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Dual Effects of MLS Antibiotics: Transcriptional Modulation and Interactions on the Ribosome

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Summary

The macrolide-lincosamide-streptogramin (MLS) antibiotics are an important group of translation inhibitors that act on the 50S ribosome. We show that, at subinhibitory concentrations, members of the MLS group modulate specific groups of bacterial promoters, as detected by screening a library of promoter-*luxCDABE* reporter clones of *Salmonella enterica* serovar Typhimurium. The patterns of transcription permit identification of classes of promoters having differential responses to antibiotics of related structure and mode-of-action; studies of antibiotic synergy or antagonism showed that eukaryotic translation inhibitors may act on the 50S ribosome. The mechanism of transcriptional modulation is not known but may involve bacterial stress responses and/or the disturbance and subsequent compensation of metabolic networks as a result of subtle interference with ribosome function. Transcriptional patterns detected with promoter-*lux* clones provide a novel approach to antibiotic discovery and mode-of-action studies.

Introduction

Antibiotics are naturally occurring organic molecules of low molecular weight (<3000 D) that have been isolated by virtue of their ability to inhibit (or kill) living organisms; in most cases they act by binding to specific cellular targets [1]. It is estimated that tens of thousands of such molecules have been isolated from bacteria, fungi, and plants since the beginning of the antibiotic era (around 1950); the major source has been the Streptomycetes [2]. A significant number of antibiotics target the bacterial ribosome, and two major classes have been identified by their ability to bind and interfere with the function of either the 30S or 50S subunits during translation [3–5]. In recent years, genetic analysis, chemical foot-printing, nuclear magnetic resonance, and X-ray crystallography have permitted high-resolution studies of antibiotic-ribosome interactions, and specific binding sites have been defined at the level of single nucleotides in 16S and 23S rRNA molecules [6–8]. These studies provided conclusive evidence for rRNA molecules as the key functional components of the ribosome in the process of peptide bond formation and the targets for antibiotic action.

The MLS group was defined on the basis of cross-resistance patterns and certain synergistic and antagonistic interactions [9], which showed that they act at the peptidyltransferase center (PTC) of the 50S subunit; binding involves domains II and V of the 23S rRNA, blocking peptide bond formation in subtly different ways [10–12]. Ribosomal proteins also play roles in MLS binding, presumably by influencing rRNA folding and structure [13, 14]. Recent high-resolution X-ray analyses have shown that the macrolide members of this family interfere principally with the transit of the newly synthesized polypeptide chain through the peptide exit channel of the ribosome [15, 16].

Clinically, the MLS antibiotics are used primarily for the treatment of a variety of Gram-positive infections, especially methicillin-resistant staphylococci [17]. The increasing appearance of MLS-resistant strains has compromised these applications in recent years, and as a result many synthetic derivatives of the macrolides have been made [18]. New and improved variants with enhanced stability and pharmacokinetic behavior have been developed for the treatment of a variety of emerging bacterial infections, including *Helicobacter pylori*; macrolide antibiotics also have favorable activity in alleviating bacterial infections associated with cystic fibrosis [19–21].

Use of libraries of promoter-*lux* fusion constructions has shown that most antibiotics demonstrate typical hormetic responses [22]. At subminimal inhibitory concentrations (sub-MIC) these compounds may modulate the transcription of some 5%–10% of bacterial genes in the cell, often inducing 10- to 100-fold up- or downregulatory responses, with only limited effects on growth. At higher concentrations the compounds exhibit their well characterized inhibitory or cidal activities through target-related responses, with few transcription changes [23]. Antibiotics of dissimilar structural classes and modes of action affect different groups of promoters. Thus, inhibitors of specific steps involved in the complex process of translation modulate the activity of distinct sets of promoters, presumably due to their interaction with different sites within the ribosome. Our previous studies indicated that different classes of inhibitors of 30S or 50S function might be distinguished in this way [23].

Since the promoters affected at sub-MIC depend to a large extent on the nature of the antibiotic class being used, it seems likely that in each case only transcripts associated with particular metabolic networks are affected. The MLS antibiotics provide a good test for this proposition. Since the early work of Vazquez it has been known that these structurally different molecules (Figure 1) act at nearby or overlapping sites on the 50S ribosome, as manifested by synergistic or competitive bind-

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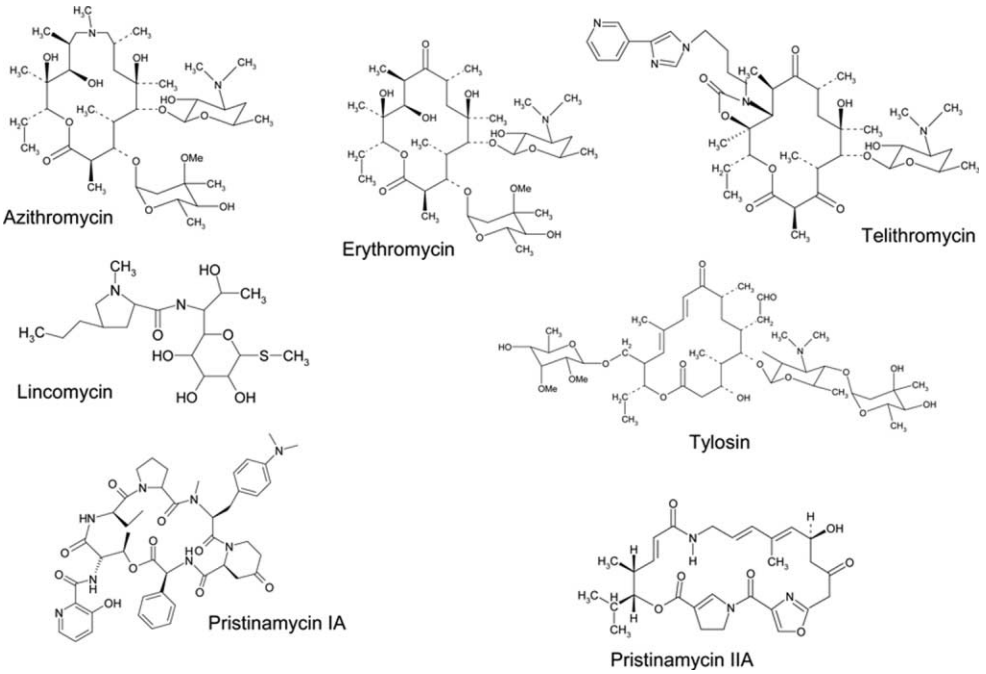

Figure 1. The Structural Diversity of the MLS Antibiotics

ing to ribosomes and cross-resistance patterns of bacterial mutants, and have subtle differences in mode of action and interaction [4]. We demonstrate that antibiotic-induced transcription patterns provide a convenient method to discriminate between members of the MLS family and related inhibitors, by providing activity "fingerprints" or "signatures" for the peptidyltransferase and peptide exit tunnel inhibitors. We suggest that the analysis of sub-MIC-induced transcription patterns could provide the basis for high-throughput screens to identify novel inhibitory compounds with defined modes of action. The *luxCDABE* reporter system is especially effective for such assays [24, 25], which could be used with crude mixtures containing low concentrations of bacterial metabolites, since alterations in *lux* gene expression can be detected with very high sensitivity in liquid or on solid culture media.

Results

Screening the Promoter-*lux* Reporter Library with MLS Antibiotics

A 6500 clone library of promoter-*lux S. typhimurium* constructs [23, 26] was used to monitor transcriptional changes in the bacterial host on exposure to subinhibitory concentrations of different MLS antibiotics. The transcriptional profile of *S. typhimurium* in response to a subinhibitory concentration of azithromycin is shown in Figure 2A. Each data point represents the response of a single clone from the library. Points above the diagonal indicate upregulated clones, those below are downregulated.

After the initial screening, a total of 589 clones from the library were found to be at least 3-fold modulated up or down by one or more of the five antimicrobials. These positive hits were rescreened, and 193 were selected for an additional overnight rescreen, which identified 169 clones giving a consistent response (3-fold activated or repressed). The distribution of upregulated and downregulated clones identified with the different MLS antibiotics is summarized in Table 1.

Comparison of Transcription Responses among Different Antibiotics

To investigate further the changes in transcription patterns, luminescence responses from the primary screening were reanalyzed to compare directly the activities of any two different compounds. These data were obtained by taking the $\log_{10}$ of the ratio of the values from the control (LB) and experimental (LB plus antibiotic) groups. In those cases where the ratio was greater than zero, the antibiotic-treated group produced stronger luminescence than the control group. When less than zero, the antibiotic-treated group had reduced luminescence compared to the control group. The collected values obtained from each antibiotic treatment were then plotted against a reference antibiotic for comparison purposes. Each data point represents the activities of a single clone in response to two separate antimicrobials. For example, in Figure 2B, coordinates (1, 0.5) represent clones that are more strongly activated by the reference azithromycin (on the X-axis) than by pristinamycin (on the Y-axis). Results with different antibiotics were plotted and the relative distributions can serve as response signatures for each drug. Thus, when the transcription profile of azithromycin was plotted against the profile of pristinamycin, most of the points were scattered, with subpopulations located in sectors repre-

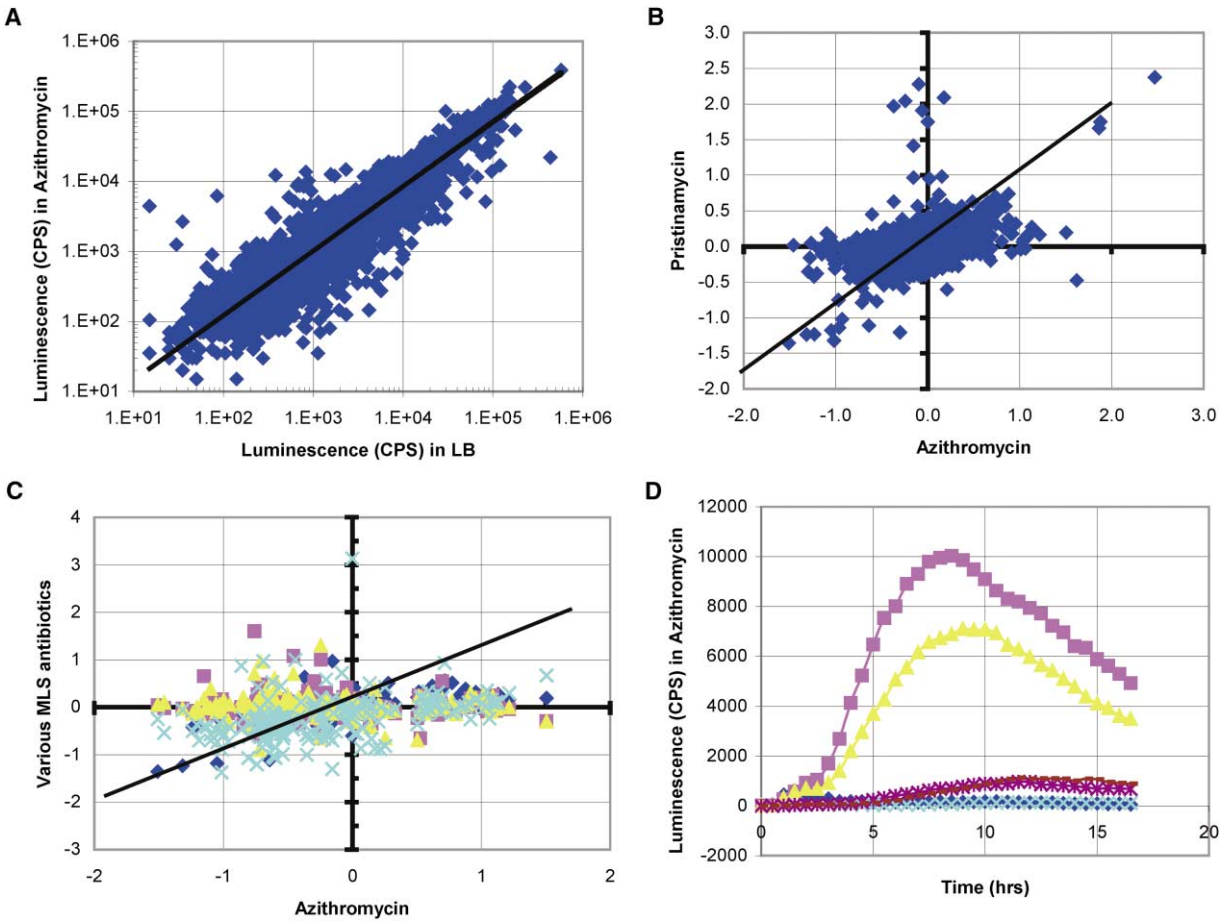

Figure 2. Identification and Analysis of MLS-Modulated *lux*-Promoter Clones

(A) Scatter plot showing luminescence in response to azithromycin at 1 μg/ml, measured in counts per second (cps), plotted against response without antibiotic, determined using a 6500-clone *S. typhimurium* random promoter-*lux* library, incubated in microtiter plate liquid cultures for 22 hr at 37°C. Points above the diagonal indicate promoter-activated strains and below the diagonal, clones in which promoter activity was repressed.

(B) Scatter plot of pristinamycin activity (1.25 μg/ml) against azithromycin (1 μg/ml) after 22 hr, determined using the *S. typhimurium* library. Activity calculated from $\log_{10}$ of luminescence of antibiotic-treated cultures over luminescence in LB without antibiotic.

(C) Combined scatter plot of the response to pristinamycin (1.25 μg/ml) incubated 22 hr, lincomycin (6.25 μg/ml) incubated 24 hr, tylosin (6.25 μg/ml) incubated 24 hr, and telithromycin (1.25 μg/ml) incubated 21 hr against azithromycin; results from 196 MLS responsive clones were used.

(D) Luminescence response curves of MLS sensitive- and resistant- (p*ermC*) p*ilvL/G-lux S. typhimurium* grown in the presence of azithromycin at 1.25 μg/ml (▲, –), at 2.5 μg/ml (■, ⋆), and in the absence of antibiotic (◆, ×).

senting higher levels of activation and repression by azithromycin compared to pristinamycin. Overall, the results show that azithromycin at 0.31 μg/ml induces more potent induction or repression of certain promoters than pristinamycin at 1.25 μg/ml; however, a number of unique promoter-dependent responses were identified. When the results comparing other MLS antibiotics were compiled in the same way, distinct spectra of promoter-modulation activity for each antibiotic became readily apparent (Figure 2C). For example, telithromycin,

Table 1. *S. typhimurium* Clones Activated/Repressed by Different MLS Antibiotics

Drugs	Classes	Activated	Repressed	Total
Erythromycin	14-membered Macrolide	62	37	99
Azithromycin	15-membered Macrolide	60	32	92
Tylosin	16-membered Macrolide	58	42	100
Telithromycin	Ketolide	92	34	126
Pristinamycin	Streptogramin	73	37	110
Lincomycin	Lincosamide	64	48	112
All 6 antibiotics		12	9	21

Table 2. Identification of Active Library Clones Responding to Different MLS Antibiotics

Gene	Erythromycin	Azithromycin	Telithromycin	Tylosin	Lincomycin	Pristinamycin	Description
STM1547			+++			+++	putative mar-R family transcriptional regulator
STM1678				+		−	putative 2′-hydroxylisoflavone reductase
ybeL			+	− − −	−	+	putative cytoplasmic protein
cpxA	−	−	+++	+++	− −	+++	sensory kinase in 2-component regulatory system with CpxR
yfcY	+++		++		− − −		putative acetyl-CoA acetyltransferase
yrbC					+		putative ABC superfamily transport protein
yedO		+		+			putative 1-cyclopropane-carboxylate deaminase
marT	+++		+++		+		putative transcriptional regulator MarT
STM1560		++		++		−	putative alpha amylase
yqjA	+++	++			++		putative DedA family, membrane protein
fadL	+	++	+++	++		+++	transport of long-chain fatty acids
STM4316	+		++		+		putative cytoplasmic protein

a ketolide, clearly has a pattern similar, but distinct from, the related 14- and 16-membered macrolides; it shows similarities to the transcriptional response of lincomycin, which might suggest some congruence in 23S rRNA binding sites.

Identification of Signature Promoter Sequences

MLS-activated promoter inserts were PCR amplified and their nucleotide sequences determined. The results (Table 2) indicate that promoters for different genes vary in their sensitivity to MLS-modulation (with responses ranging from 3× to 100×) and that the MLS compounds modulate different transcripts with significant overlap. This promoter sequence information did not reveal any patterns of functional metabolic clustering associated with the different antibiotic classes. A significant number of the promoter responses were *S. typhimurium* genes of unknown function. Thus, discernable antibiotic signatures were identified, but they do not appear to be associated with any specific biochemical networks, such as the well characterized stress responses.

Transcriptional Responses in MLS-Resistant Strains

Clinically significant resistance to the MLS antibiotics has been known for some time; mutations in the bacterial chromosome or the acquisition of resistance plasmids/transposons can lead to a number of resistance phenotypes [20]. Cross-resistance between the MLS compounds (the MLS_B phenotype) is common, and the early studies of Vazquez and collaborators [28] provided the first convincing evidence that this class of compounds interacts at overlapping site(s) on the 50S ribosome at the PTC. We tested MLS-resistant strains for their response to subinhibitory antibiotic concentrations to establish that transcription modulation requires known ribosome binding sites. Studies with macrolide-resistant strains carrying mutant *rplV* (L5) and *rplD* (L22) alleles showed that ribosome mutations alter MLS responses [23]. Here we employed *S. typhimurium* carrying a plasmid encoding an *ermC* gene under the control of the *tac* promoter and inducible by IPTG [18] that confers resistance to certain macrolides by methylation of A2058 in domain V of the 23S rRNA [29]. The *ermC*-containing *lux*-reporter strains showed reductions in transcriptional activation caused by erythromycin and its close structural relatives (e.g., clarithromycin or azithromycin) (Figure 2D). On the other hand, macrolide derivatives that possess a secondary binding site on 23S rRNA, such as telithromycin, still elicited a transcription response in erythromycin-resistant hosts.

Responses to Other 50S Subunit Inhibitors

In addition to the MLS, there are a number of antibiotics with diverse structures with similar modes of action binding to the 50S ribosome to block translation. The 169 MLS-active promoter-*lux S. typhimurium* clones were screened against these antibiotics and representative transcription/modulation results are shown in Table 3. As expected, considerable differences in promoter responses were observed. The resolving power of the *lux*-reporter response effectively discriminates between 50S inhibitors acting at different target sites on the ribosome. Some promoter-*lux* constructs had broad-spectrum responses (*tsr*) while others had very limited responses (*ybfE*). Two antibiotics acting specifically on the 60S ribosome of eukaryotic cells, anisomycin and cycloheximide, were included in this screening. Anisomycin at a high concentration (200 μg/disc) elicited a weak luminescence response with the p*tsr-lux* construct (with no effect on growth of the bacteria). On the other hand, cycloheximide had no detectable activity. However, both eukaryotic inhibitors were found to influence transcription effects when used in combination with MLS antibiotics (see below.)

Interactions between Inhibitors Acting on the 50S Subunit

In the mid-1970s David Vazquez and his collaborators studied synergistic or antagonistic interactions between

Table 3. Responses of Active Library Clones to MLS and Other 50S Ribosome Antibiotics

	MLS 50S inhibitors						Miscellaneous 50S inhibitors							
Gene	Erythromycin 15 μg	Telithromycin 15 μg	Clarithromycin 20 μg	Azithromycin 10 μg	Carbomycin 40 μg	Lincomycin 50 μg	Pristinamycin 20 μg	Tiamulin 20 μg	Chloramphenicol 30 μg	Sparsomycin 20 μg	Anisomycin 200 μg	Linezolid 200 μg	Blasticidin 100 μg	Everninomicin 100 μg
ilvC	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	+ + +	0	0	0	+ + +
ilvLG	+ + +	+	+ + +	+ + +	+	+ +	+	+	+ + +	+ + +	0	NT	+	
tsr	+ +	+	+ +	+ +	+ +	+ +	+	+	+ +	+ +	+ +	+ +	+ +	+ +
ybfE	+	+	+	+	+	+	NT	+ +	+		0	0	0	0
prpB	+ +	+	NT	+ +	+	+ +	+ +	+ +	NT	+ +	+	+	+ +	+
sopB	+	+ +	NT	+ + +	+	0	0	+	NT	+ +	0	0	+ +	0
rpsL	0	0	NT	+ + +	0	0	0	NT	NT	+	0	0	0	+
ndk	0	0	NT	+	0	0	0	NT	NT	0	0	0	0	0
Zone of inhibition	5 mm	8 mm	5 mm	15 mm	0 mm	0 mm	7 mm	0 mm	21 mm	13 mm	0 mm	0 mm	10 mm	0 mm

"0" indicates tested but no activity; "NT" indicates not tested.

various translation inhibitors [28] as determined by measuring competition (or enhancement) of ribosome binding using radio-labeled antibiotics; these results were interpreted in terms of overlapping binding sites on the 50S ribosome. Subsequent work using sensitivity-disc and tube-dilution studies have demonstrated frequent synergistic and antagonistic interactions between antibiotics; a number of these interactions are of clinical significance [30]. The *lux*-reporter strains provide an exquisitely sensitive method for the detection of interactions between MLS antibiotics and other 50S inhibitors. Some typical experiments are shown in Figure 3: they reveal a range of interactions between translation inhibitors acting on the 50S ribosome. The mechanisms underlying these interactions are not well defined, and it is not known if any given interaction illustrates overlaps in binding sites, allosteric interactions, or some other biochemical effect. Nonetheless such screening may be of value in mechanism-of-action studies and in indicating potential positive or negative effects in the therapeutic use of antimicrobials.

Anisomycin and cycloheximide are considered to be primarily inhibitors of translation in eukaryotes [31], although some Archaea are susceptible to anisomycin. Using the more sensitive transcription modulation response, we examined the possibility that the eukaryotic inhibitors might have functional interactions with bacterial inhibitors at the ribosome level. Anisomycin and cycloheximide were found to have concentration-dependent effects on activation induced by macrolide (erythromycin and azithromycin) and ketolide (telithromycin) antibiotics. At high (but noninhibitory) concentrations (100 μg/ml), they antagonized the antibacterial compounds, as indicated by reduced luminescence responses (not shown). However at lower concentrations (25 μg/ml), anisomycin and cycloheximide markedly enhanced the activation of certain promoter fusions by azithromycin and telithromycin. (Figure 4).

Discussion

Screening for the targeted inhibition of biochemical processes in microbes has provided a rich collection of naturally derived antibiotics that have been the foundation of the success of the pharmaceutical industry in the treatment of infectious diseases [32]. Mode-of-action studies began early in the antibiotic era [33] and led to the identification of a variety of specific macromolecular targets in bacterial cells. Increasingly sophisticated molecular studies of antibiotic/ribosome interactions have provided antibiotics with improved pharmacologic properties and broadened spectrum of activity (including resistant strains). For these applications, attention was focused on use of antibiotics at growth-inhibitory concentrations.

It has been known for some time that antibiotics at sub-MIC have diverse physiological effects on bacteria and their eukaryotic hosts [34]; however, such activities were considered to be secondary issues in the therapeutic use of antibiotics (for example, the post-antibiotic effect [35]) and have been largely ignored in mode-of-action studies. We have shown that subinhibitory con-

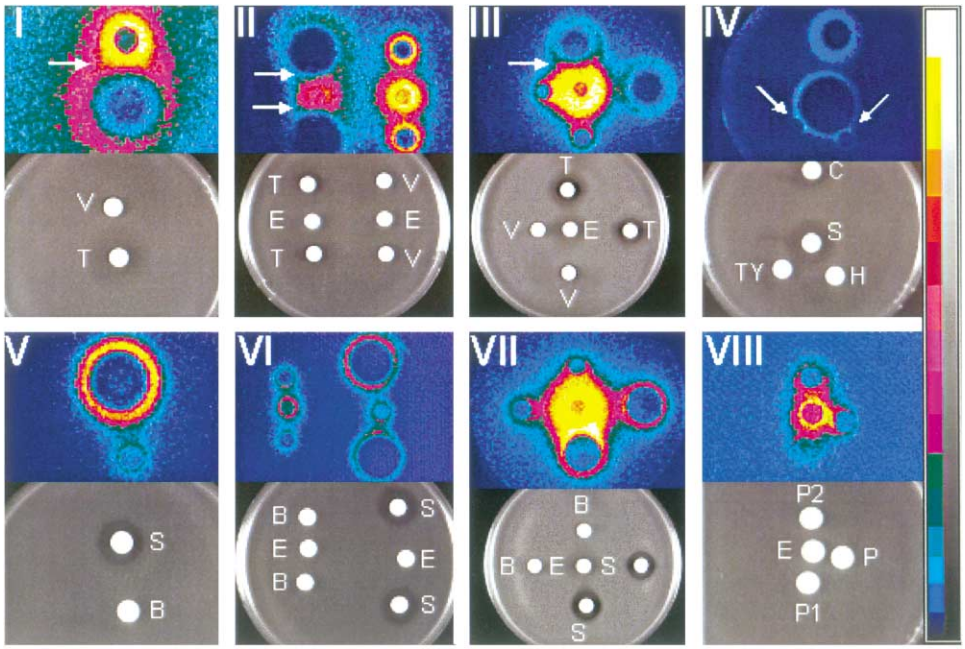

Figure 3. Use of p*ilvL/G-luxCDABE S. typhimurium* to Identify Interactions (Synergy and Antagonism) between MLS Antibiotics and Other 50S Inhibitors

Virginiamycin, (V), telithromycin (T), erythromycin (E), sparsomycin (S), blasticidin (B), tylosin (TY), hygromycin A (H), pristinamycin IA (P1), pristinamycin IIA (P2) and pristinamycin complex (P).

Top row of each panel: luminescence from each plate has been converted to the scale indicated on the right, white being high *lux* expression and dark blue being low *lux* expression. Antagonistic interactions are indicated by arrows.

(I) Antagonism between (V) and (T) appears as a flattening between the (V) ring and the (T) ring.

(II and III) Antagonism between (T) and (E) is shown as a flattening between the (E) ring and the (T) rings. Synergy between (V) and (E) is seen by fusing of the (E) ring with the (V) rings.

(IV) Antagonism between (TY), (H), and (S) is shown by semicircular reductions of the (S) ring caused by (TY) and (H).

(V) Synergy between (S) and (B) causes fusion of the two rings.

(VI and VII) Synergies between (B), (S), and (E) are shown by fusions of the rings.

(VIII) Synergies between (P1), (P2), (P), and (E) cause fusion of the four rings to form a leaf-shaped luminescence pattern.

centrations of antimicrobials modulate global cellular metabolism by fine-tuning the activity of different sets of promoters that are related to antibiotic function. The fact that different antibiotics activate or repress different groups of promoters provides a novel approach to the identification of classes of antibiotics and their mode of action. Transcription modulation thus proves to be a more sensitive test of small molecule activity than growth inhibition.

Among the most valuable and widely used antibiotics are the macrolides that inhibit bacterial protein synthesis by interfering with the peptide exit tunnel function on the 50S ribosome. Erythromycin is the oldest of these compounds, and its related chemically modified derivatives dominate the therapeutic market [21, 36]. Extensive studies of macrolide binding to the 50S subunit, employing cross-linking, fingerprinting, nuclear magnetic resonance, and more recently high-resolution crystallographic studies, have revealed the interactions determining the functional binding of macrolides (and other MLS antibiotics) to a small number of sites in the 23S rRNA. The most important interaction is with base A2508, which was first indicated from studies of MLS-resistant bacteria [11, 15, 19, 37]. All macrolides bind to the same site on the ribosome within the PTC, interacting principally with the polypeptide exit tunnel and influencing peptide egress from the ribosome. Different macrolides (14-, 15-, 16-membered rings) interact with other domains in the 23S rRNA that may enhance binding. For example, telithromycin, which is active on some erythromycin-resistant strains, interacts with A2508 (domain V) and with the loop of helix 35 of the 23S RNA [38, 39]. The MLS component lincomycin binds to the PTC but directly inhibits peptide bond formation, whereas the streptogramins (pristinamycin) interfere with peptide export.

The transcription-modulating effects of the different classes of macrolides allow fine discrimination between their structural analogs; defined panels of promoter/reporter fusions have utility in antibiotic discovery and identification, to assign antibiotics with unknown mode of action to specific classes, for example. Screening can be carried out directly with crude cell supernatants or extracts of producing strains in a high-throughput manner, thereby distinguishing inhibitors with novel modes of action from known compounds at an early stage of the drug discovery process. Since the promoter/reporter constructs vary in their responses depending on the promoter, bacterial host, and culture medium (minimal or rich, solid or liquid), panels could

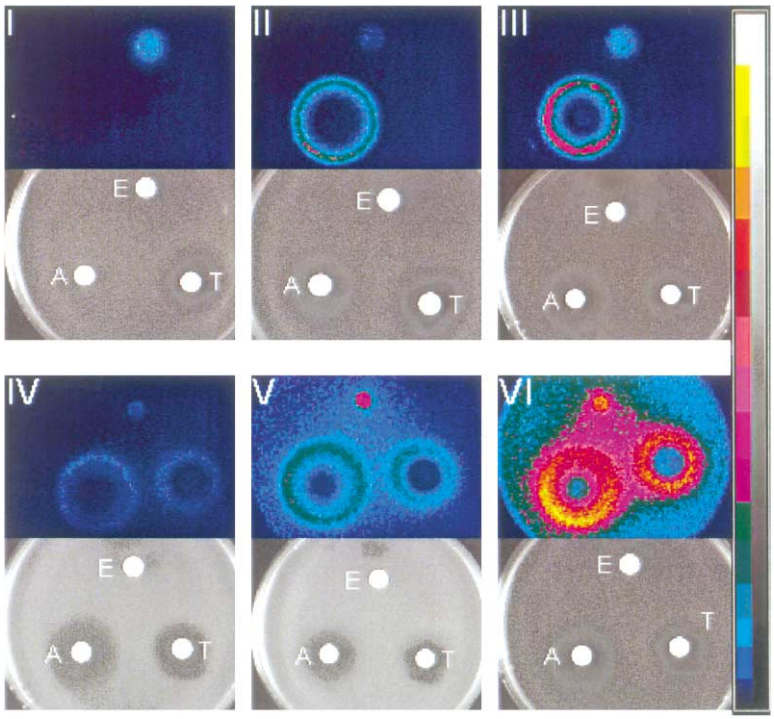

Figure 4. The Effect of Anisomycin and Cycloheximide on Luminescence Induced by Erythromycin (E), Telithromycin (T), and Azithromycin (A)

Filter discs containing the antibiotics were placed on lawns of p*ilvL/G* (I–III) and p*tsr* (IV–VI) *S. typhimurium*. Plates I and IV: LB; II and V: LB plus 25 μg/ml of cycloheximide; III and VI: LB plus 25 μg/ml of anisomycin. Top row of each panel: luminescence produced from each plate has been converted to the scale indicated on the right, white being high *lux* expression and dark blue being low *lux* expression.

On LB alone (I), although there is an inhibition zone around (T), only (E) induces *lux* expression; (A) and (T) do not induce *lux* expression. With cycloheximide (II) and anisomycin (III) added to the LB, (A) is upregulated compared to LB alone (I). Similarly, the response of p*tsr* to all three drugs (A, T, E) is increased in the presence of cycloheximide (V) and anisomycin (VI) compared to no antibiotics (IV).

be designed to discriminate between compounds on the basis of mode of action or structural class. Very low concentrations of both Gram-negative and Gram-positive active compounds can be detected, even when using a Gram-negative screening host; employing hypersensitive hosts [40] would enhance this capability. Nonetheless, reporter libraries developed with Gram-positive hosts such as *Staphylococcus aureus* would be essential to examine the full spectrum of all antibiotic activities.

Comparisons of the use of promoter-*lux* fusions to identify functional interactions between ribosomally active antibiotics in vivo may be expedient for mode-of-action studies and in the screening of chemically synthesized derivatives; crude reaction products could be tested rapidly for their activity profiles in the presence of compounds with known target specificity. 60S (eukaryotic) ribosome inhibitors showed only weak stimulatory activity on bacterial *lux* reporter strains, but they stimulated or antagonized transcription modulation by MLS antibiotics (Figure 4). This suggests that compounds such as anisomycin and cycloheximide may interact functionally with bacterial 50S ribosomes; anisomycin is known to inhibit protein synthesis in the halobacteria. From an evolutionary standpoint, this cross-reactivity would be expected if the ribosomal targets for these inhibitors were conserved [41]. In any event, these observations raise the possibility that even compounds considered as eukaryotic inhibitors could be used as lead molecules in chemical modification programs to identify novel classes of antibacterial compounds active on the 50S ribosome. Similar studies with a selection of antibiotics that bind to the bacterial 30S ribosome subunit are in progress. We note that several groups have applied comparable approaches to obtain transcription signatures for a variety of antibiotic classes [42–45]. Using DNA bacterial arrays, these workers have tested MIC or higher concentrations of antibiotics and found a range of transcription modulation more limited than what is reported here. In addition, earlier studies did not report the in-depth examination of an antibiotic class such as MLS. In several instances only stress responses were detected. Our studies confirm the value of transcription modulation induced by antibiotics as a simple experimental approach to discriminating between structurally different inhibitors of translation.

The mechanism of antibiotic-induced transcription modulation is not known. We suggest that antibiotics at sub-MIC bind to their known target sites on the ribosome (albeit transiently), causing minor perturbations in ribosome function. These effects must be responsible for a mechanism coupling translation to transcription, resulting in promoter-selective modulations of the latter. The transmission of signals from ribosome to RNA polymerase due to subinhibitory MLS could involve the release of small amounts of incomplete polypeptides [46], interference with ribosome assembly [47], induction of translation errors [48], or possibly interactions of small molecules with RNA [49]. The sequelae of all these events may be low-level stress responses that act through one of the many bacterial sigma factors to activate or repress specific sets of transcripts [50, 51]. These changes might also result in compensating effects on the transcription of nodes of linked metabolic networks.

It is clear that antibiotic inhibitors (and possibly other small molecules) exhibit hormesis, a phenomenon characterized by distinctly different responses at low concentrations (transcription modulation) compared to high concentrations (growth inhibition) [22]. We believe that subinhibitory concentrations identify responses that

more accurately reflect antibiotic mode of action and have suggested that these effects might represent the "natural" role of antibiotics, since in the environment the concentrations of these molecules rarely attain inhibitory levels [23].

Significance

The action of subinhibitory concentrations of antibiotics on promoter-*lux* fusions provides a highly sensitive and discriminatory approach for the study and classification of protein synthesis inhibitors. Transcriptional effects detected with selected promoter fusions provide gene expression "signatures" and permit the identification of compounds on the basis of mode of action.

Interactions between different structural and functional classes of antibiotics can be readily detected in vivo, and both synergies and antagonisms are common. Studies with combinations of antibiotics showed that inhibitors of translation in eukaryotic cells altered the transcriptional activity of antibacterial antibiotics. This is consistent with the evolutionary relationships between ribosome structures, indicating that antibiotic target sites are conserved between kingdoms [41]. The implications for the evolution of natural antibiotic inhibitors of the ribosome are apparent; did these molecules coevolve with the translation system [52]? The interactions between antibiotics which act on the ribosome, as detected with promoter-*lux* fusions, can be employed to identify ribosome-active compounds that could be of value as lead molecules in structure-based chemical modification programs to provide novel classes of ribosome-targeted antibacterial compounds. The eukaryote inhibitors anisomycin and cycloheximide might be candidates for just such a program.

The mechanism(s) by which inhibitors of ribosome function can modulate a plethora of transcription events is not known. The ribosome is an extremely complex target and has pleiotropic activities; the effect of a translation inhibitor on cell function cannot be easily interpreted. The ease, flexibility, and wide dynamic range of promoter-*lux* reporter libraries permit the identification of unsuspected activities of small molecules that may reflect the roles of these molecules in the environment.

Experimental Procedures

Antibiotics and Growth Conditions

Cultures were grown aerobically in Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/ml) at 37°C (unless otherwise noted). Other antibiotics were added as appropriate. Antibiotics were kindly donated by industry, obtained from Sigma, or taken from the laboratory collection.

Screening of Promoter Responses to MLS Antibiotics and Other 50S Subunit Inhibitors

Salmonella enterica serovar Typhimurium strain ATCC 14028 (referred to as *S. typhimurium*) was used in this study. A random promoter library was constructed by cloning *Sau3A* restriction endonuclease fragments of genomic DNA into the expression vector pCS26 upstream of a promoterless *luxCDABE* operon, as described by Bjarnason [26]. The library consists of 6,528 clones (17 × 384 microtiter plates). *Salmonella* reporter clones were grown aerobically at 37°C in solid or liquid LB medium with kanamycin at 50 μg/ml to maintain the plasmids.

Screening was conducted in 384-well opaque wide plates. A 384-pin replicator was used to inoculate overnight cultures into the wells of screening plates containing LB broth with various antibiotics at a range of subinhibitory concentrations (typically 10-fold lower than the MIC). Plates were then incubated at 37°C and luminescence was measured (at 5 hr and 20 hr) using a Victor II 1420 Multilabel counter (Perkin-Elmer, Boston, MA). Based on differential expression of 3-fold or greater luminescence, positive clones were selected, rearrayed, and vigorously rescreened using the same conditions, with an additional concentration inserted into each antibiotic tested. Overall, the fall-off rate was 67.2% for the first screen and 12.4% for the second screen. To obtain more detailed profiles of the response of the clones to specific antibiotics, they were rescreened again in an overnight assay with continuous hourly monitoring.

Characterization of promoter inserts in active clones was done by PCR-amplification and sequencing of insert DNA fragments using vector primers pCSFor (5′-TGGCAATTCCGACGTCTAAG-3′) and pCSRev (5′-CACTAAATCATCACTTTCGG-3′). Sequencing was performed by Certigen (Lubbock, TX). The promoters were identified by comparison with the GenBank database using the standard BlastN program (NCBI), then analyzed using VECTOR NTI software (Informax, Bethesda, MD).

Solid Media Assay

Overnight LB cultures from single colonies of reporter strains were diluted 1000-fold and inoculated into 0.7% agar (containing kanamycin); appropriate dilutions were made into soft agar and overlaid on LB plates. Etest^{R} strips (AB Biodisk, Solna, Sweden) or antibiotic sensitivity discs were placed on top of the overlay. Plates were incubated at 37°C overnight and luminescence was detected with a Luminograph LB980 photon camera (Berthold Technologies, Bad Wildbad, Germany).

Assaying the Effects of Antibiotic Resistance on Transcription Modulation

Duplicates of 2-fold serial dilutions of MLS antibiotics were made in the wells of black clear-bottom 96-well plates. Overnight liquid cultures of sensitive reporter strains and reporter strains transformed with pCT*ermC* (a plasmid encoding a *tac*-regulated erythromycin resistance gene [18, 27]) were diluted 1:100 in LB medium. Isopropylthio-β-galactoside (IPTG) to a final concentration of 1 mM was added to all wells, and luminescence was recorded after overnight incubation at 37°C in the multilabel counter. Other plasmid-determined resistance mechanisms were used in similar fashion. Tests were also carried out on solid medium as indicated above. In some cases, *E. coli* MG1655 carrying resistance plasmids was used as a host strain to analyze the effect of resistance mechanisms.

Competition Experiments

Competition experiments were conducted using agar medium assays. Antibiotic sensitivity discs containing, for example, 15 μg of erythromycin were placed in the middle of the plate and discs containing appropriate concentrations of other antibiotics were placed in close proximity to the central disc; tests at various displacements were necessary. In some cases, one of the antibiotics being tested was incorporated into the agar medium at a subinhibitory concentration. Luminescence was monitored using the Luminograph camera; the shape of the light zone between neighboring antibiotics indicated the type of interaction (or lack thereof) between the two drugs.

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