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Antibiotics that Inhibit 30S or 50S Ribosomal Subunit Formation: Hygromycin B, Quinupristin-Dalfopristin and XRP 2868.

Susan Mabe McGaha

East Tennessee State University

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Antibiotics That Inhibit 30S or 50S Ribosomal Subunit Formation: hygromycin B, quinupristin-dalfopristin and XRP 2868

A dissertation

presented to

the faculty of the Department of Biochemistry and Molecular Biology

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Doctor of Philosophy in Biomedical Sciences

by

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December 2006

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Keywords: Ribosomal subunit assembly, Antibiotics, Translational inhibitors
Several antibiotics that prevent translation by binding to ribosomal subunits have been shown to also inhibit ribosomal subunit assembly (Champney and Tober 2003). The aminoglycoside hygromycin B was examined in *Escherichia coli* cells for inhibitory effects on translation and ribosomal subunit assembly. The streptogramin antibiotics quinupristin-dalfopristin and XRP 2868 (NXL 103) were examined for similar effects on these 2 cellular functions in antibiotic-resistant strains of *Haemophilus influenzae, Staphylococcus aureus*, and *Streptococcus pneumoniae*.

Pulse chase experiments were performed which verified slower rates of ribosomal subunit formation in drug treated cells. Hygromycin B exhibited a concentration dependent inhibitory effect on viable cell number, growth rate, protein synthesis and 30S and 50S subunit formation. 16S rRNA specific probes hybridized to rRNA fragments in cells treated with hygromycin B. RNase II and RNase III deficient strains of *E. coli* exhibited the most accumulation of 16S rRNA fragments upon treatment with hygromycin B. Examination of total RNA from
treated cells showed an increase in RNA corresponding to precursor to the 16S rRNA while 16S rRNA decreased. There was also an increase in small fragment RNA. Hygromycin B was a more effective inhibitor of translation than ribosomal subunit formation in *E. coli*.

Two streptogramin antibiotics were compared for inhibitory effects in antibiotic-resistant *Haemophilus influenzae, Staphylococcus aureus*, and *Streptococcus pneumoniae*. IC$_{50}$ values for XRP 2868 were several fold lower than those of quinupristin-dalfopristin for inhibition of cell viability, protein synthesis, and ribosomal subunit formation. Both antibiotics revealed a concentration dependent inhibitory effect on cellular functions including 50S ribosomal subunit formation in the three organisms examined.

XRP 2868 inhibited both 50S ribosomal subunit assembly and translation. XRP 2868 was effective against MRSA and was a better inhibitor in each of the antibiotic resistant strains examined compared with quinupristin-dalfopristin.
ACKNOWLEDGEMENTS

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CHAPTER 1
INTRODUCTION

Ribosomes are the biological structures responsible for the manufacture of proteins within cells. The bacterial ribosome is formed from two subunits having sedimentation coefficients of 30S (small subunit) and 50S (large subunit). These subunits are ribonucleoprotein complexes, consisting of ribosomal RNA (rRNA) and ribosomal proteins. Figure 1 illustrates the prokaryotic ribosome. For many years the ribosome function in translation has been a target for antimicrobial drugs. Overuse and negligent use of antibiotics coupled with the fast evolutionary rate of bacteria and their ability to adapt to new onslaughts have resulted in an increase in antimicrobial resistance creating a major problem for human welfare.

Currently, only a small number of antibiotics are available to treat multi-drug resistant strains of bacteria, but resistance to even the newest antimicrobial agents is appearing (Hancock 2005). Increasing antibiotic resistance in microbial populations has necessitated the search for alternate cellular targets for new and existing antimicrobial agents. One novel target is ribosomal subunit assembly. There are many antibiotics available that inhibit translation in bacterial cells by binding to the 30S or 50S ribosomal subunit. In several cases it has been observed that these drugs also posses a second inhibitory activity that is stopping ribosomal subunit assembly. The formation of a functional ribosome is
a vital cellular process and is therefore also an important cellular drug target (Champney 2003).

Figure 1  The Prokaryotic Ribosome. The prokaryotic ribosome is assembled in cells from 30S subunits made from 16S rRNA and proteins and 50S subunits consisting of 23S and 5S rRNA and proteins.
Several studies have been carried out with inhibitors of 50S and 30S subunit assembly. Recent work with the macrolides, ketolides, lincosamides and streptogramin B compounds has revealed that these antibiotics affect 50S subunit assembly specifically (Champney and Tober 1998; Champney and Tober 2000). Studies by Mehta and Champney (2002; 2003) also found that aminoglycosides have a second inhibitory target that is preventing 30S subunit formation. Figure 2 (A-B) illustrates a proposed model for inhibition of ribosomal subunit formation by inhibitors of 30S and 50S ribosomal subunits. Comparison studies with several antibiotics from each class have provided useful insights into structure-activity relationships that could lead to novel drug development targeting ribosomal subunit assembly. To broaden our understanding of subunit assembly inhibitors, it is important to examine new or poorly studied antibiotics for their possible effects on inhibition of subunit formation. Identifying and comparing these antimicrobial agents will aid in the search to find more suitable targets for future antibiotics.
Figure 2  Models for 30S and 50S ribosomal subunit assembly inhibition by antibiotics. Inhibition of 30S (A) and 50S (B) ribosomal subunit assembly. (i) Subunit formation in control cells (ii) Subunit formation in antibiotic treated cells. Antibiotics may bind to one of two inhibitory sites, the fully formed ribosomal subunit inhibiting translation (top panel), or an intermediate particle halting assembly of the ribosomal subunit (bottom panel).
There are many antibiotics that target translation by binding to ribosomal subunits that have not been examined for possible inhibitory effects on ribosomal subunit assembly. Among those antimicrobial agents are the aminoglycoside hygromycin B, a 30S subunit inhibitor, and the streptogramin compounds quinupristin-dalfopristin (Synercid) and XRP2868 (NXL 103) affecting the 50S subunit.

Hygromycin B has a structure that is different from most aminoglycosides. Classic aminoglycosides are characterized by two differential amino-sugar rings that are connected to a 2-deoxystreptamine ring (Figure 3A). The position at which the two variable rings are attached determines the class of aminoglycoside to which the compound belongs. Hygromycin B is unusual in its structure having instead two ether linkages connecting two of its three sugar residues resulting in a fourth, 5-membered ring (Figure 3B). This extended structure has been shown recently to bind to the 30S ribosomal subunit near the A, P and E sites and spans a 13 Å distance on the small subunit (Figure 4) (Brodersen and others 2000).

Hygromycin B has been in use since 1957 as the first additive in livestock feed to be approved by the Food and Drug Administration. It has been used extensively since then as a growth aid and parasite control agent in the production of swine and poultry. The compound removes parasites by disrupting the egg laying process and then killing the adults. Eliminating ova production ensures that pens and lots will not be contaminated with eggs, a problem associated with other one-dose parasite removal options (Kelly and Olsen 1960;
Biehl 1986; Lamb and others 1999). Hygromycin B has also been used in the laboratory to select for recombinant cells containing a hygromycin B resistance marker (Gritz and Davies 1983). Importantly it may also be an effective inhibitor of ribosomal subunit formation.

Figure 3 Chemical structures of aminoglycoside antibiotics. (A) The classical 2-deoxystreptamine aminoglycosides. (B) Hygromycin B has two ether linkages connecting two of its three sugar residues resulting in a fourth, 5-membered ring.
Figure 4  Crystal structure of hygromycin B bound to the small subunit. Hygromycin B is shown bound to *Thermus thermophilius* 30S subunit, yellow stick with color corresponding red (oxygen) and blue (nitrogen) balls. Ring structures are labeled 1-4. Green nucleotides represent the proposed decoding center, orange nucleotides represent mutation sites causing resistance to hygromycin B (*Cell* 2000; 103:1145).

Recent studies with the aminoglycosides neomycin and paromomycin have revealed that these drugs are capable of inhibiting 30S ribosomal subunit formation by stalling assembly at an intermediate phase of 30S formation (Mehta and Champney 2002; Mehta and Champney 2003). Previous Northern hybridization analysis with inhibitors of 50S ribosomal subunit assembly have
shown that these antibiotics cause a decrease in the amount of mature 23S rRNA and an accumulation of partially degraded 23S particles in antibiotic treated cells (Champney and Burdine 1996; Champney and Burdine 1998a; Champney and Miller 2002; Silvers and Champney 2005). This accumulation of rRNA fragments is the result of antibiotic binding to an intermediate particle in ribosomal subunit assembly. These particles are normally degraded by RNases as illustrated in Figure 2B. In strains deficient in RNases important for particle turnover this process is retarded and a build up of ribosomal RNA fragments from the stalled particle occurs. The resulting fragments are large enough to be examined by Northern hybridization analysis. Previous work has shown that RNase II and III and PNPase are heavily involved in the turnover of 23S rRNA in the precursor which forms in the presence of the 50S inhibitor azithromycin (Silvers and Champney 2005). The accumulation of 16S rRNA fragments during treatment with 30S ribosomal subunit inhibitors has never been examined. One goal of this study was to determine if the 30S ribosomal subunit inhibitor hygromycin B had any effect on accumulation of 16S rRNA fragments in E. coli cells with loss of function mutations in RNase I, RNase III, RNase II, and PNPase mutant strains. The structure and mechanism of binding of hygromycin B sets the compound apart from other aminoglycosides; therefore, it is of interest to examine the effect these differences have on inhibition of subunit assembly and its effects on turnover of 16S rRNA oligonucleotide accumulation in hygromycin B treated cells.
Other antibiotics with the potential to be specific inhibitors of ribosomal subunit formation are the streptogramin compounds quinupristin-dalfopristin and XRP 2868. The chemical structures of these antibiotics are shown in Figure 5. Streptogramin compounds have been isolated from naturally occurring sources and have been used clinically in parts of Europe as antimicrobial agents for more than 20 years (Leclercq and Courvalin 1998). Streptogramin antibiotics are a combination of two chemically distinct compounds that are administered clinically in a 30:70 weight-to-weight ratio of group B to group A. Each streptogramin antibiotic is composed of a group B (or type I) and a group A (or type II) streptogramin. Each group has a unique binding site. Each compound alone has a bacteriostatic effect, but when combined the two groups act synergistically to produce bactericidal activity (Allignet and others 1996; Malbruny and others 2002). Group B streptogramins have been shown to bind to a site on the 50S ribosome and block the peptide exit tunnel. Group A streptogramins bind within the peptidyl transferase center and interfere with positioning of substrate in the A and P sites. The binding of group A streptogramins also increases the binding affinity of group B streptogramins giving a synergistic inhibitory effect. The crystal structure of quinupristin-dalfopristin binding to the 50S subunit of Deinococcus radiodurans has been resolved and is pictured in Figure 6 (Harms and others 2004).

Historically streptogramin compounds have been limited in their use because of limited solubility; however, two new streptogramin compounds
(quinupristin-dalfopristin and XRP2868) have been introduced that have increased solubility and may be administered intravenously and orally respectively. Quinupristin-dalfopristin is a combination of quinupristin (derived from pristinamycin IA) a group B streptogramin, and dalfopristin (derived from pristinamycin IIA) a group A streptogramin that have been approved for clinical use in the United States. Quinupristin-dalfopristin has been indicated for treatment of vancomycin-resistant Enterococcus faecium and treatment of complicated skin infections cause by Staphyloccus aureus or Streptococcus pyogenes (Lamb and others 1999). Quinupristin-dalfopristin has been shown to be effective in treatment of antibiotic resistant infections where few other treatment options exist (Low 1995; Drew and others 2000). A recent study using a S. aureus biofilm model has shown quinupristin-dalfopristin to be more effective than many other antibiotics against bacteria in biofilms (Pfeil and Wiedemann 2000). XRP 2868 has also been shown to have good inhibitory activity against a wide range of Gram-positive pathogens and some Gram-negative respiratory tract pathogens. XRP 2868 is one of only three antibiotics from new classes of antimicrobial agents that have been released in the past 40 years (Lamb and others 1999).

XRP 2868 is a mixture of 2 streptogramin compounds, RPR 132552A and RPR 202868, that are chemically modified forms of quinupristin and dalfopristin respectively (Figure 5). The structural changes of XRP 2868 from quinupristin-dalfopristin lead to better water solubility and efficacy of an oral administration
route. Pankuch and others (2003) compared XRP 2868 with quinupristin-dalfopristin in Gram-positive pneumococci and *Haemophilus*. Minimum inhibitory concentrations for XRP 2868 were 2 fold lower than quinupristin-dalfopristin for the inhibition of pneumococcal growth and 4-8 fold lower for inhibition of *Haemophilus* strains (Pankuch and others 2003). XRP 2868 is not yet available clinically but shows superiority over quinupristin-dalfopristin in its inhibitory activities against important resistant strains of pathogenic bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), erythromycin-resistant *Streptococcus pneumoniae* and β-lactamase-positive *Haemophilus influenzae* (Pankuch and others 2003; Eliopoulous and others 2005; Goldstein and others 2005; Mabe and Champney 2005). XRP 2868 has also been shown to be 4 fold more effective than quinupristin-dalfopristin for inhibition of *Enterococcus faecium* and exhibited greater efficacy in inhibition of *Enterococcus faecalis* (Eliopoulous and others 2005). In a murine lung and thigh infection model the antibiotic has shown great effectiveness in clearing infection by multi-resistant strains of *S. pneumoniae* and *S. aureus* (Andes and Craig 2006).

Quinupristin-dalfopristin is already proving to be an important antibiotic in the last line of defense against multi-drug resistant microbes. XRP 2868, pending clinical trials and approval from the Food and Drug Administration, will likely also be an important resource in the clinical arsenal against the increasing threat of multi-drug-resistant strains of pathogenic bacteria. It is possible that inhibition of subunit formation could play a role in the success of both
quinupristin-dalfopristin and XRP 2868 in inhibiting the growth of these organisms. More potent subunit assembly inhibition is also a possible mechanism for the increased activity of XRP 2868 against microorganisms in comparison to quinupristin-dalfopristin. Understanding the underlying mechanisms behind the inhibitory effects of these new compounds is crucial for improved drug design. Furthermore, it is important to determine the effects of other antimicrobials on subunit formation in order to realize the potential of this new antibiotic target.

Figure 5  Structures of streptogramin antibiotics. (a) quinupristin, (b) dalfopristin, (c) RPR 202868, and (d) RPR 132552A.
Figure 6  Crystal structure of quinupristin-dalfopristin bound to the large subunit. Quinupristin-dalfopristin is shown bound to the 50S ribosomal subunit of *Deinococcus radiodurans* in relation to the P-site and the ribosomal exit tunnel (gold) (*BioMed Central* 2004; 2:4).

The goals of this study were to determine the effects of XRP 2868 and quinupristin-dalfopristin upon cellular functions in methicillin-resistant *Staphylococcus aureus*, β-lactamase-positive *Haemophilus influenzae*, and erythromycin-resistant *Streptococcus pneumoniae*. The study also examined the effects of hygromycin B upon cellular functions in *Escherichia coli* including the effect of hygromycin B on the accumulation of 16S rRNA fragments in *E. coli*
strains treated with the antibiotic. Hygromycin B, quinupristin-dalfopristin, and XRP 2868 have not been examined for inhibitory activity against ribosomal subunit assembly. I hypothesized that the translational inhibitors examined here also possess a second target in inhibiting ribosomal subunit formation. The streptogramin compounds XRP 2868 and quinupristin-dalfopristin that bind to the 50S ribosomal subunit target the 50S ribosomal subunit in assembly. The aminoglycoside hygromycin B binds to the 30S and specifically targets this subunit in assembly. I also hypothesized that hygromycin B would cause an accumulation of 16S rRNA fragments in cells treated with the antibiotic and that this accumulation could be detected by using a Northern blotting procedure. An additional expectation was that of 16S rRNA fragments would be higher in strains missing the RNases that are necessary for turnover of stalled 16S rRNA particles. An increase in 16S precursor and small fragment RNA and a decrease in 16S rRNA in total RNA isolated from cells treated with hygromycin B was also hypothesized.

Several methods were used to test these hypotheses. Each drug’s mechanism of action was measured via a four-part assay experiment that was used to determine effects on cellular processes including growth rate, rate of protein synthesis by $^{35}$S-methionine incorporation, rate of subunit formation by $^3$H-uridine incorporation into rRNA and cell viability. Pulse chase kinetic analysis with $^3$H-uridine labeling was used to measure rates of subunit formation in cells. An examination of rRNA turnover in cells treated with hygromycin B was also
performed. Northern blotting techniques with a 16S rRNA specific biotin labeled probe were used to show accumulation of 16S rRNA in cells treated with hygromycin B. RNase deficient strains were also examined to determine the enzymes that may be involved in turnover of stalled 16S precursor particle. Total RNA was also examined using an Agilent Bioanalyzer 2100. For each assay, comparisons were made between inhibitory effects on protein synthesis relative to subunit assembly inhibition. Information from these studies will aid in understanding structure-activity relationships for ribosomal subunit assembly inhibition and can also help elucidate the mechanisms cells have for recycling rRNA that has been bound by antibiotic. Comparing inhibitory activities of different types of antibiotics also provides information useful in determining the value of ribosomal subunit assembly inhibition as a possible target for future drug development.
CHAPTER 2
MATERIALS AND METHODS

Materials

XRP 2868 and quinupristin-dalfopristin were supplied by Aventis Pharmaceuticals and were used as a 70:30 mixture of the A- and B-streptogramins. Stock solutions of all antibiotics were made at 10 mg/ml in distilled H$_2$O. Lysozyme, thymine, lipo-proteins, hemin, nicotinamide adenine dinucleotide, and hygromycin B were purchased from Sigma Chemical Corporation. Tryptic soy broth, tryptone peptone, agar, agarose, sucrose, Scintisafe Gel scintillation fluid, sodium dodecyl sulfate, 3-(N-Morpholino)-propanesulfonic acid, trichloro-acetic acid, 20X saline-sodium citrate, formaldehyde, formamide, methanol, deoxycholic acid sodium salt, phenylmethanesulfonyl fluoride, isopropanol, Mirus Label-IT® biotin labeling kit, Fuji medical X-ray film, and Kodak GBX developer and fixer were purchased from Fisher. AquaPure RNA isolation kit was purchased from Bio-Rad. Nytran SPC nylon transfer membranes were purchased from Scheicher & Schuell. The North2South® chemiluminescent nucleic acid hybridization and detection kit was purchased from Pierce. Washing & Pre-Hybridization solution and background quencher were purchased from Molecular Research Center, Inc. PCR primers
were obtained from Life Technologies. The PCR Super-mix was purchased from Gibco BRL. The GF/A glass fiber filters and blotting paper were purchased from Whatman International. $^3$H-uridine (45 Ci/mmol) was purchased from New England Nuclear. $^{35}$S-methionine (TRANS$^{35}$S-LABEL 1175 Ci/mmol) was purchased from MP Biomedical. Low range, ready-to-use RNA ladder was purchased from Fermentas.

Cricket Graph III software (Computer Associates) was used to construct graphs and curves and for calculations. Curve fitting was also performed through Cricket Graph software. Curve fits were chosen according to best fit.

**Media**

Tryptic Soy Broth: 30 g tryptic soy broth in 1 L dH$_2$O.

Tryptic Soy Broth Plates: 1 L tryptic soy broth and 15 g agar.

5X A-salts: 52.5 g K$_2$HPO$_4$, 22.5 g KH$_2$PO$_4$, 5 g (NH$_4$)$_2$SO$_4$, and 2.5 g NaCitrate·(2H$_2$O) to 1 L dH$_2$O

Buffers

S-Buffer: 10 mM Tris-HCl (pH 8.0), 50 mM NH$_4$Cl and 0.5 mM Mg Acetate.

10X MOPS Buffer: 0.2 M MOPS (pH 7.0), 20 mM sodium acetate, 10 mM EDTA (pH 8.0).
RNA Resuspension Buffer: 150 µl formamide, 36 µl formaldehyde, 30 µl 10X MOPS buffer.

RNA Running Buffer: 900 ml sterile dH₂O, 100 ml 10X MOPS buffer.

Alkaline Transfer Buffer: 3 M NaCl, 8 mM NaOH, and 2 mM Sarkosyl.

5X Neutralizing Buffer: 79.25 g Na₂HPO₄, 60.25 g NaH₂PO₄ in 1 L dH₂O

Formamide Hybridization Buffer: 50% formamide, 5X SSC, 0.1 % sarkosyl, 0.02% SDS, 200 µg/ml BSA, with 1X background quencher.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

**Bacterial Strains**

Several bacterial strains were used in this study. Those strains are listed in Table 1.

**Table 1** Strains of bacteria used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
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<td><em>Escherichia coli</em> D10</td>
<td>HfrH <em>met</em> <em>rnr</em> 1</td>
<td>RNase 1†</td>
<td>(Gesteland 1966)</td>
</tr>
<tr>
<td></td>
<td><em>relA</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>F* Δpnp::kanR</td>
<td>PNPase †</td>
<td>(Reuven and Deutscher 1993)</td>
</tr>
<tr>
<td>CA244</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Name</td>
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<td>Phenotype</td>
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<tr>
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<tr>
<td><em>Escherichia coli</em> SK7622</td>
<td>F^- thyA715</td>
<td>RNase III^-</td>
<td>(Babitzke and others 1993)</td>
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<tr>
<td><em>Escherichia coli</em> SK4803</td>
<td>mcD38::kanR</td>
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<td></td>
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<tr>
<td><em>Escherichia coli</em> SK4803</td>
<td>gal thi ton sup</td>
<td>RNase II^-</td>
<td>(Donovan and Kushner 1986)</td>
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<tr>
<td><em>Staphylococcus aureus</em> A1024</td>
<td>hasdR4</td>
<td>Methicillin-resistant</td>
<td>(Champney and Burdine 1998b)</td>
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<tr>
<td><em>Streptococcus pneumoniae</em> 11591</td>
<td>ErmB^+</td>
<td>Erythromycin-resistant</td>
<td>(Champney and others 2004)</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em> G79-84</td>
<td>□-lactamase^+</td>
<td>Methicillin-resistant</td>
<td>(Champney and Tober 2003)</td>
</tr>
</tbody>
</table>
Methods

MIC Determination

The minimal inhibitory concentration for each antibiotic was determined by a broth dilution method as described (Champney and Burdine 1998a). Six test tubes were filled with 1 ml of TSB. Each tube received 50 µl of an overnight culture of bacterial cells and antibiotic over a range of concentrations. The tubes were incubated at 37°C overnight and the absorbance at 600 nm was measured.

Analysis of Cell Growth and Cell Viability

Cell cultures except *S. pneumoniae* were grown in a water bath at 37°C in TSB in the presence or absence of antibiotic according to the method of Champney and Burdine (1998a). *S. pneumoniae* was grown in TSB at 37°C in 8 ml screw cap tubes. Media were supplemented with 50 µg/ml of thymine for *E. coli* strain SK7622 and with 10 µg/ml of hemin and nicotinamide adenine dinucleotide and 0.7% lipo proteins for *H. influenzae*. Growth was initiated by adding cells from an overnight culture (~0.1-0.2 ml) to TSB growth media. Growth rates were measured by recording the increase in cell density over time using a Klett-Summerson colorimeter. Cell growth was monitored over two cell doublings at which point 10 µl was removed from the growing culture and added to 990 µl of A-salts. A serial dilution was performed to achieve a final dilution of $10^{-5}$ and 10 µl was plated on square TSB agar plates (Jett and others 1997).
TSB agar plates for *E. coli* SK7622 and *H. influenzae* were supplemented with Thymine at 50 µg/ml. Plates were incubated for 24-48 hours at 37°C. Colonies were counted from control and drug treated samples to determine the effect of the antibiotics on the viable cell number.

**Analysis of the Rate of Protein Synthesis**

Cell cultures were grown as described above. After 2 cell doublings in the presence or absence of antibiotic, $^{35}$S-methionine was added to the culture at a concentration of 1 µCi/ml. Three samples of 0.4 ml were removed at 5 minute intervals after addition of the $^{35}$S-methionine. Cells were precipitated in 20% TCA and collected on Whatman GF/A glass fiber filters. Filters were washed with 10% TCA and 97% ethanol to ensure there was no free $^{35}$S-methionine on the filters. Filters were air dried under a heat lamp and placed in vials with 3 ml Scintisafe fluid before being measured for radioactivity by liquid scintillation counting.

**Analysis of Ribosomal Subunit Assembly**

All bacterial cells were grown as described above. For all strains except *S. pneumoniae*, a 30 ml culture of bacterial cells in TSB was started from an overnight culture. Cells were grown to a Klett of approximately 20 and the 30 ml culture was split into six 5 ml cultures. *S. pneumoniae* was grown in TSB at 37°C in 8 ml screw cap tubes. Antibiotic was added in different concentrations to
appropriate cultures. After 15 minutes of growth in the presence of antibiotic,
$^3$H-uridine (1 µCi/ml) and 1 µg/ml uridine was added to the control and antibiotic
treated samples. In order to halt further isotope incorporation after 2 cell
doublings, uridine (50 µg/ml) was added in excess to each culture. After a 15-
minute chase period, cells were spun in a Beckman centrifuge (J2-21) at 6,000
rpm for 12 minutes in a JA21 rotor. Cell pellets were washed with sterile S-buffer
or SAS-buffer ($S. aureus$, and $S. pneumoniae$). Washed pellets were spun again
at 6,000 rpm for 12 minutes. The cell pellets were stored at -70°C before cell
lysis.

Different lysis procedures were used for the various strains of bacteria.
Washed cell pellets were thawed at room temperature. $E. coli$ and $H. influenzae$
cell pellets were re-suspended in 200 µl of S-buffer and 10 µl of a 5 mg/ml
solution of lysozyme was added. $S. aureus$ was re-suspended in 200 µl of SAS-
buffer with 20 µg lysostaphin and 15 µl of 0.1M phenylmethanesulfonyl fluoride in
isopropanol. $S. pneumoniae$ was re-suspended in 300 µl of SAS-buffer, 300 µg
lysozyme and 30 µl of 0.1M PMSF. Suspended $E. coli$, $H. influenzae$, and $S.$
aureus cells were allowed to incubate at room temperature for 15 to 20 minutes
and were then subject to a freeze-thaw process. $S. pneumoniae$ was incubated
for 30 minutes at 37°C. After the incubation, 100 mg 0.1 mm sterile glass beads
and 15 µl of 10% deoxycholic acid sodium salt were added. Cells were frozen for
5 minutes at -70°C and then thawed at room temperature. This procedure was
repeated twice. The $S. pneumoniae$ cells were vortexed for 1.5 minutes after
each freeze thaw until the lysate was viscous. DNA was digested in cell lysates by adding 1-2 units of RNase free DNAses to each sample. The samples were spun at 6,000 rpm for 12 minutes to clear the lysate of cell debris.

To examine the amount of subunits in cells and the effects of antibiotics on subunit assembly, cell lysates were centrifuged on linear sucrose gradients. Linear sucrose gradients of 5-20% were prepared to separate ribosome particles. The cleared supernatant was loaded on the top of the 5-20% linear sucrose gradients. Gradients were made using a Buchler gradient maker and 6 ml each of 5% and 20% sucrose in S- buffer for *E. coli* and *H. influenzae* and SAS-buffer for *S. aureus* and *S. pneumoniae*. The prepared gradients were placed in a SW40 swinging bucket rotor and centrifuged in a Beckman LE80K Ultracentrifuge at 39,000 rpm for 4.5 hours or 18,000 rpm for 18 hours. An ISCO Model UA-5 absorbance monitor was used to measure and record the absorbance at 254 nm of the gradients as they were pumped through. Fractions of equal amounts were collected into vials and mixed with 3 ml of Scintisafe gel. The incorporation of $^3$H-uridine into RNA was then measured by liquid scintillation counting set for dual labeled samples to distinguish $^3$H from $^{35}$S.
3H-uridine Pulse-Chase Kinetic Analysis of Ribosomal Subunit Formation

*E. coli* cells were grown in 24 ml of TSB to approximately 10 Klett units at 27°C. 50 µg/ml of hygromycin B was added to the culture. The control sample was grown in the absence of the antibiotic. When the cell density reached a Klett reading of 30, cells were pulse-labeled with 3H-uridine (1 µCi/ml) for 3 minutes and then chased with uridine at 50 µg/ml. Samples of 4 ml were taken from the cultures at intervals and centrifuged at 6000 rpm for 12 minutes. Cell pellets were washed and stored at -70°C before cell lysis for sucrose gradient centrifugation and liquid scintillation counting as described above.

Construction of Biotinylated 16S and 23S rRNA Specific Probes

Polymerase chain reaction (PCR) was used to amplify the 16S (241 base pair) and 23S (146 base pair) specific probes from plasmid pKK3535 DNA. PCR reaction mixtures contained 45 µl PCR Supermix High Fidelity reagent mixture (Gibco BRL), 1 µl of plasmid DNA (6.5 ng), 1 µl (10 pmol) of either the 16S or 23S forward primer, 1 µl (10 pmol) of either 16S or 23S reverse primer, and 2 µl sterile dH₂O. Primer sequences are given in Silvers and Champney (2005). Samples were placed in a MJ Research PTC-100 programmable thermocycler for 35 cycles under the following conditions: denaturation at 94°C for 30 seconds, annealing of primers to target DNA at 57°C for 30 seconds, and extension of the primers at 72°C for 30 seconds. The PCR products were purified by extraction with an equal volume of phenol:CHCl₃ and precipitated with
2 volumes of pure ethanol. The pellets were dried at 44°C for 15 minutes then resuspended in 30 µl of sterile dH₂O. Purity of the PCR products was examined by running 1 µl on a 2% agarose gel. The purified DNA probes were labeled with biotin using the Label-IT biotin labeling kit (Mirus) following the manufacturer’s instructions. Specificity of each probe was tested by hybridization with 23S and 16S rRNA as shown in Figure 7. Detection of the biotin label is carried out by using a streptavidin horseradish peroxidase conjugate as illustrated in Figure 8.

![Figure 7](image)

Figure 7  Assay for specificity of 16S and 23S biotin labeled probes. (A) Hybridization of 16S rRNA specific probe with 16S and 23S rRNA. (B) Hybridization of 23S rRNA specific probe with 16S and 23S rRNA.
Figure 8 Detection of rRNA via biotin labeled 16S internal probe. Streptavidin horseradish peroxidase conjugate is used to illuminate biotin labeled probe positions upon addition of peroxide substrate.

Isolation of Small RNA and Total RNA

*E. coli* cells were grown in 10 ml of TSB. Cells were harvested and lysed, and lysates were centrifuged through S-buffered sucrose gradients as described above. The top fractions of sucrose gradients were taken and RNA was isolated from these fractions by phenol and chloroform extraction. One volume of absolute ethanol and Mg acetate to 0.01 M was added to the pool of fractions and was allowed to stand at -20°C for 15 minutes to precipitate the RNA. Samples were spun at 7,000 rpm for 30 minutes and the supernatant was
discarded. The RNA pellet was resuspended in 0.5 ml TE buffer and transferred to a 1.5 ml tube. The samples were vortexed with 5 µl 10% SDS and 0.5 ml phenol and spun in a microcentrifuge at 10,000 rpm for 5 minutes. The aqueous layers were extracted and placed into new microcentrifuge tubes and the phenol extraction repeated. The aqueous layers were placed into new tubes and 0.5 ml of chloroform was added. The samples were vortexed and spun at 10,000 rpm for 5 minutes in a microcentrifuge. The chloroform extraction was also performed twice. Aqueous layers were removed into new tubes and 5 µl of 5 M NH₄ acetate was added and the tubes were filled with absolute ethanol, mixed, and allowed to stand at -70°C for 30 minutes. Samples were spun in a cold room at 10,000 rpm for 10 minutes. After the addition of 0.5 ml of 70% ethanol, the samples were spun again at 10,000 rpm for 10 minutes. Ethanol was decanted and RNA pellets were dried until no ethanol was detectable. RNA was re-suspended in 25 µl of sterile water.

Total RNA was isolated from cell pellets using the AquaPure RNA isolation kit from Bio-Rad according to the manufacturer’s directions.

Northern Analysis of 16S rRNA

RNA was isolated as described above. The RNA (2.5-5 µg) was mixed with 15 µl RNA resuspension buffer, heated at 55°C for 15 minutes, and then quickly cooled on ice. Loading dye 5 µl (80% glycerol, 1% bromo-phenol blue) was added to the samples before loading on 1.5% agarose gels. Top samples
from gradients were run for 2 hours, while total RNA samples were run for 4 hours at 50 volts. A low range RNA ladder (Fermentas) was biotin labeled using the LabelIt Kit from Mirus according to the manufacture’s directions and added to one well for determining size of RNA fragments. RNA in the gels was examined by soaking in 1% ethidium bromide solution and examination by UV light.

After electrophoresis, RNA from the gels was blotted onto nylon membranes (Nytran) using a Turboblot apparatus. Following the manufacturer's directions, alkaline transfer buffer was used to carry out the transfer. After the transfer, gels were checked for RNA. The membranes were neutralized in 1X neutralizing buffer and the RNA was cross-linked to the membranes using a UV oven (Fisher-Biotech). The membranes were placed in 50 ml plastic corex tubes with 15 ml of 1X prehybridization solution (MRC, Inc.) and allowed to incubate at 42°C for 30 minutes in a hybridization incubator (Fisher-Biotech). The pre-hybridization buffer was discarded and the membranes were hybridized overnight at 42°C in the hybridization incubator with hybridization buffer, with 1X background quencher (MRC Inc.) and 4 pmol of denatured 23S or 16S probe. The probe was denatured by mixing with 0.1 volume of Mirus Denaturation Buffer D1 and incubating at room temperature for 5 minutes. The solution was chilled on ice and mixed with 0.1 volume Mirus Neutralization Buffer N1 and incubated at room temperature for 5 minutes.

Following hybridization, the membranes were washed and the probe detected using Pierce’s North2South chemiluminescent hybridization and
detection kit according to the manufacturer’s instructions. The membrane was covered with plastic wrap on a glass plate and exposed to Fuji Medical X-ray film. X-ray film was developed by soaking the film in Kodak GBX developer for 1-5 minutes, rinsing in H₂O, soaking in fixer and replenisher for 1-5 minutes, followed with a final rinsing in H₂O.

**Analysis of Total RNA Via Aligent Bioanalyzer**

*E. coli* cells were grown as described previously with various concentrations of hygromycin B and without hygromycin B. Total RNA was extracted as described above. Total RNA was examined using an Aligent Bioanalyzer 2100 and the RNA 6000 lab on a chip. Five microliters (200 ng/µl) of sample from total RNA was loaded onto each well of the RNA 6000 chip. Sample prep, loading procedure, and run were carried out according to manufacturer’s recommendations for total RNA analysis.
CHAPTER 3

RESULTS

Effects of Hygromycin B on Growth Rate, Protein Synthesis, Cell Viability, and Ribosomal Subunit Formation in *Escherichia coli*

**MIC Determination of Hygromycin B in *E. coli***

Minimal inhibitory concentration (MIC) represents the minimal concentration of antibiotic that will halt visible cell growth. An MIC value was determined prior to other experiments in order to find a suitable concentration range of antibiotic. The MIC value for hygromycin B in *E. coli* D10-1 growing in TSB at 37°C was 150 µg/ml (Table 2). Sub-inhibitory concentrations of drug were chosen based on the results of the MIC. Sub-inhibitory amounts of drug suppress cell growth without halting growth completely. The sub-inhibitory concentrations chosen based on the MIC value spanned a range from 0-100 µg/ml.

**Effects of Hygromycin B on Protein Synthesis**

Aminoglycoside antibiotics are well-known inhibitors of translation in bacteria cells. The rate of protein synthesis in growing *E. coli* cells was examined by measuring the incorporation of $^{35}$S-methionine into cellular proteins. Figure 9A shows the rate of incorporation of $^{35}$S-methionine with increasing concentrations of hygromycin B. The inhibitory effect on protein synthesis is
represented in Figure 9B as a percent of control \[^{35}\text{S}-\text{methionine incorporation at 15 minutes graphed as a percent of control protein synthesis}].\] The IC\(_{50}\) is given in Table 2.

![Graph A: Time-dependent inhibition of \[^{35}\text{S}-\text{methionine incorporation in E. coli D10-1 cells treated with hygromycin B at (■) 0 µg/ml, (□) 15 µg/ml, (□) 30 µg/ml, (□) 45 µg/ml, (□) 60 µg/ml, (●) 75 µg/ml.}]

![Graph B: Concentration-dependent inhibition of \[^{35}\text{S}-\text{methionine incorporation in E. coli D10-1 cells at 37 °C treated with hygromycin B graphed as a percent of control protein synthesis. Arrow indicates IC}_{50}\] value. Bars indicate standard error. Results are the mean of two experiments.}
Table 2  MIC and IC\textsubscript{50} values for hygromycin B in \textit{E. coli} cells.

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>\textsuperscript{a}Cell Number</th>
<th>\textsuperscript{b}Growth Rate</th>
<th>\textsuperscript{c}Protein Synthesis</th>
<th>\textsuperscript{d}30S subunit amount</th>
<th>\textsuperscript{d}50S subunit amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>20</td>
<td>25</td>
<td>16</td>
<td>65</td>
<td>45</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Cell number was determined by colony counting after dilution of $10^{-5}$ and plating on TSB agar plates. 
\textsuperscript{b} Growth rate was determined by cell density (measured in Klett units) over time. 
\textsuperscript{c} Protein synthesis rates was determined by $^{35}$S-methionine incorporation. 
\textsuperscript{d} Subunit formation was determined by $^3$H-uridine incorporation.

Effects of Hygromycin B on Growth Rate and Cell Viability.

The inhibition of cell viability and cellular growth rate is consistent with the demonstrated inhibitory effects of hygromycin B on translation. As shown in Figures 10A and 10B, hygromycin B diminished the number of viable cells and increased the doubling time in a concentration dependent fashion. Hygromycin B inhibited viable cell numbers by 50\% at a concentration of 20 µg/ml. Growth rate was reduced by half at a concentration of 25 µg/ml.
Figure 10  Inhibition of growth rate and cell viability in *E. coli* cells with increasing concentrations of hygromycin B. (A) Percent decrease in growth rate with increasing concentrations of hygromycin B (■). (B) Percent decrease in viable cell numbers with increasing concentrations of hygromycin B (■). Arrows indicate IC$_{50}$ values. Bars indicate standard error. Results are the mean of two determinations.

**Inhibition of Ribosomal Subunit Formation by Hygromycin B**

The effect of hygromycin B on ribosomal subunit formation was examined in growing *E. coli* cells. The concentration dependence of ribosomal subunit assembly was measured by examining sucrose gradient profiles of ribosomal subunits labeled with $^3$H-uridine during growth in the presence and absence of hygromycin B. Figures 11A-F show sucrose gradient profiles of lysates from cells grown without antibiotic or in the presence of the drug. The 30S ribosomal subunit amount was reduced more so than the 50S amount initially. There was
also an increased amount of fragmented RNA that accumulated in the top fractions of the gradients from cells treated with hygromycin B. Figure 12 shows the concentration dependent inhibitory action on assembly of the 30S ribosomal subunit by hygromycin B. Increasing concentrations of the antibiotic lowered 30S particle amounts in growing *E. coli* cells. The amounts of 50S ribosomal subunits were also reduced. The decline in 50S ribosomal subunit amounts was most apparent at higher concentrations of hygromycin B. IC$_{50}$ values for inhibition of ribosomal subunit formation are given in Table 2.

**Pulse-chase kinetic analysis of ribosomal subunit assembly**

Pulse-chase kinetic analysis was also used to measure the rates of ribosomal subunit synthesis in growing cells. Figures 13A and 13B show the pulse chase kinetic analysis of ribosomal subunit formation in control and hygromycin B treated cells. In the absence of the antibiotic 30S ribosomal subunit formation was complete in 15 minutes and 50S subunit formation reached a plateau in 30 minutes. When cells were treated with hygromycin B, 30S subunit formation did not reach control levels until 60 minutes and 50S ribosomal subunit amounts were estimated to reach control levels in approximately 120 minutes. 30S and 50S ribosomal subunit formation rates were inhibited equally relative to control rates.
Figure 11  Effect of hygromycin B on ribosomal subunit amounts.  (A) Sucrose gradient profiles of *E. coli* grown in the absence of antibiotic and in the presence of (B) 15 µg/ml hygromycin B (C) 30 µg/ml hygromycin B.  (D) 45 µg/ml hygromycin B (E) 60 µg/ml hygromycin B  (F) 75 µg/ml hygromycin B.
Figure 12  Concentration dependent inhibition of ribosomal subunit assembly in *E. coli* cells at 37°C treated with hygromycin B. Inhibition of 30S assembly (●), and inhibition of 50S assembly (○). Arrows indicate IC$_{50}$ values for 30S (red arrow) and 50S (green arrow) ribosomal subunit formation. Results are the mean of two determinations. Bars indicate standard error.
Figure 13 \(^3\)H-uridine pulse and chase labeling kinetic analysis of ribosomal subunit formation. Ribosomal subunit assembly kinetics in *E. coli* at 27 °C. (A) Assembly of 30S subunits (●) and 50S subunits (▲) without hygromycin B. (B) Assembly of 30S subunits (●) and 50S subunits (▲) with hygromycin B (50 µg/ml). Results are the mean of two determinations. Bars indicate standard error.

**Northern Hybridization Analysis of 16S rRNA Fragmentation**

Previous work with 50S ribosomal subunit inhibitors has shown that 23S rRNA fragments accumulate upon drug treatment. These are in greater amounts in *E. coli* strains containing one or more mutations in specific RNase genes that may be involved in the turnover of antibiotic-stalled rRNA (Silvers and Champney 2005). RNase II, RNase III, and PNPase were shown to be involved in the turnover of stalled 23S rRNA. Studies examining paromomycin and neomycin have revealed an accumulation of fragmented RNA in the top portion of gradients from cells treated with these aminoglycoside antibiotics (Mehta and Champney
Broken down rRNA as well as smaller tRNA and mRNA sediment in the top fractions of sucrose gradients. It is apparent from Figure 11(A-F) that in cells treated with hygromycin B there are substantially higher amounts of RNA oligonucleotides in the top fractions compared to 30S and 50S subunit amounts. My goal was to determine if the 30S ribosomal subunit inhibitor hygromycin B had any effect on accumulation of 16S rRNA fragments and to determine the enzymes involved in turnover of 16S rRNA.

Analysis by Northern hybridization with 16S and 23S rRNA-specific probes was performed on RNA isolated from the top fractions of sucrose gradients of lysates from *E. coli* strains grown with hygromycin B. The ribonuclease mutant strains examined in this study included D10-1 (RNase I−), SK4803 (RNase II−), CA244 (PNPase−), and SK7622 (RNase III−) deficient strains. Table 1 lists the strains of *E. coli* used in this experiment along with their respective phenotype and genotypes. Figure 14 shows the autoradiograph of the Northern blots hybridized with the 16S and 23S rRNA specific probes. Hybridization of 16S rRNA specific probe with rRNA isolated from the top fraction occurred in each strain that had been treated with hygromycin B. SK7622 and SK4803 strains contained larger amounts of 16S fragments in comparison to D10-1. D10-1 exhibited very little accumulation of 16S oligonucleotides with or without the presence of hygromycin B. RNase III deficient SK7622 had particularly high amounts of 16S fragmentation. The size of RNA fragments hybridized to 16S rRNA specific probes were estimated to be 900-200
nucleotides. Size was calculated using a low range RNA ladder (Fermentas) that was biotin labeled using the Mirus Label-It kit. No accumulation of 23S RNA was visualized in the top fractions (Figure 14B).

Figure 14  Northern hybridization analysis of rRNA. RNA was isolated from the top regions of sucrose gradients from cells grown without and with hygromycin B (50 µg/ml). The RNA was separated on a 1.5% agarose gel and a blot of the gel was hybridized with a biotin labeled 16S specific DNA probe (A) or a 23S specific DNA probe (B). 16S rRNA was used as a standard along with a biotin labeled low-range ladder.
Analysis of Total RNA Via Agilent Bioanalyzer

Total RNA was examined in *E. coli* cells treated with hygromycin B at various concentrations of antibiotic and in control cells using the RNA 6000 lab chip kit and Bioanalyzer 2100 (Agilent). With increasing concentrations of hygromycin B there was an increase in the amount of RNA accumulation corresponding to a 16S rRNA precursor. There also was a concentration dependent decrease in the amount of 16S ribosomal RNA present in the antibiotic treated cells along with an increase in the amount of small fragments of RNA. Table 3 lists the percentage of total area for each peak corresponding to small RNA, 16S rRNA, precursor to the 16S rRNA, and 23S rRNA. Figure 15(A-B) shows the fluorescent chromatograph and virtual gel from the Agilent Bioanalyzer analysis.

Table 3  Percent of total area for fluorescent chromatograph peaks representing small RNA, 16S rRNA, precursor for the 16S rRNA, and 23S rRNA from *E. coli* D10-1 with increasing concentrations of hygromycin B. Results are the mean of two determinations. Standard error of the mean is represented.

<table>
<thead>
<tr>
<th>Concentration of hygromycin B (µg/ml)</th>
<th>Small RNA</th>
<th>16S</th>
<th>Precursor to the 16S</th>
<th>23S</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>60.9±0.1</td>
<td>9.6±0.1</td>
<td>0.3±0.1</td>
<td>13±1.2</td>
</tr>
<tr>
<td>25</td>
<td>59±1.0</td>
<td>10.4±0.1</td>
<td>0.5±0.04</td>
<td>13.5±0.2</td>
</tr>
<tr>
<td>50</td>
<td>62.8±0.8</td>
<td>10.4±0.5</td>
<td>0.75±0.2</td>
<td>12.5±0.6</td>
</tr>
<tr>
<td>75</td>
<td>64.5±1.5</td>
<td>7.3±0.05</td>
<td>1.1±0.2</td>
<td>12.7±0.3</td>
</tr>
<tr>
<td>100</td>
<td>68.8±0.8</td>
<td>4.8±0.05</td>
<td>1.2±0.1</td>
<td>10±0.15</td>
</tr>
</tbody>
</table>
Figure 15  Agilent bioanalyzer analysis of total RNA from *E. coli* cells treated with hygromycin B.  (A) Fluorescent chromatograph showing the intensity of labeled RNA peaks in samples of total RNA extracted from control *E. coli* cells (red line) and cells treated 100 µg/ml of hygromycin B (blue line).  (B) Virtual gel produced by the Agilent software based on the results from the fluorescent chromatograph.
The Effect of Quinupristin-Dalfopristin and XRP 2868 on Cellular Growth Rates, Cell Viability, Protein Synthesis, and Ribosomal Subunit Formation in *H. influenzae, S. aureus* and *S. pneumoniae*.

**MIC Determination of Quinupristin-Dalfopristin and XRP 2868.**

Minimal inhibitory concentrations (MIC) for both antibiotics were determined for each organism and are listed in Table 4. The values were in good agreement with those found by Pankuch and others (2003).

**Effects of Quinupristin-Dalfopristin and XRP2868 on Protein Synthesis**

Streptogramin antibiotics are well-known inhibitors of translation in bacteria cells. Quinupristin-dalfopristin kills cells by inhibiting protein synthesis and 50S subunit formation (Lamb and others 1999; Champney and Tober 2000). Protein synthesis rates in growing cells were measured by the incorporation of $^{35}$S-methionine into cellular proteins. Figure 16 A-C illustrates the effect of increasing concentrations of each drug on the rate of translation. Both drugs inhibited translation at a lower concentration in *S. aureus* and *S. pneumoniae* compared with *H. influenzae*. XRP 2868 was the more effective inhibitor of protein synthesis in all three organisms examined. IC$_{50}$ values for translational inhibition are presented in Table 4. Inhibition of protein synthesis by each drug is represented as a percent of control $^{35}$S-methionine incorporation.
Figure 16 Inhibition of protein synthesis by quinupristin-dalfopristin and XRP 2868. Streptogramin concentration dependent inhibition of protein synthesis rates. Protein synthesis rates were measured by $^{35}$S-methionine incorporation in (A) *H. influenzae*, (B) *S. aureus* and (C) *S. pneumoniae* in the presence of increasing concentrations of XRP 2868 (○, dashed line) and quinupristin-dalfopristin (□, solid line). Power best fit lines are shown for *S. aureus* and *S. pneumoniae*. Linear best fit lines are shown for *H. influenzae*. Bars indicate standard error. Arrows indicate IC$_{50}$ values for quinupristin-dalfopristin (open arrows) and XRP 2868 (filled arrows). Results are the mean of three determinations.
Table 4  MIC and IC$_{50}$ values for streptogramin antibiotic inhibition of cell viability, protein synthesis and 50S ribosomal subunit formation in three microorganisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antibiotic</th>
<th>$^a$Cell Number</th>
<th>$^b$Protein Synthesis</th>
<th>$^c$50S subunit amount</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\textit{H. influenzae}$</td>
<td>Quinupristin-dalfopristin</td>
<td>2.2</td>
<td>3</td>
<td>3.6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>XRP2868</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>$\textit{S. pneumoniae}$</td>
<td>Quinupristin-dalfopristin</td>
<td>0.07</td>
<td>0.12</td>
<td>0.24</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>XRP2868</td>
<td>0.07</td>
<td>0.07</td>
<td>0.11</td>
<td>0.5</td>
</tr>
<tr>
<td>$\textit{S. aureus}$</td>
<td>Quinupristin-dalfopristin</td>
<td>0.13</td>
<td>0.11</td>
<td>0.2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>XRP2868</td>
<td>0.05</td>
<td>0.07</td>
<td>0.4</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ Cell number was determined by colony counting after dilution of $10^{-5}$ and plating on appropriate nutrient agar plates.

$^b$ Growth rate was determined by cell density (measured in Klett units) over time.

$^c$ Protein synthesis rates was determined by $^{35}$S-methionine incorporation.

$^d$ Subunit formation was determined by $^{3}$H-uridine incorporation.

Effects of quinupristin-dalfopristin and XRP 2868 on cell viability

The inhibition of cell viability is consistent with the demonstrated inhibitory effects of each antibiotic on translation. Quinupristin-dalfopristin and XRP 2868
both reduced the viable cell count in each organism tested in a concentration dependent manner, consistent with the inhibitory effects seen on protein biosynthesis. XRP 2868 exhibited the greatest effect on each organism examined. The results are shown in Figure 17 A-C and IC$_{50}$ values for the antibiotics are shown in Table 4. The growth rate of each organism also declined in proportion to the drug concentration (data not shown).

![Graphs showing concentration dependent inhibition of cell viability](image-url)

Figure 17 Streptogramin concentration dependent inhibition of cell viability. Inhibition of cell viability by colony counting in (A) *H. influenzae*, (B) *S. aureus* and (C) *S. pneumoniae* in the presence of XRP 2868 (○, dashed line) and quinupristin-dalfopristin (■, solid line). Linear best fit lines are shown for *H. influenzae* and power best fit lines are shown for *S. aureus* and *S. pneumoniae*. Bars indicate standard error. Arrows mark IC$_{50}$ values for XRP 2868 (filled arrows) and quinupristin-dalfopristin (hollow arrows). Results are the mean of three determinations.
Inhibition of ribosomal subunit formation by quinupristin-dalfopristin and XRP 2868

Each antibiotic was also examined for its effects upon ribosomal subunit formation. The concentration dependence of ribosomal subunit assembly was measured by examining sucrose gradient profiles of ribosomal subunits labeled with $^3$H-uridine during growth in the presence and absence of each drug in each organism. Both drugs showed a concentration dependent inhibition of 50S ribosomal subunit formation. XRP 2868 was more effective than quinupristin-dalfopristin at inhibiting 50S ribosomal subunit formation, inhibiting assembly by half at 2-4 fold lower concentrations than quinupristin-dalfopristin, with the exception of S. aureus that was nearly equivalent. The results for inhibition of 50S particle synthesis are illustrated in Figure 18A-C. IC$_{50}$ values for each antibiotic are shown in Table 4.
Figure 18 Streptogramin concentration dependent inhibition of 50S ribosomal subunit assembly. Inhibition of 50S subunit amounts from $^3$H-uridine labeled ribosomal subunits separated by sucrose gradient centrifugation in (A) *H. influenzae*, (B) *S. aureus* and (C) *S. pneumoniae* in the presence of XRP 2868 (●, dashed line) and quinupristin-dalfopristin (■, solid line). Linear best fit lines are shown for *H. influenzae* and power best fit lines are shown for *S. aureus* and *S. pneumoniae*. Arrows mark IC$_{50}$ values for XRP 2868 (filled arrows) and quinupristin-dalfopristin (hollow arrows). Results are the mean of three determinations. Bars indicate standard error.
The aminoglycoside hygromycin B and the streptogramin compounds quinupristin-dalfopristin and XRP 2868 are all well known translational inhibitors. Hygromycin B acts specifically on the 30S subunit and the streptogramin compounds quinupristin-dalfopristin and XRP 2868 specifically inhibit 50S ribosomal subunit function. The mechanism for their inhibition of translation has been well studied; however, these antibiotics have not been examined prior to this study for their ability to inhibit ribosomal subunit formation (Brodersen and others 2000; Harms and others 2004). Ribosomal subunit formation is a critical life process that would make an ideal target for antimicrobial agents (Champney 2003). This work has shown that hygromycin B inhibits 30S ribosomal subunit assembly specifically and that quinupristin-dalfopristin and XRP 2868 act to inhibit 50S ribosomal subunit synthesis. Studies of this nature provide a better understanding of the classes of antibiotics that specifically target ribosomal subunit formation and the discrepancies within each class will aid future drug development in this novel target area.

It is known that aminoglycoside antibiotics inhibit translation by binding to the 30S ribosomal subunit. Recent studies have shown that the aminoglycoside antibiotics neomycin and paromomycin posses a secondary inhibitory target,
preventing the formation of functional 30S ribosomal subunits (Mehta and Champney 2002; Mehta and Champney 2003).

Previous studies have shown that these antibiotics prevent translation and ribosomal subunit formation with near equal action in growing E. coli cells (Mehta and Champney 2002). An interesting difference was seen in the effect of hygromycin B on protein synthesis and ribosomal subunit assembly with this antibiotic. Hygromycin B was a stronger inhibitor of translation than 30S ribosomal subunit formation with IC\textsubscript{50} values of 16 µg/ml and 65 µg/ml respectively. Inhibition of growth rate and total viable cell numbers were in accordance with the reduction of protein synthesis rates (Table 2).

Hygromycin B has recently been shown to affect a ribosomal ATPase RbbA that is required for protein synthesis (Ganoza and Kiel 2001). RbbA is an ATPase that binds to the 30S subunit near the E-site and functions to aid ejection of tRNA from the E-site (Xu and others 2006). Hygromycin B binds near the binding site for RbbA disrupting the binding of the ATPase. Hygromycin B has been shown to inhibit 70-80% of the ATPase activity of 70S ribosomes whereas similar aminoglycoside antibiotics did not have the same effect; neomycin and streptomycin inhibited only 10-25% of ribosomal ATPase activity (Ganoza and Kiel 2001). The effects of hygromycin B on this enzyme may contribute to the marked difference seen in its inhibitory effect on translation compared to other aminoglycosides.
Inhibition of 30S and 50S ribosomal subunit assembly by hygromycin B was also examined. Inhibition of 50S ribosomal subunit assembly by most 50S protein biosynthesis inhibitors does not affect 30S subunit synthesis (Champney 2001). However, 50S ribosomal subunit formation may be affected by inhibitors of 30S ribosomal subunit assembly. Both neomycin and paromomycin have been shown to cause a reduction in 50S ribosomal subunit formation in *E. coli* and *S. aureus* at higher concentrations of drug (Mehta and Champney 2002; Mehta and Champney 2003). Hygromycin B demonstrated a similar reduction pattern in the amounts of 50S ribosomal subunit. The indirect action of these antibiotics on the 50S subunit has been attributed to a downstream effect (Mehta and Champney 2002; Mehta and Champney 2003). Small subunit (30S) particle biosynthesis precedes 50S ribosomal subunit synthesis; therefore, any effect on 30S formation could have a nonspecific downstream action of slowing or halting 50S biosynthesis. The effects are similar to that found with polar effect on genes transcribed from a multi-gene transcript. When transcription of a gene upstream is affected, all of the genes downstream are also affected while any genes farther upstream remain unaffected. Figure 19A-B illustrates the tandem synthesis of the 30S and 50S particles.
Figure 19  Tandem synthesis of the 30S and 50S ribosomal subunits in *E. coli* cells. (A) Transcripts for 16S and 23S rRNA genes are linked. (B) Formation of 30S and 50S ribosomal subunits in *E. coli* cells.

Pulse-chase kinetic analysis of ribosomal subunit formation in cells treated with hygromycin B supports a nonspecific effect on 50S particle synthesis. In control cells there was approximately twice the amount of 50S ribosomal subunits compared to 30S subunits and the amount of time required to
synthesize a 50S particle was about twice as long as a 30S subunit. This ratio in quantity and synthesis rate for the ribosomal subunits was consistently seen. In drug-treated cells the rate for synthesis of the 30S and 50S increased to 60 minutes and ~120 minutes before reaching control amounts, respectively. The results for the pulse-chase kinetic analysis indicated an equal inhibition of the rate of 30S and 50S ribosomal subunits (Figure 13A and 13B). An equivalent effect on 30S and 50S subunit assembly rates is expected if only the 30S particle formation is being affected by an antibiotic. An equal decline in 50S subunit formation has been observed in previous studies with neomycin and paromomycin (Champney and Mehta 2002; Champney and Mehta 2003). The theory for this indirect effect on the 50S has been explained above.

Northern blot analysis with 16S rRNA specific probe on small RNA fragments isolated from hygromycin B treated cells showed increased accumulation of fragmented 16S rRNA (Figure 14A-B). Samples from SK7622 (RNase II’) and SK4803 (RNase III’) strains treated with drug contained larger amounts of 16S fragments in comparison to D10-1 (RNase I’). Previous work with 23S assembly inhibitors has also shown these enzymes to also be important in the turnover of stalled 50S intermediate (Silvers and Champney 2005). There were only trace amounts of 16S fragments in D10-1 treated with hygromycin B. The small amount of fragment build up in these cells indicates that RNase I is not an important enzyme for the turnover of stalled 16S particle. Silvers and Champney (2005) also found this to be the case with 50S intermediate turnover;
D10-1 behaved similarly to wild type cells. One interesting difference between 23S and 16S turnover was the size of rRNA fragments produced. Turnover of azithromycin stalled 50S subunit intermediates gave 23S rRNA in the sizes of 500 and 1000 base pairs were observed (Silvers and Champney 2005). The 16S fragments that were produced during hygromycin B treatment were in the size of 200-900 base pairs. The difference in size is likely because of differences in the 16S and 23S rRNA, the differences in the proteins interacting with the rRNA in the precursor forms of the 30S and 50S subunits and how these are recognized by RNases involved in turnover.

The large amounts of 16S fragmentation in *E. coli* strains SK7622 and SK4803 could be an indication of the importance of RNase II and III in the breakdown of ribosomal subunit precursor that forms during treatment with antibiotics which inhibit assembly. The model in Figure 2 predicts that RNase enzymes are necessary for the removal/recycling of stalled precursor from cells treated with assembly inhibiting antibiotics. RNase III deficient SK7622 had particularly high amounts of 16S particle accumulation implicating this RNase as an especially important factor in the turnover of 30S ribosomal subunit precursor that is formed during treatment with hygromycin B. RNase III is an important endoribonuclease that plays a role in the processing of both 16S and 23S rRNA (Srivastava and Schlessinger 1990). Hybridization with a 23S specific probe showed no accumulation of 23S fragments in any of the drug treated samples indicating that
hygromycin B is specifically targeting only the 30S ribosome in assembly and translation (Figure 14B).

Examination of total RNA from hygromycin B treated *E. coli* cells using the Agilent bioanalyzer also supported specific inhibition of 16S rRNA. Total RNA analysis showed an increase in small fragments of RNA and a decrease in the amount of RNA corresponding to 16S rRNA. Meanwhile the amount of fragments of RNA corresponding to the 16S rRNA precursor increased (Table 3, Figure (15A-B). A decrease in the amount of RNA corresponding to the 23S was also seen.

Hygromycin B binds with specificity only to the 30S ribosomal subunit and inhibits translation by interfering with the A, P, and E sites only on this subunit (Brodersen and others 2000). Hygromycin B has been shown to bind to a part of helix 44 (H44) that changes position during translocation and it is believed that hygromycin B binding to H44 restricts the movement of this helix during protein synthesis (Frank and Agrawal 2000). It is possible that hygromycin B binding to this helix could also restrict movement necessary to form functional 30S subunits and in so doing prevent conformational rearrangement required for processing of the 16S rRNA transcript and subsequent 30S ribosomal subunit maturation.

The binding of hygromycin B can be interrupted by modification of the antibiotic by phosphotransferases or mutation of target rRNA bases. The hygromycin phosphotransferase enzyme is found in the hygromycin B producing organism *Streptomyces hygroscopicus* (Malpartida and others 1983; Bilang and
others 1991). The gene for hygromycin phosphotransferase has also been located on plasmids in bacteria found in animals treated with the antibiotic. The resistance gene has also been discovered on R plasmids in 2 clinical strains of bacteria, *Escherichia coli* and *Klebsiella pneumoniae* (Gomez-Lus R 1998).

Mutation of rRNA is another mechanism of resistance to hygromycin B. It has been shown in single rRNA allelic derivatives of *Mycobacterium smegmatis* that point mutations at or near the hygromycin B binding site in helix 44 of 16S rRNA confer resistance to the antibiotic (Pfister and others 2003). Studies with *E. coli* and *Tetrahymena thermophila* mutants concur (Spangler and Blackburn 1985; De Stasio and Dahlberg 1990).

Several crystal structures of aminoglycosides complexed with 30S subunits or 16S fragments have been resolved and are the basis for new avenues of structure based drug design (Brodersen and others 2000; Vicens and Westhof 2003). A group at Anadys Pharmaceuticals, Inc. has designed hybrid aminoglycoside ligands. These ligands combine components of hygromycin B and paromomycin or neomycin B and are designed to make the most of subtle differences in binding of these antibiotics onto the 30S ribosomal subunit near helix 44 and the decoding site (Zhou and others 2005; Murray and others 2006).

Crystal structures of antibiotic bound to intermediate particles in ribosomal subunit assembly have not yet been resolved. Antibiotic hybrids designed based on their ability to bind these intermediate structures could be more effective antibiotics than their parent drugs. Inhibition of subunit assembly would halt
protein synthesis before it began by preventing the formation of functional ribosomes. Targeting of ribosomal subunit formation should create an even greater bactericidal effect in comparison to inhibition of translation. It is important to examine other antibiotics for their inhibitory effects on this possible target.

This study also examined the effects of several new streptogramin compounds for their effects on ribosomal subunit formation. The development of new antimicrobial agents is critical for overcoming the problem of microbial drug resistance. Both the development of new antibiotics like linezolid (Livermore 2003) and the modification of existing compounds such as the aminoglycoside hybrid ligands are required. Quinupristin-dalfopristin is a streptogramin antibiotic that has been in clinical use since 1999 for the treatment of multi-resistant infections of Gram-positive bacteria. XRP 2868 (also known as NXL 103) is a new oral streptogramin derived from quinupristin-dalfopristin that is showing great promise as a treatment option in multi-resistant Gram-positive infections. XRP2868 has been shown to be more inhibitory than nine other antibiotics tested against a variety of Gram-positive clinical isolates (Goldstein and others 2005). The new antibiotic has also shown better activity against \textit{H. influenzae} and \textit{S. pneumonai}e than its parent drug combination of quinupristin-dalfopristin in MIC assays (Pankuch and others 2003).

Antibiotics that bind to the large 50S ribosomal subunit of bacterial ribosomes inhibit both the translational activity of the subunit and specifically prevent its formation in cells (Champney 2003). Both A and B-type
streptogramins have this dual activity (Champney and Tober 2000). The goal of this study was to compare the inhibitory effects of XRP 2868 with quinupristin-dalfopristin on cellular functions including ribosome function and assembly in β-lactamase-positive Haemophilus influenzae, methicillin-resistant Staphylococcus aureus, and erythromycin-resistant S. pneumoniae strains. These resistant strains were chosen because they are commonly found in human infections and they often respond to streptogramin antibiotics (Lamb and others 1999). Minimal inhibitory concentrations (MIC) for both antibiotics were determined for each organism and are listed in Table 4. The values for S. pneumoniae and H. influenzae are in good agreement with those found by Pankuch and others (2003).

Quinupristin-dalfopristin kills cells by inhibiting protein synthesis and 50S subunit formation (Lamb and others 1999; Champney and Tober 2000). Both drugs inhibited translation at a lower concentration in S. aureus and S. pneumoniae compared with H. influenzae. XRP 2868 was the more effective inhibitor of protein synthesis in all three organisms examined (Figure 16). IC50 values for translational inhibition are summarized in Table 4.

Each antibiotic was also examined for its effects upon ribosomal subunit formation. Previous work has shown that the streptomycin compound virginiamycin caused a specific decrease in the amount of 23S rRNA in Bacillus subtilis cells treated with the antibiotic (Cocito 1969; Cocito 1971; Cocito 1973). It has been demonstrated that the streptogramin B component is responsible for
the reduction in 50S ribosomal subunit formation by several streptogramin compounds in \textit{B. subtilis} and \textit{S. aureus} while the A component functions to inhibit protein synthesis (Cocito 1969; Cocito 1971; Cocito 1973; Champney and Tober 2000). Quinupristin-dalfopristin and XRP 2868 showed a concentration dependent inhibition of 50S ribosomal subunit formation with XRP 2868 being as effective as quinupristin-dalfopristin at a 2-4 fold lower concentration of drug with the exception of \textit{S. aureus} (Figure 18A-C). The major effect of both drugs in \textit{S. aureus} was inhibition of translation and XRP 2868 reduced protein biosynthesis by half at a concentration of drug two fold lower than quinupristin-dalfopristin. There was not a specific decline in 30S subunit amounts in cells treated with each antibiotic (data not shown).

Resistance to quinupristin-dalfopristin, XRP 2868 and other streptogramins is incurred by enzymatic modification of the compound, active transport or efflux by an ATP powered pump, and alteration of the target site. Because there are different binding sites for each of the components, the mechanisms of resistance are also different. Resistance to group B components most often occurs though cross resistance provided by \textit{erm} genes encoding erythromycin methyl transferases. These enzymes transfer methyl groups onto an adenine residue in the 23S rRNA that results in decreased binding of macrolide, lincosamide, and streptogramin B antibiotics. Other mechanisms of resistance to group B streptogramins are rare and specific for certain strains of Gram-positive bacteria. Group A resistance is most often mediated by genes that encode
acetyltransferases that inactivate the antibiotic or by genes which encode efflux pumps that actively transport the antibiotic out of the cells (Thal and Zervos 1999). Quinupristin-dalfopristin has been available clinically only since 1999. Resistant isolates containing one or more of the above mentioned resistance factors have already begun to appear in the clinical setting (Bozdogan and Leclercq 1999; Lina and others 1999; Malbruny and others 2002). The need to expand the antibiotic arsenal is ever present. Improving existing antibiotics and seeking out new targets for existing antimicrobial agents are ways to strengthen our stand in the war against antibiotic resistant pathogens.

The results show that XRP 2868 is a more effective inhibitory agent against methicillin-resistant S. aureus, erythromycin-resistant S. pneumoniae, and ß-lactamase-positive H. influenzae than quinupristin-dalfopristin. This study is the first to test the inhibitory effects of this new compound on cellular functions in a MRSA strain and demonstrates that this drug is effective against this resistant organism. The IC$_{50}$ values for inhibition of protein synthesis by XRP 2868 in all bacteria examined were 2-5 fold lower than that of quinupristin-dalfopristin. 50S subunit amounts were reduced in proportion to the decline in protein synthesis in all organisms. The inhibition of 50S subunit amounts indicates that cell killing was a result of the antibiotics’ effect on translation and the assembly of 50S subunits. Understanding the relationship between structural variance and differential inhibition of cellular function in target organisms is important for the development of effective antibiotics (Chu and others 1996).
More studies of this nature with other modified streptogramin compounds would be helpful in this regard.

This work is a continuation of studies examining possible ribosomal subunit assembly inhibitors. More studies such as this one are needed to elucidate the potential of ribosomal subunit assembly as an antibiotic target and increase our understanding of the mechanism behind specific inhibition of this vital cellular function.


aminoglycoside mimetics as antibacterial translation inhibitors.

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ABBREVIATIONS

30S .......................... Small subunit of ribosome
50S .......................... Large subunit of ribosome
DNA .......................... Deoxyribonucleic acid
MIC .......................... Minimal inhibitory concentration
RNA .......................... Ribonucleic acid
rRNA .......................... Ribosomal RNA
S-buffer, R-buffer ................. Subunit buffer, Ribosome buffer
IC$_{50}$ .......................... 50% inhibitory concentration
TSB .......................... Tryptic soy broth
MRSA .......................... Methicillin resistant *Staphylococcus aureus*
RNase, PNPase ................. Ribonuclease, Polynucleotide Phosphorylase
TCA .......................... Trichloro-acetic acid
SDS .......................... Sodium lauryl sulfate
DOC .......................... Deoxycholic acid sodium salt
EDTA .......................... Disodium ethylenediamine-tetraacetate
PMSF .......................... Phenylmethanesulfonyl fluoride
MOPS .......................... 3-(N-Morpholino)-propanesulfonic acid
SAS-buffer ....................... *S. aureus* subunit buffer
PCR .......................... Polymerase chain reaction
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