16S rRNA fragmentation as shown previously [15]. However, when VRC and the antibiotics were used together, the fragmentation of both 16S and 23S rRNA species was enhanced and there was an increase in the smaller RNA fragments (Figure 5). Fragment sizes ranged from 130 to 525 nucleotides of RNA. These results confirm the degradation of rRNAs indicated by the gradient centrifugation and Agilent gel results.

Discussion

Bacterial ribosomes are essential structures in all prokaryotic cells. Expression of genetic information relies on accurate and rapid translation of mRNA sequences. A very large number of antimicrobial agents target the translation process causing either bacterial stasis or bacteriocidal effects [8, 23]. Ribosomal subunit formation is an essential process for bacterial cell growth and viability and is also a target for many of these same translational inhibitors [8].

The present work builds on several of our previous findings regarding various aspects of antibiotic inhibition of bacterial ribosome formation [5, 7, 12, 15, 24, 25, 32]. A large number of different translational inhibitors can stall ribosomal subunit formation in growing cells [9–11]. Mutant strains of *E. coli* deficient in specific RNases showed an enhanced sensitivity to both macrolide [29, 32] and aminoglycoside [16] antibiotics, and subunit formation was impaired in each case while rRNA degradation was stimulated. The present work extends the evidence showing that impairment of subunit formation by mutation or small molecule inhibitors is detrimental to cell growth. The identification of RNases as an antibiotic target has recently been reviewed [14]. It was suggested that, while not a broad spectrum target, inhibition of RNases could be used to target specific microorganisms.

The specific ribonuclease(s) inhibited by VRC in these cells are not known. RNase III is one possible target since the formation of both subunits was impaired to the same extent. This enzyme generates both precursor 16S and 23S rRNAs from the primary transcript [35] and is a endoribonuclease, the likely substrate for VRC [2]. Other processing endoribonucleases like RNase E, G, R or PH could also be affected by VRC [16]. VRC may certainly have other effects on tRNA or mRNA metabolism in cells in addition to the inhibitory effect on subunit formation. These remain to be investigated.

The present findings are generally similar to our previous results seen after VRC treatment of both methicillin-susceptible (MSSA) and methicillin resistant (MRSA) *S. aureus* cells [17]. A comparison of the findings is given in Table 4. In both organisms, VRC reduced the rate of subunit synthesis by 2 to 4 fold. In addition, the amounts of both subunits were reduced and turnover of rRNA was stimulated. Interestingly, protein synthesis rates were stimulated to some extent in both organisms. The largest difference seen was the effect on cell viability. In the gram-negative *E. coli* cells, VRC treatment resulted in a small decline in CFUs, while

a much larger killing effect was seen in the gram-positive *S. aureus* cells. VRC may be bacteriocidal in *S. aureus*.

A separation of the inhibition of subunit formation and subunit function in translation is a finding from this research [8]. A large reduction in the rate of synthesis of both subunits was revealed without a concomitant reduction in translation rates. Preexisting ribosomes in VRC treated cells seem capable of normal rates of protein synthesis. This data further indicate that VRC is reducing the rate of ribosomal subunit formation without affecting the function of the bacterial ribosomes.

The results described here can be interpreted as follows. Either of the two antibiotics is capable of binding to a subunit precursor particle and stalling subunit maturation, as we have previously demonstrated [15, 24]. VRC alone likely impairs the processing of rRNA precursor sequences, a process essential for subunit maturation as well [17]. Combining VRC with an antibiotic will generate a stalled subunit precursor in a cell with a reduced rRNA processing capability, thus enhancing the effectiveness of the antimicrobial agent.

Inhibition of rRNA processing RNases indicates a new antimicrobial target in cells. A search for other small molecule inhibitors of RNase activity or the application of current RNA interference methods may promote the development of more effective current or novel antimicrobial therapies.

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Fig. 1. Ribosomal subunit synthesis rates measured by ^3H uridine pulse-chase labeling assays. (a) Rates of formation of 30S subunits in cells growing with or without 5mM VRC. (b) Rates of formation of 50S subunits in cells growing with or without 5mM VRC. Results are the means $\pm$ standard error of 2 independent experiments.

Fig. 2. Protein synthesis rates for cells growing without and with VRC. ³⁵S-Methionine incorporation into total cellular proteins was measured. Results are the means $\pm$ standard error of 2 independent experiments.

Fig. 3.

Sucrose gradient profiles of ^3H uridine labeled ribosomal subunits. (a) Gradient profile for subunits from cells grown without and with 5mM VRC. (b) Gradient profile for subunits from cells grown with 5µg/mL paromomycin and/or VRC. (c) Gradient profile for subunits from cells grown with 5µg/mL azithromycin and/or VRC. Note the differences in the left (without VRC) and right (with VRC) axis.

Fig. 4. Agilent gel analysis of total RNA. Lane 1, RNA from control cells; lane 2, RNA from cells grown with paromomycin; lane 3, RNA from cells grown with azithromycin; lane 4, RNA from cells grown with 5mM VRC; lane 5, RNA from cells grown with paromomycin and 5mM VRC; lane 6, RNA from cells grown with azithromycin and 5mM VRC. A ladder of RNA standards is shown on the left. The larger precursor to 16S rRNA (P16S) is indicated.

Fig. 5. Northern hybridization analysis of rRNA fragmentation. RNA was isolated from cells grown in the presence of 10 μ g/mL antibiotics and/or 5mM VRC. The top of the gel containing the intact 16S and 23S rRNAs was removed in order to allow probe hybridization to only rRNA fragments. (A) 16S rRNA hybridization. The samples are lane 1, RNA from control cells; lane 2, RNA from cells grown with 5 mM VRC; lane 3, RNA from cells grown with paromomycin and VRC; lane 4, RNA from cells grown with azithromycin and VRC. (B) 23S rRNA hybridization. Lanes as in (A).

Table 1

VRC and antibiotic effects on cell viability

	CFU ($\times 10^7/\text{mL}$) ^a
Control	301 $\pm$ 52.6
Paromomycin	23 $\pm$ 13.3(7.7) *
Azithromycin	53 $\pm$ 7.5 (17.4) *
VRC	261 $\pm$ 46.9 (86.7)
Paromomycin + VRC	18 $\pm$ 2.5 (6.0) *
Azithromycin + VRC	15 $\pm$ 7.6 (5.0) *

^aColony forming units for the control and VRC or antibiotic treated cells. The percentage reduction in viability compared with the untreated control cells is listed in parenthesis. Results are the means $\pm$ standard error of 3 independent experiments.

(*) statistically significant with a *P* value <0.05.

Table 2

Distribution of ribosomal subunits in sucrose gradient regions

Treatment	% Total gradient radioactivity ^a		
	Top gradient region	30S region	50S region
Control	29.09±2.6	23.83±0.7	41.19±2.5
Paromomycin	38.34±4.3 (131.8)	32.12±7.1 (134.8) *	24.92±3.7 (60.5) *
Azithromycin	42.45±7.6 (145.9)	30.74±4.1 (129.0) *	22.27±3.5 (54.1) *
VRC	55.99±2.2 (192.5) *	17.84±1.5 (74.9) *	22.66±1.6 (55.0) *
Paromomycin + VRC	71.33±2.6 (245.2) *	12.29±2.6 (51.6) *	13.89±2.4 (33.7) *
Azithromycin + VRC	61.90±1.8 (212.8) *	23.4±1.40 (98.2)	10.05±0.3 (24.4) *

^aRadioactivity in sucrose gradient fractions corresponding to the top, 30S and 50S regions was summed and calculated as a percent of the total radioactivity in the gradient. Percentage of the control amount in the top, 30S and 50S regions is shown in parentheses. Results are the means ± standard error of 2 independent experiments.

(*) statistically significant with a *P* value <0.05.

Table 3

The amount and distribution of 23S, 16S, and small RNA species as determined by Agilent gel electrophoresis analysis

Treatment	% Total area ^a			
	RIN	Small RNA	16S rRNA	23S rRNA
Control	9.2±0.7	22.63±13.1	31.47±4.5	43.73±5.7
Paromomycin	6.5±0.1 (71.2)	23.30±2.3 (103.0)	15.35±0.2 (48.8)	13.05±0.2 (29.8) *
Azithromycin	6.1±0.1 (65.8) *	27.25±3.7 (120.4)	15.10±0.1 (48.0)	7.10±0.1 (16.2) *
VRC	9.6 ±0.5 (104.3)	13.53±3.0 (59.8)	24.57±2.6 (78.1)	35.93±10.1 (82.2)
Paromomycin + VRC	5.7±0.1 (62.0) *	38.55±0.2 (170.3)	12.00±0.1 (38.1)	6.35±0.1 (14.5) *
Azithromycin + VRC	5.6±0.1 (60.9) *	38.90±2.3 (171.9)	8.65±0.2 (27.5) *	4.50±0.1 (10.3) *

^aThe amount of RNA in each gel region from Figure 4 was computed by analysis of the electropherograms using Agilent software. Percentage of the total RNA compared to the control in each gel region is shown in parentheses. The RNA integrity number (RIN) is indicated. Results are the means ± standard error of 2 independent experiments.

(*) statistically significant with a *P* value <0.05.

Table 4

Comparison of VRC effects on *E. coli* and *S. aureus* cells

Organism	Cell viability ^a	Top of gradient ^b	30S subunits ^b	50S subunits ^b	30S synthesis ^c	50S synthesis ^c	Protein synthesis ^d
<i>E. coli</i> Control	100	29.1	23.8	41.2	60	60	100
<i>E. coli</i> +VRC	86.7	56 (192)	17.8 (75)	22.7 (55)	240	240	150
<i>S. aureus</i> Control (MSSA)	100	33.2	21.7	41.6	60	60	100
<i>S. aureus</i> + VRC (MSSA)	5.3	57 (172)	14.6 (67)	24.5 (59)	90	120	116
<i>S. aureus</i> Control (MRSA)	100	30.7	17.5	47.9	60	60	100
<i>S. aureus</i> + VRC (MRSA)	1.6	44 (145)	17 (101)	33 (70)	120	120	200

^aPercent reduction in CFU for *E. coli* or *S. aureus* cells treated with 5mM VRC.^bPercent of the total radioactivity in sucrose gradient regions (top fractions, 30S and 50S). Percent of control value in parenthesis.^cSubunit synthesis times (min) for control and VRC treated cells from pulse and chase assays.^dRelative rates of protein synthesis for control and VRC treated cells.