Assays to Detect and Characterize Synthetic Agents that Inhibit the ErmC Methyltransferase

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High throughput chemical file screening with an enzymatic assay to detect inhibitors of the ErmC methyltransferase enzyme from macrolide-lincosamide-streptogramin B (MLS_B) resistant pathogenic bacteria identified low molecular weight compounds that had IC_{50s} (50% inhibitory concentration) in the nMolar to μMolar range. These same inhibitors were assessed *in vitro* for their capacity to inhibit the liver enzyme, cathechol-*O*-methyltransferase and the prokaryotic enzyme, *EcoR*I methylase. Selective inhibitors of the ErmC methyltransferase were tested in tertiary assays to determine their minimal inhibitory concentrations (MICs), as single agents and in combination with the macrolide, azithromycin, against strains of pathogenic bacteria expressing MLS_B-resistance. Compounds that were active *in vitro*, alone or in combination with azithromycin, against strains of macrolide-resistant pathogens were tested in a mouse model of infection using an MLS_B-resistant strain of *Staphylococcus aureus* or a macrolide-susceptible strain of *Streptococcus pyogenes*.

Macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics have distinct chemical structures but have been shown to have a similar mechanism of action against bacteria. They inhibit peptidyltransferase reactions *in vitro* and are inhibitors of peptide bond formation in whole cells[1,2]. The first macrolides, largely fermentation products, were narrow to medium-spectrum antibiotics with *in vitro* potency mainly against Gram-positive bacteria, including streptococci, staphylococci and some anaerobes. The lincosamide, clindamycin, was also used extensively against anaerobic bacteria. The newer macrolides, azithromycin and clarithromycin, are semi-synthetic derivatives with expanded activities against certain Gram-negative bacterial species, especially *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Resistance to these antibiotics emerged within a few years of their introduction as therapeutic agents. The most widespread mechanism of macrolide and lincosamide-resistance in pathogenic bacteria results from a base-specific dimethylation of bacterial 23S ribosomal RNA near or within the macrolide binding site such that antibiotics of the macrolide, lincosamide, or streptogramin B chemical classes now fail to bind. Bacterial cells with such modifications become insensitive to inhibitors of these chemical classes. This phenotype is designated MLS_B-resistance[3,4].

Specific dimethylation is achieved in the bacterial cell through the action of a family of closely related methyltransferases encoded by genes designated *erm*, for *e*rythromycin-*r*esistance *m*ethylase. There are at least eight classes of *erm* genes as determined by nucleic acid hybridization analyses and nucleotide sequence comparisons; they show a significant amount of amino acid identity, suggesting that they are derived from a common ancestor. The methylases from Gram-positive bacterial pathogens, for example, are more than 50% identical in amino acid sequence[5]. Consensus primers to conserved regions of the *erm* methylase genes are sufficient to allow detection of all *erm* genes in populations of bacteria by the polymerase chain reaction[6].

The ErmC enzyme has been most extensively characterized. It exists in solution as a 29 kDa monomeric protein which, in the bacterial cell, is largely bound to ribosomes. Methylation in whole cells specified by several related Erm methylases (ErmC, ErmD, ErmAm, and ErmE) inhibits methylation *in vitro* by the ErmC methylase, suggesting that these enzymes modify the same site on rRNA[7]. While there are few published studies of the effect of inhibitors, these enzymes, like many transmethylases, have an absolute requirement for the methyl donor, S-adenosyl methionine (AdoMet). Analogues of AdoMet such as adenosine and S-adenosylhomocysteine (SAH) are highly inhibitory[8,9]. The ErmC methylase also appears to be highly specific for domains of its second substrate, the 23S rRNA. Studies suggest that the enzyme-23S rRNA complex has a dissociation constant of 4×10^{-9} M with a k_{on} and k_{off} of $4 \times 10^{6}\ M^{-1}\ s^{-1}$ and $6.8 \times 10^{-2}\ s^{-1}$, respectively[10].

High throughput screening of synthetic compounds was initiated in order to detect specific inhibitors of the ErmC methylase that could be used in combination with a broad-spectrum macrolide antibiotic for treatment of infections caused by MLS_B-resistant pathogenic bacteria. Such a combination could be used in human populations against macrolide-susceptible and resistant bacteria to extend the efficacy of macrolide, lincosamide and streptogramin B antibiotics, in much the same way that β-lactam, β-lactamase inhibitor combinations are presently used. Such inhibitors were detected and their IC_{50s} for the ErmC methylase determined, as was their selectivity for this, as opposed to two other transmethylation enzymes, the catechol-*O*-methyltransferase from rat liver and the *EcoR*I methylase from *E. coli*. Finally, their capacity to inhibit MLS_B-resistant pathogenic bacteria *in vitro*, and in some cases *in vivo*, was assessed.

Materials and Methods

Chemicals and Other Reagents

P11 cellulose phosphate fibrous cation exchanger (#21111) for column chromatography was from Whatman. *E. coli* rRNA and dithiothreitol (DTT) were from Boehringer-Mannheim. 3,4-dihydroxybenzoic acid, sinefungin, and ethylene glycol-bis (β-aminoethyl ether) N,N′-tetraacetic acid (EGTA) were from Sigma. RNasin ribonuclease inhibitor (#N2111) was from Promega. Catechol-*O*-methyltransferase (COMT; EC 2.2.1.6; NEE-160) and [Methyl-^{3}H] AdoMet; (0.55 mCi/ml; NET-155H) were from NEN. Plasmid BR322 was from Pharmacia. S-adenosyl methionine (AdoMet), *Pvu*II, *EcoR*I, and *EcoR*I methylase were from New England Biolabs. Azithromycin (CP-62,993-42; lot #17419-200-4F) was from the Pfizer pharmacy. Brain heart infusion broth (BHI) was from Remel, Lenexa, Kansas. CP and UK compounds were synthesized by standard methods at Pfizer Central Research, Groton, CT and Sandwich, U.K.

Strains and Plasmids

Bacillus subtilis BD170 (*trp*C2 *thr*-5), containing plasmid pE194 *cop-6* a derivative of pE194 which carries a constitutive mutant of the *erm*C gene from *S. aureus*[8,11] was used as a source of ErmC methylase. *S. aureus* 01A110 and 01A129 are clinical strains with the MLS_B-resistant phenotype. The former is constitutive and the latter, inducible to MLS_B-resistance with 14- and 15-membered macrolides. *E. faecalis* 03A1029 is also an inducibly-resistant MLS_B-resistant clinical strain, while *E. faecalis* RH110 (Tn916ΔE) and the Group B streptococcal strain COH31-46 (Tn916ΔE) were produced by transformation of clinical strains with a transposon carrying a constitutive MLS_B-resistance gene[12]. *E. coli* V854 contains a plasmid which expresses constitutive MLS_B-resistance[13]. *S. pyogenes* 12384 was obtained from ATCC.

Preparation of the ErmC Methyltransferase

Cultures of BD170 (pE194 *cop-6*) were grown with erythromycin selection and enzyme was prepared as previously described[7,8]. Briefly, cells from exponential cultures of this strain were washed once in a low salt buffer, passaged through a French pressure cell, and the cell-free extract digested with DNase. Ribosomes were separated by centrifugation, washed with high salt solutions, and the supernatant solution was dialyzed for 4 hours with several changes of fresh buffer. Magnesium acetate was omitted and the preparation was chromatographed on a phosphocellulose P11 column[7]. Active fractions were pooled and stored at −70°C.

Erm Methyltransferase Assay

Buffers and solutions were prepared with glass distilled DEPC-treated water. 10× reaction buffer containing 500 mM Tris-HCl (pH 7.5), 40 mM $MgCl_2$ and 400 mM KCl was frozen in 50 ml aliquots. Each reaction was performed in a well of a 96-well microtiter plate containing 5.0 μl of 10× reaction buffer, 5.0 μl of 100 mM DTT, 0.025 μl RNasin, 1.4 μl rRNA, and 0.75 μl [^{3}H]-AdoMet adjusted to 30 μl. Putative inhibitors were added in 10 μl volumes. 10 μl of enzyme stock was added. The reaction was incubated for 120 minutes at 37°C and terminated by adding 150 μl of cold 6.6% TCA to each well. The plates were chilled to 4°C for 30 minutes and harvested on a 96-well cell harvester (Skatron, Lier, Norway) using three ten-second wash cycles. The filters were dried in a 50°C drying oven for 45 minutes. TCA-precipitable counts on the filters were quantitated in a LKB beta-plate counter. Sinefungin, an AdoMet analogue, was used as a positive control. IC_{50s} were determined by graphing percent inhibition, as compared to the 'no inhibitor' control, *versus* increasing concentrations of inhibitor.

Catechol-*O*-methyltransferase Assay

Inhibitory activity was determined in a reaction mixture consisting of 225 mM TES, pH 8.0, 10 mM EGTA, 2 mM DTT, 3 mM magnesium chloride, 0.2 mM [^{3}H]-AdoMet, and 2 mM 3,4-dihydroxybenzoic acid. 15 μl of this mixture was added to each well of a microtiter plate. 12.8 μl [^{3}H]-AdoMet [0.153 μCi], 15 μl COMT (0.16 units) and 15 μl inhibitor were added. A 'no enzyme' and a 'no inhibitor' well served as controls in each experiment. The reaction mixture was incubated, extracted and centrifuged as specified in the catechol-*O*-methyltransferase protocol (NEN). The product was quantitated with a Packard Tri-Carb Liquid Scintillation Analyzer. Sinefungin was used as a positive control.

*Eco*RI Methylase Assay

For each assay, 2.0 μl (0.5 μg) linear pBR322 was added to 2.5 μl of 320 μM AdoMet in 5× methylase buffer

(250 mM NaCl, 250 mM Tris-HCl pH 8.0 and 50 mM EDTA), 3.5 μl of inhibitor diluted to desired concentration, and 2.0 μl (0.5 unit) of *EcoR*I methylase in a final volume of 10 μl. The assay tube was incubated at 37°C for 1 hour. A 'no inhibitor' and a 'no methylase' control were included in each assay. The reaction was terminated by heating to 65°C for 10 minutes. The pBR322 template DNA was digested with *EcoR*I restriction endonuclease by standard methods. 17 μl of 3 × agarose gel buffer with 0.25% bromphenol blue and 3% glycerol[14] was added to each reaction tube. 20 μl of this mixture was electrophoresed in a 1.0% agarose gel using 1 × TAE running buffer[14]. 20 μl of *Hind III*-digested λ DNA was added to a separate well to serve as size marker. The samples were electrophoresed for 1 hour at 50 mA and the DNA samples were visualized by photography of ethidium bromide-stained gels. For this assay, complete inhibition of the methylation reaction resulted in the formation of two bands at 2.30 Kb and 2.06 Kb, and no appearance of a 4.36 Kb band. No inhibition of the methylation reaction resulted in the complete inaccessibility of the *EcoR*I site on pBR322, and the appearance of a single band of 4.36 Kb. Inhibition was measured as a decrease in the intensity of the 4.36 Kb linear band on a GDS 5000 UVP densitometer.

Minimum Inhibitory Concentrations of Test Compounds +/− Azithromycin

Preparation of Bacterial Strains

Working stocks of each of the bacterial strains were prepared by growth of isolated colonies in BHI. The presence of desired phenotypes was confirmed. *E. faecalis* RH110 (Tn916ΔE) and Group B streptococcal strain COH31-46 (Tn916ΔE) were grown in 5 μg/ml erythromycin to select against loss of the macrolide-resistance phenotype, while *E. coli* V854 was grown in 10 μg/ml erythromycin. Frozen stocks were prepared by mixing 600 μl of an overnight culture of each strain with 400 μl of 50% glycerol. Replicate stocks were frozen at −70°C. 25 μl of thawed working stock was inoculated into 10 ml of BHI broth and incubated overnight at 37°C with shaking. *E. faecalis* RH110 (Tn916ΔE) and the streptococcal strain COH31-46 (Tn916ΔE) were grown in BHI with 5 μg/ml erythromycin. In the morning, strains were removed from the incubator and their turbidities at A600 were determined, using BHI as a blank. Each of these strains achieved turbidities of 0.3 to 0.5 under these conditions.

Preparation of Microtiter Plates

The MIC determinations were performed in microtiter plates, using a separate plate for each test compound. A Cetus Pro/Pette (Perkin-Elmer) was used to serially dilute the azithromycin in wells A1 to H1 across the wells through column 7. Test compounds were two-fold serially diluted in BHI in test tubes. With a multichannel pipettor, 50 μl of the first dilution of test compound was added to A1 through A7. Additions were continued in this manner through well G7. One well was filled with 200 μl BHI to serve as a media sterility control well. A second well was filled with 100 μl BHI and 100 μl of the test strain to serve as a bacterial growth control. Additional wells contained 150 μl BHI and 50 μl of azithromycin or test compound stock solution as controls for compound and azithromycin sterility. Microtiter plates were either inoculated immediately or wrapped and frozen at −70°C.

Inoculation of Strains into Microtiter Plates

Bacteria were prepared as indicated above and diluted 1/1000 in BHI. The diluted test strain was poured into a sterile trough or petri dish. A multichannel pipettor was used to introduce 100 μl of this suspension to wells A1 through H8. 100 μl of this inoculum was also added to the appropriate control wells (see above). The final inoculum in the wells was $2 \sim 7 \times 10^5$ viable cells/ml, as determined by dilution and plating. The plates were shaken for 3 to 5 minutes on a Sarstedt TPM-2 shaker at 500 rpm. They were incubated overnight (approximately 15 hours) in a 37°C incubator. The turbidity in each well was observed with a mirrored plate reader (Titertek). For each plate, the lowest concentration of azithromycin that produced no growth was scored as the azithromycin MIC. The MIC of the test compound was similarly defined. For the wells with test compound plus azithromycin, the MIC was considered to be the combination which contained the lowest total concentration that inhibited growth of the test strain. The control wells were observed for appropriate growth or absence of growth. The solvent control wells in these assays produced growth of test organisms comparable to the growth control well. Synergy in these assays was defined as a ≥ 2 dilution difference between the MIC of azithromycin plus test compound as compared to the MIC of test compound or azithromycin alone[15].

Acute Systemic Infections

The acute systemic infections were produced by ip administration of bacterial challenge to CF1 (Charles River) mixed sex mice (11 ~ 13 g). The number of organisms injected was adjusted to provide an inoculum one to ten times the LD_{100}. *S. pyogenes* ATCC 12384 was grown overnight at 37°C in BHI broth, appropriately diluted and administered as a suspension in BHI broth. *S. aureus* 01A129 was grown overnight on BHI agar containing erythromycin (5 μg/ml), washed off with sterile phosphate buffered saline, adjusted to a standard turbidity and suspended in sterile 5% hog gastric mucin prior to administration to mice. Survival was recorded over a 4-day period and the PD_{50} calculated from data obtained from a dose range consisting of four different antibiotic concentrations in a two-fold (*S. aureus* studies) or four-fold (*S. pyogenes* study) dilution series. Mice (10 per group) were treated subcutaneously at 0.5 and 4 hours

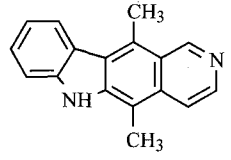
after challenge. The 50% protective dose (PD_{50}) was expressed in mg/kg/dose and calculated by the probit method[16].

Results and Discussion

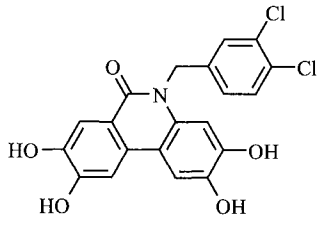
Compounds of several structural types (Table 1) were shown to be potent inhibitors of the ErmC methyltransferase reaction, producing IC_{50s} of 450 nM to 22.1 μM against the ErmC methylase. No structural similarities were apparent among them which might indicate their mode of action. Nor were these compounds known to be inhibitors of methylase enzymes or ribosome components. Many of these compounds were highly specific for the Erm methylase, producing little or no inhibition of COMT or the *EcoR*I methylase (Table 2). Note that the IC_{50s} of the ErmC methylase are expressed in μM concentrations, while the IC_{50s} for COMT and the *EcoR*I methylase are expressed in mM concentrations. CP-112,842-03 and UK-80,882 were the least selective, being 50 and 6.2 times more inhibitory for the ErmC methylase than for COMT, respectively. Only CP-117,519-01 produced any inhibition of the *EcoR*I methylase, generating an IC_{50} of 800 μM. Increasing concentrations of AdoMet in the Erm methylase assay did not affect the IC_{50s} of any of these compounds, suggesting that their inhibition was not due to competition for the AdoMet site on the enzyme (data not shown). UK-105,730 was among the most potent, producing an IC_{50} of 450 nM against the Erm methylase while the β-lactam, CP-29,474-22 was the least potent, with an IC_{50} of 22.1 μM.

The MIC of these inhibitors for a panel of known MLS_B-resistant bacterial strains was determined (Table

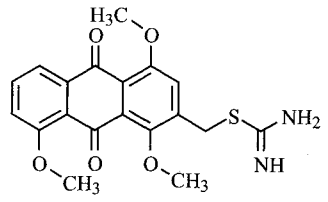
Table 1. Chemical structures of ErmC methylase inhibitors.



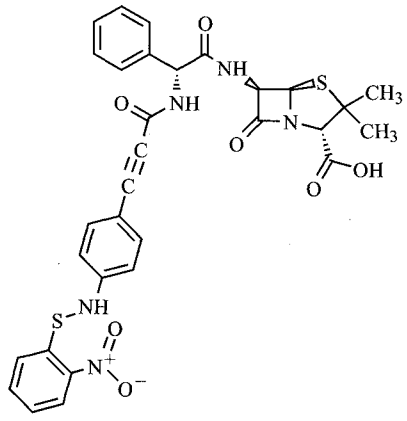
CP-3025 (Replaces P 3025)



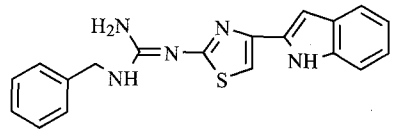
CP-112842-3 hemihydrate



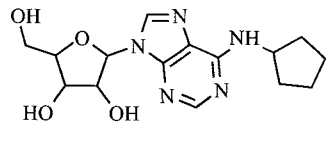
CP-117519-1 hydrochloride salt



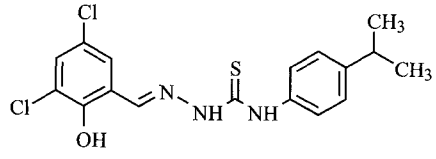
CP-29474-22 triethylamine salt



CP-74932-4 hydrobromide salt



UK-80882



UK-105730

Table 2. IC_{50s} of synthetic agents for the ErmC methyltransferase, the cathechol-*O*-methyltransferase and the *Eco*R I methyltransferase.

Compound	ErmC methyltransferase IC_{50} (μM)	Catechol-*O*-methyltransferase IC_{50} (mM)	*EcoR* I methyltransferase IC_{50} (mM)
P-3025	4.1	>1.0	>1.0
CP-29,474-22	22.1	>1.0	>1.0
CP-74,932-04	0.75	>1.0	>1.0
CP-112,842-03	0.9	0.045	>1.0
CP-117,519-01	6.6	>1.0	0.800
UK-80,882	63	0.390	>1.0
UK-105,730	0.45	>1.0	>1.0
Sinefungin	5.0	2.0	0.080

Table 3. Checkerboard synergy MIC determinations in BHI broth (μg/ml).

Bacterial strain	P-3025	CP-29,474-22	CP-74,932-04	CP-112,842-03	CP-117,519-01	UK-80,882	UK-105,730
S. aureus 01A110	6.2/0.39	>25/>25	3.1/0.39	>25/>25	>25/>25	0.78/0.39	3.12/12.5
	6.2/>50	>50/>50	3.1/>50	>50/>50	>50/>50	1.5/>50	6.25/50
S. aureus 01A129	6.2/0.39	>25/>25	3.1/0.39	>25/>25	6.2/12.5	0.78/0.39	1.5/1.5
	6.2/>50	>50/>50	3.1/>50	>50/>50	>50/50	0.78/>50	>50/>50
E. faecalis 03A1029	3.1/0.39	6.25/0.78	6.2/0.39	>25/>25	0.39/25	3.1/0.39	12.5/0.39
	3.1/>50	6.25/>50	6.2/>50	>50/>50	>50/50	6.2/>50	50/>50
E. faecalis	6.2/0.39	1.56/1.56	6.2/0.39	>25/>25	>25/>25	3.1/0.39	12.5/0.78
RH110 (Tn916ΔE)	6.2/>50	1.56/>50	6.2/>50	>50/>50	>50/>50	1.5/>50	25/>50
GBS COH31-46	3.1/0.39	<0.39/<0.39	6.2/0.39	>25/>25	25/25	0.78/0.39	12.5/0.39
(Tn916ΔE)	3.1/>50	<0.20/>50	6.2/>50	>50/>50	50/>50	0.78/>50	25/>50
E. coli 854 MLS_BR	6.2/0.39	6.25/1.56	6.2/0.39	>25/>25	>25/>25	>25/>25	>25/>25
	6.2/>50	12.5/>50	12.5/>50	>50/>50	>50/>50	>50/>50	>50/>50

For each strain, the top row of numbers is the concentration of the test compound plus the concentration of azithromycin that produce an MIC. The bottom row is the MIC of the compound alone/MIC of azithromycin alone.

3). In these assays, the MIC of the inhibitor in combination with the macrolide, azithromycin, was compared to the MIC of the inhibitor alone and azithromycin alone. CP-29,474-22, did not inhibit the MLS_B-resistant staphylococcal strains, but was active against the enterococcal and Group B streptococcal strains. It was also moderately active against the recombinant *E. coli* strain expressing MLS_B resistance. Azithromycin did not have a synergistic effect on the potency of CP-29,474-22 against these strains. CP-117,519-01 showed synergistic or additive activity with azithromycin against *S. aureus* 01A129 and *E. faecalis* 03A1029 in the assay. In contrast, ErmC inhibitors P-3025 and CP-74,932-04 were active as single agents against each of the bacterial strains. Both compounds had MICs of 3.1 to 6.25 μg/ml, and neither had a synergistic or additive effect with azithromycin. This was an unexpected result, since inhibition of the ErmC methylase would not *a priori* be expected to inhibit the growth of bacterial cells expressing this enzyme. Although these compounds did not inhibit the bacterial *Eco*RI methylase, which methylates an internal adenine residue within a specific hexanucleotide palindromic sequence on DNA, it is possible that they inhibit other bacterial transmethylases and that this effect is responsible for their potency as single agents. P-3025 and CP-74,932-04 were also inhibitory to macrolide-sensitive bacterial strains (data not shown). Since their effect on these strains could not have been due to inhibition of ErmC methylase, these inhibitors must have additional effects on bacterial cells. Sinefungin produced no inhibition in these assays, presumably because it was not able to penetrate into bacterial cells (data not shown).

Some of the selective ErmC methylase inhibitors that showed *in vitro* activity against *Staphylococcus aureus* were evaluated for activity against a generalized, fatal infection in mice caused by a *Staphylococcus aureus* strain, 01A129, expressing inducible MLS_B-resistance. None of these compounds protected mice at subcutaneous doses of $\leq$100 mg/kg. CP-29,474-22 was not active *in vitro* against staphylococcal strains, but showed activity against enterococcal and streptococcal

Table 4. Parenteral protective dose 50 (PD_{50}) determinations.

Compound	PD_{50} (mg/kg/dose)	
	S. aureus (01A129)	*S. pyogenes* (ATCC 12384)
P-3025	>100	NT*
CP-29,474-22	NT	29.2**
CP-74,932-04	>100	NT
UK-80,882	>100	NT
UK-105,730	>100	NT
Vancomycin	14.8	NT
Azithromycin	>100	<1.56

* No test.
** MIC = 3.1 μg/ml.

strains. It was progressed to a PD_{50} determination in a mouse model of infection with a macrolide-susceptible *S. pyogenes* strain, and produced a PD_{50} of 29.2 mg/kg (Table 4). This β-lactam derivative was shown in separate experiments to have *in vitro* potency against several β-lactam-resistant bacterial strains, suggesting that it had additional modes of action against bacterial cells (data not shown).

It is relevant to considerations of the effectiveness of putative Erm methylase inhibitors to observe that Erm enzymes in the cell are maintained at relatively low levels by two independent regulatory mechanisms. Where they are inducible, it has been shown that one level of regulation occurs at the level of the ribosome by a process called translational attenuation[17]. However, in recent years in areas where macrolide use has increased many MLS_B-resistant strains have become constitutive, which usually occurs by a deletion in the 5′ regulatory region[5]. A second aspect of regulation may limit the production of methylase in cells with both inducible and constitutive MLS_B-resistance. It has been observed that expression of the protein is autoregulated at the level of its own translation, and that certain methylase-deficient variants of ErmC with mutations in the structural gene overproduce truncated forms of inactive methylase. This is consistent with a model in which active methylase regulates its own production by a feedback regulatory loop. The net effect of this is to bring the number of methylase molecules in the cell to a steady state which is approximately equal to the number of ribosomes per cell, $2 \sim 3 \times 10^4$[18]. Competitive or non-competitive inhibitors of enzymes produced constitutively would have to be present in higher concentrations to be effective. One hundred and sixty thousand synthetic compounds were screened and small-molecule inhibitors of various structural types were identified as specific inhibitors of the dimethylation of 23S rRNA catalyzed by the ErmC methylase. Four of these had $IC_{50s} < 5\ \mu M$ and appeared to have little or no inhibitory activity against two unrelated methyltransferases. Some of these were able to penetrate into Gram-positive, and sometimes into Gram-negative, MLS_B-resistant bacterial strains and inhibit growth (Table 3), either alone or in synergy with a macrolide. Although most of these compounds had no activity in mouse models of infection, they represent synthetic leads which may be utilized to design more potent, selective agents with pharmacokinetic properties suitable for use in human disease.

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