A Novel Imidazoline Antimicrobial Scaffold that inhibits DNA replication with Activity Against Mycobacteria and Drug-Resistant Gram-Positive Cocci

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SUPPLEMENTARY METHODS

Strain Growth Conditions. All M. smegmatis strains were isogenic to mc^2155 and were cultured at 37°C in Luria Broth (LB) media supplemented with 0.5% (v/v) glycerol, 0.5% (v/v) dextrose, and 0.05% (v/v) Tween 80 for liquid cultures, or on LB agar plates supplemented with 0.5% glycerol and 0.5% dextrose. M. tuberculosis Erdman strain was cultured as follows: 37°C in 7H9 broth supplemented with 10% (v/v) oleic acid/dextrose/catalase (OADC), 0.5% glycerol, and 0.05% Tween 80, or on 7H10 agar supplemented with 10% oleic acid/dextrose/catalase (OADC) and 0.5% glycerol. DH5α grown in LB with appropriate antibiotic selection was used for all recombinant DNA manipulations.

Reagents, Synthesized Compounds, and Antibiotics. Schemes for the synthesis of the 14 derivatives of SKI-356313 are provided as Supplementary Schemes S1 to S6. Derivatives characterization is provided in Supporting Information. Antibiotic concentrations used were as follows: Kanamycin 40 μg/ml and hygromycin 150 μg/ml in E.coli. Kanamycin 20 μg/ml, hygromycin 50 μg/ml and ATc 50 ng/ml in M. smegmatis. MMS (Sigma), C2FDG (Molecular Probes), EtBr (Sigma), vancomycin (Sigma), cyclophosphamide (Sigma) and [5,6-^3^H]uracil (PerkinElmer, NET368) were used at the indicated concentrations.

Disk Diffusion Assays, Minimal Inhibitory Concentration Assays and Survival Curves. Disk diffusion assays were conducted as follows: M. smegmatis grown to an OD600 of 0.5 was diluted 1:5 in Mueller Hinton broth and
plated for lawn growth on Mueller Hinton agar plates. Sterile 6mm discs (Bio-101 #248712) were placed on top of the cells and 5μl of 10mM compound, or DMSO as a negative control, was added on top of each disc. Plates were incubated at 37°C for 3 days before measurement of the zone of growth inhibition. *M. tuberculosis* disk diffusion assays were conducted in a similar manner except *M. tuberculosis* was plated for lawn growth on 7H10 agar supplemented with 10% oleic acid/dextrose/catalase (OADC) and 0.5% glycerol. Plates were incubated at 37°C for 21 days before measurement of the zone of growth inhibition.

*Broth microdilution MIC assays for* *M. Smegmatis* and *M. tuberculosis* were conducted via serial dilution in 96 well plates. Mid-log cultures were diluted to an OD600 of 0.1 in LB media (for *M. smegmatis*) or 7H10 media (for *M. tuberculosis*). 96 well plates were set up with serial 2-fold dilutions of drug across all wells, with the last well containing no drug as a positive control for growth. *M. smegmatis* MIC broth dilution 96 well plates were incubated at 37°C observed for growth on day 4 and 7 by visual inspection. *M. tuberculosis* MIC broth dilution 96 well plates were incubated at 37°C observed for growth on day 6 and 12 by visual inspection. For all other organisms, broth microdilution MIC assays were conducted at NAEJA ([www.naeja.com](http://www.naeja.com)) as follows: Isolated colonies from 18–24 hour agar plates (Mueller Hinton, Blood Agar or SDA plates) were suspended in a sterile water and each bacterial suspension was adjusted to read between 0.09 and 0.11 absorbance at 620 nm (0.5 McFarland Standard). These were subsequently diluted 1/100 in appropriate broth and inoculated into 96-well plates. For *C. albicans* the suspension was adjusted to read between 70 and 75% transmittance at 530nm and then diluted 1/500 in RPMI+MOPS broth. Aerobic
and fungal plates were incubated at 35°C for 18 hours. *S. pneumoniae* plates were incubated in the presence of 5% CO₂. Plates were read using a Beckman Automated Plate Reader at 650 nm. Readings were confirmed by visual examination of plates.

MIC assays on solid media for *M. smegmatis* overexpression library and MMS library MIC determination were conducted as follows: Log-phase *M. smegmatis* was harvested by centrifugation, and 5x10^6 CFU (for kanamycin-selected strains) and 1x10^8 CFU (for all other strains) spotted onto LB plates supplemented with appropriate antibiotic for plasmid selection and SKI-356313 at the indicated concentration. Plates were incubated at 37°C and observed for growth at Day 5, 6 and 7.

For survival curves, *M. tuberculosis* or *M. smegmatis* cultures grown to an OD600 of 0.5 were treated with SKI-356313 and incubated at 37°C. Aliquots were removed at designated time points and plated onto solid media for determination of CFU/ml.

**Non-Replicating Bacillus Assays (Wayne Model).** Wayne Model experiments were performed according to previously published protocols, and described in brief here(1). Mid-log cultures of *M. tuberculosis* were diluted in Dubos Tween-albumin broth to a final OD600 0.005. Culture was then aliquoted, 17ml each, into sterile borosilicate 20x125mm glass tubes to yield a headspace ratio (HSR) of 0.5, each with a 1.5x8mm Teflon-coated stir bar and either loosely capped (for oxygenated culture conditions) or tightly sealed with rubber stoppers (for deoxygenated culture conditions) and stirred at 150rpms at 37°C. To prevent the
introduction of oxygen into deoxygenated culture tubes, a 1ml tuberculin syringe and intradermal bevel 26G 3/8 needle was used for the addition of compounds or the removal of culture aliquots. Drugs were added at indicated time points, with the exception of metronidazole which was added at 0 hours due to its inactivity on actively growing cultures(1). Stocks of isoniazid, metronidazole and methylene blue were diluted in dH₂O, and SKI-356313 was diluted in DMSO, such that total volume of compound added was less than 500μl. Methylene blue was added to separate culture tubes at a final concentration of 1.5μg/ml. Aliquots of cultures were diluted in 7H9/TB media and plated for CFU.

**Genomic Fragment Overexpression Libraries.** MGM 5000 and MGM 5001 (M. smegmatis and M. tuberculosis genomic fragment overexpression libraries, respectively) were created as follows. A 5149 bp PacI fragment from pTE-mcs1(2) and a 1284bp PacI fragment from pTX-2MIX(3) were jointed to create pMSG417, in which a ClaI site is downstream of the TetO regulated promoter. Insertion of annealed tetRBSHAClaIS and tetRBSHAClaIL oligonucleotides into the SphI/ClaI sites of pMSG417 introduces a ribosomal binding site and Hemagglutinin tag to create the final library vector pMSG419. M. smegmatis or M. tuberculosis genomic DNA were separately partially digested with HpaII, TaqI, and HinPI. Partial digests were separated on agarose gels and 1–3 kb chromosomal fragments were gel purified, mixed, and ligated into ClaI digested and dephosphorylated pMSG419. Transformation into E.coli and selection on hygromycin allowed the isolation of 8600 E. coli clones from the pMSG419-M. smegmatis library, and 113,700 E.coli clones from the pMSG419-M. tuberculosis
library. Multiple *M. smegmatis* transformations with each plasmid library were performed and approximately $2 \times 10^7$ *M. smegmatis* clones to create strains MGM5000 and 5001. Primers pMSG419up and pMSG419sequp were used for sequencing of genomic fragments in pMSG419 *M. smegmatis* and *M. tuberculosis* library constructs.

**Individual ORF Overexpression Constructs.** All open reading frames were amplified from *M. smegmatis* gDNA. Msmeg_5185 was amplified using primers oKH9 and oKH10, and inserted into the MscI/EcoRI digested pMV261 vector to produce pKH03. The hicB ORF was amplified using primers oKH11 and oKH12 and inserted into the MscI/EcoRI digested pMV261 vector to produce pKH04. The ORF of tet(V) was amplified using primers oKH13 and oKH15 and inserted into the MscI/EcoRI digested pMV261 vector to produce pKH05. The ORF of Msmeg_3815 was amplified using primers oKH18 and oKH19 and inserted into the MscI/EcoRI digested pMV261 vector to produce pKH07. The ORF of Msmeg_1953 was amplified using primers oKH35 and oKH38 and inserted into the MscI/EcoRI digested pMV261 vector to produce pKH08. All ORFs inserted into the pMV261 vector were verified by sequencing to be free of mutations.

**MMS Mutagenized M. smegmatis Libraries.** The protocol for the creation of MGM 5008 and MGM 5009 (0.2% and 0.4% MMS libraries, respectively) was adapted from the literature and is summarized here(4-7). Liquid *M. smegmatis* cultures were collected by centrifugation and re-suspended in 7H9 media supplemented with 0.5% (v/v) Tween80, then treated with MMS at either 0.2%
(v/v) MMS or 0.4% (v/v) MMS and incubated with shaking at 37°C for one hour. Cultures were collected by centrifugation, washed three times with 7H9 supplemented with 0.5% Tween 80, and cultured on LB agar media and surviving bacteria pooled and frozen. Library mutagenesis efficiency was determined by screening for the frequency of total amino acid auxotrophy according to protocols adapted from the literature (8). MGM 5008 and MGM 5009 were cultured on minimal agar media (8) supplemented with a solution of total amino acids (1.14g/ml BSA dropout powder, 20mg/L uracil, 20 mg/L histidine, 100mg/L leucine and 50 mg/L tryptophan). These plates were then replica plated onto minimal media plates lacking total amino acid supplementation and incubated at 37°C for three days. Amino acid auxotrophy frequency was calculated as the frequency of colonies capable of growth on minimal media supplemented with amino acids but incapable of growth on plates lacking total amino acid supplementation. Approximately 1000 colonies were screened for each strain. The amino acid auxotrophy frequencies of MGM 5008 and MGM 5009 were 1.75x10^{-2} and 2.5x10^{-2}, respectively.

**Isogenic HicB P12T Mutant Strain Construction.** MGM 5010 (HicB P12T SNP mutant) was created by mycobacterial recombineering (9). Briefly, Mc21659 (mc2155/pRGM18) was transformed with JCV198 (hygromycin resistance rescue oligo) and oKH66 (introduction of KpnI site and HicB P12T SNP). Hygromycin resistant colonies were selected, submitted to colony PCR using oKH67 and oKH68 to amplify the 369 nucleotide SNP-containing fragment, PCR purified and diagnostically digested with KpnI for verification of the presence of the HicB P12T
mutation, and unmarked by selecting on sucrose followed by growth without kanamycin. The presence of the HicB P12T mutation was confirmed by sequencing with oKH67 and oKH68.

**Creation of a ΔhicB Strain.** MGM 5018 (ΔhicB) was created by a sacB/galK counterselectable marker system of homologous recombination(10). Primers oKH83 and oKH84 were used to amplify a 581 bp region upstream of the 5′ start of the hicB ORF from wild-type *M. smegmatis* DNA (JCVI Comprehensive Microbial Resource database mc²155 sequence). Likewise, primers oKH85 and oKH86 were used to amplify a 623 bp region downstream of the 3′ end of the predicted hicB ORF. The oKH83–84 fragment was digested with NdeI/SspI, and the oKH85–86 fragment was digested with NdeI/XbaI and sequentially ligated into pAJF067 (sacB and galK counterselectable mycobacterial suicide vector), transformed into *M. smegmatis* and plated for growth on hygromycin, which allowed for selection of colonies in which the plasmid had successfully integrated into the chromosome via homologous recombination. Southern blot of gDNA from hygR colonies allowed verification of plasmid construct integration into the chromosome at the hicB locus, using SphiI digest yielding 3860 bp and 1056 bp fragments for 5′ integration events; and Bsal digest yielding 1671 bp and 8450 bp fragments for 3′ integration events (using a probe to the oKH83–oKH85 5′ region). Sucrose and 2-deoxy-galactose (2-DOG) resistant, hygromycin sensitive recombinants were genotyped by southern hybridization, in which digestion of gDNA with SmaI yielded a 1636 band in wild-type hicB but a 6799 bp band in ΔhicB (using a probe to the oKH85-oKH86 3′ region). The final recombination
event contains a 39 nucleotide scar of hicB and preserves the HicB translational reading frame.

**lacZ Transcriptional Fusions.** The construction of lacZ transcriptional fusions to hicB or tet(V) promoter regions was performed as follows: The hygromycin resistance cassette was isolated from p16R1(11) by BspHI/Smal digest. The BspHI overhang was flushed and XbaI linkers attached, followed by digestion with XbaI and ligation into pMV206(12) that had been cut with Nhel/Spel (to remove kanamycin resistance gene). The resulting plasmid was designated pMV206Hyg. Subsequently, the lacZ and RBS was cut from plasmid pJEM15 (13) with PstI/BamHI and ligated into pMV206Hyg cut with PstI/BamHI for the creation of pHMG147. Primers oKH69 and oKH70 were used to amplify a 287 bp region spanning the wild-type hicB and tet(V) intergenic region from wild-type M. smegmatis DNA. Primers oKH69 and oKH70 were also used to amplify a 287 bp region spanning the HicB P12T SNP-containing hicB and tet(V) intergenic space from strain MMS 42 gDNA. These fragments were individually digested with XbaI/SphI and separately ligated into the XbaI/SphI digested pHMG147 vector to produce pKH11 (lacZ transcriptional fusion to wild-type hicB-tet(V) intergenic region in the direction of hicB transcription) and pKH12 (lacZ transcriptional fusion to HicB P12T promoter region in the direction of hicB transcription), respectively, and subsequently transformed into M. smegmatis to generate MGM 5011 and MGM 5012. Likewise, primers oKH71 and oKH72 were used to amplify a 318 bp region spanning the wild-type hicB and tet(V) intergenic region from wild-type M. smegmatis DNA. Primers oKH71 and oKH72 were also
used to amplify a 318 bp region spanning the HicB P12T SNP-containing hicB and tet(V) intergenic space from strain MMS 42 gDNA. These fragments were individually digested with XbaI/SphI and ligated into the XbaI/SphI digested pHMG147 vector to produce pKH13 (lacZ transcriptional fusion to wild-type hicB-tet(V) intergenic region in the direction of tet(V) transcription) and pKH14 (lacZ transcriptional fusion to HicB P12T promoter region in the direction of tet(V) transcription), respectively, and subsequently transformed into M. smegmatis to generate MGM 5013 and MGM 5014. Additionally, plasmids pKH11, pKH12, pKH13 and pKH14 were all separately transformed into MGM 5018 for the creation of transcriptional fusions in the ΔhicB background (MGM 5019, 5020, 5021 and 5022, respectively). All intergenic region insertions were verified by sequencing.

**Radiometric Labeling Assays.** Nucleic acid synthesis was measured according to previously published protocols (14, 15). Briefly, [5,6-3H]uracil at 1μCi/ml plus appropriate drug treatment was added to early log phase M. smegmatis cultures and allowed to incubate for one hour with shaking at 37°C. For assessment of total nucleic acid incorporation, 3 ml of culture was removed and filtered through 0.45μM nitrocellulose filters. For assessment of DNA incorporation, 3 ml of culture was removed and KOH added to a final concentration of 0.3M and incubated for 24 hours at 37°C before filtering through 0.45μM nitrocellulose filter. Filters were dissolved in 2-ethoxyethanol for 2 hours, and samples read via scintillation counting using 96 well LumaPlates. Total RNA
incorporation was calculated as the difference between total nucleic acid incorporation minus DNA incorporation.

**Fluorescently-Tagged DNA Replication, Transcription and Translation Reporter Strains for Time-Lapse Microscopy.** MGM 6025 (DNA polymerase III α-subunit fusion to GFP) was constructed as follows: Plasmid pAJF315 is a pBluescript derivative encoding hygromycin resistance and containing linker-msfGFP (Addgene #29772). Msmeg 3187 was amplified from *M. smegmatis* gDNA with oAJF467 and oAJF468, cut with XbaI/NdeI and ligated into pAJF315 cut with the same, and subsequently transformed into *M. smegmatis*. For strain MGM 6063 (*rpoC* fusion to mCitrine and *rplA* fusion to mCherry), pAJF263 was created as a pBluescript derivative encoding hygromycin resistance and containing linker-mCitrine-streptagII. The gene *rpoC* was amplified from *M. smegmatis* gDNA with oAJF630 and oAJF631, cut with XbaI/NdeI and ligated into pAJF263 cut with the same and subsequently transformed into *M. smegmatis* to create strain MGM 6026. Subsequently, pAJF384, which lacks a mycobacterial origin of replication, was created through assembly of three PCR products: mCherry was amplified using oAF530 and oAF531; pACYCori was amplified using oAF532 and oAF535; streptomycin resistance cassette was amplified using oAF534 and oAF535. The gene *rplA* was amplified with oAF573 and oAF574, cut with XbaI/NdeI and ligated into pAJF384 cut with the same to create pAJF486, and subsequently transformed into MGM 6026 to create strain MGM 6063.
The fluorescent protein expression strains MGM 6026 and MGM 6063 express stable fusion proteins at the expected size and have wild-type growth rates (Supplementary Figure 5). Additionally, the MIC of ciprofloxacin for MGM 6026 is unchanged from that of wild-type \textit{M. smegmatis}. Likewise, the MIC of rifampin and the MIC of chloramphenicol for MGM 6063 are unchanged from that of wild-type \textit{M. smegmatis} (data not shown).

**Immunoblotting.** For protein and epitope tag detection, the following antibodies were used: msfGFP and mCitrine (Rockland Immunochemicals, Rabbit Anti-GFP polyclonal antibody, 1mg/ml, 1:20,000), mCherry (Rockland Immunochemicals, Rabbit Anti-RFP polyclonal antibody, 1mg/ml, 1:20,000), CarD (clone 10F05, Memorial Sloan Kettering Cancer Center Monoclonal Core Facility, mouse Anti-CarD monoclonal antibody, 1:10,000).

**Processing of Ion Torrent PGM Sequencing Runs.** The raw reads were first trimmed for quality using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with a minimum quality setting of Q=20 and a minimum read length after trimming of 30bp.

Reads were then aligned to the \textit{M. smegmatis} strain mc\textsuperscript{2}155, complete genome (Genbank id: CP000480.1) using the BWA aligner (version 0.5.9; ALN/SAMSE method). The mapped SAM files were then processed with Picard to: add read group information and then mark duplicates. A key step in any variant detection pipeline is to process the de-duplicated alignment files (BAM
files) via the GATK realignment and recalibration pipeline. However, there were two issues with doing this on the PGM data. First, because of the homopolymer errors in the PGM technology the then current version of the realignment pipeline was not usable; therefore we skipped this step.

To do the base quality recalibration, a file of known SNPs (variable sites) is needed for the recalibration step. Since there was no available file of known sites for this genome we used a two pass bootstrapping processing to first call a set of low quality SNP. This SNP file was then used as the knownSites parameter for the GATK CountCovariates step. This first SNP file was generated using:

```bash
samtools mpileup
bcftools view
```

Indel events were filtered out and the __output__ VCF file was then used in the CountCovariates/TableRecalibration (GATK version 1.6) __step__. We then called mutations (SNPs and INDELs) using the GATK UnifiedGenotyper. We then filtered for events that were unmutated (GT=0/0) in the wild-type sample and had at least one mutation in the derived samples.

Subsequently, sequences were analyzed for the frequency of SNPs among total reads available for a genomic locus. A cut-off of a 60% allele frequency was used to designate SNPs for further analysis. We next narrowed the list of SNPs for subsequent investigation by identifying SNPs encoding amino acid substitutions in their predicted open reading frames. SNPs, and their absence from the parental wild type strain, were all verified by sequencing of PCR amplified chromosomal DNA containing the mutations.
**C2FDG β-galactosidase Assay.** At indicated time points aliquots of *M. smegmatis* cultures were removed, normalized to 10⁶ CFU/well by OD600 and added to a black 96 well plate. C2FDG was added to each well at a final concentration of 33μM. PBS was added to control wells instead of C2FDG to enable calculation of background fluorescence. Plates were incubated at 37°C for three hours, then moved to a Wallace 1420 plate reader with temperature control set to 37°C, and read using plate reader filter set: Ex/Em 485±20 nm/530±25 nm.

**Microscopy parameters.** Images were acquired using a Zeiss Axio Observer Z1 microscope equipped with Definite focus, Stage top incubator (Insert P Lab-Tek S1, TempModule S1), Colibri.2 and Illuminator HXP 120 C light sources, a Hamamatsu ORCA-Flash4.0 CMOS Camera and a Plan-Apochromat 100x/1.4 oil DIC objective. Zeiss Zen software was used for acquisition and image export. The following filter sets and light sources were used for imaging: YFP (46 HE, Colibri2.0 505 LED, 40% bulb intensity and 250 ms exposure), GFP (38 HE, Colibri2.0 470 LED, 60% bulb intensity and 500 ms exposure).

**Microscopic observation of Hoechst co-localization.** Images were acquired using a Zeiss Axioplan 2 imaging microscope equipped with an AxioCam HR camera and a Plan-NEUFLUAR 100x/1.3 oil DIC objective. AxioVision software was used for acquisition and image export. The following filter sets and light sources were used for imaging: CFP (47 HE, HXP 120 C), Hoescht 33342 (DAPI
filter cube), and FM 4-64 (Rhodamine filter cube). Wild-type *M. smegmatis* was treated with 0.048μM SKI-356313 or derivatives or equal volume DMSO for four hours. For cell staining 100μl of culture was used. A final concentration of 1μg/ml FM 4-64 (Invitrogen) and 10μg/ml Hoescht 33342 (Invitrogen) was added, or 1μg/ml FM 4-64 alone was added. Cells were pelleted by centrifugation at 5000g for 1 minute and resuspended in 50μl of LB media. For single time point live cell imaging, 7μl of culture was spotted onto a No. 1.5 coverslip and pressed to a slide.

**Fluorescent Intercalator Displacement Assays.** The fluorescent intercalator displacement (FID) assay was adapted from a previously published protocol with minor adjustment(16). In an FID assay the characteristic shift of excitation and emission maxima of ethidium bromide (EtBr) intercalated into DNA compared to free EtBr in solution is measured at an excitation and emission of 545 and 595nm, respectively. When a compound is added to the EtBr-DNA mixture, a loss of the shift in excitation and emission maxima, evidenced by a decrease in relative fluorescence units (RFU), is indicative of the compound displacing EtBr from DNA and is suggestive of DNA binding capabilities of that compound. 63-base pair single-stranded oligonucleotides of complimentary forward and reverse orientation were diluted to 1.2x10^{-4}M base pair in annealing buffer (10mM Tris, pH 7.5, 50mM NaCl, 1 mM EDTA) and annealed by mixing equal volumes of both complimentary oligonucleotides, heating at 90°C for four minutes, and allowing the mixture to cool slowly to room temperature. Annealed oligonucleotides were stored at 4°C prior to use. EtBr was diluted to 1.2x10^{-5}M in
buffer (0.1 M Tris, 0.1M NaCl, pH8). Compound SKI-356313, SKI-7, SKI-22 and SKI-23 were diluted in distilled water. Black 96-well plates were used for all experiments. To each well was added 88 μl of 1.2x10^{-5}M ethidium bromide (final concentration 1.05x10^{-5}M), 10μl of annealed oligonucleotides (final concentration 1.2 x 10^{-5}M base pair), and 2 μl of compound (final concentrations between 0.0029–50.4 μM) and allowed to incubate at room temperature for 30 minutes. Each well was read using a SpectraMax Multi-Mode Microplate Reader at an excitation/emission of 545nm/595nm, with 30 reads per well. In order to determine the change in RFU dependent upon EtBr displacement from DNA, the RFU of control wells lacking DNA was subtracted from the RFU of wells with DNA. Data is reported as the percent fluorescence relative to control wells with no compound, which represent 100% fluorescence.

**Mouse Neutropenic Thigh Inoculation Model.** The study was performed with MRSA (ATCC 33591) and *S. pneumoniae* (ATCC 6303) (American Type Culture Collection) at NAEJA ([www.naeja.com](http://www.naeja.com)). The organisms were grown in Mueller-Hinton agar (MHA) and Brain Heart Infusion agar (BHIA) plates, respectively. For growth in liquid media, cat-ion adjusted Mueller-Hinton broth (MHB) and Brain Heart Infusion (BHIB) broth were used, respectively. MRSA and *S. pneumoniae* were grown fresh from the frozen stock (at –80°C) onto MHA or BHIA plates at 37°C. After checking the purity, few pure single colonies were picked and inoculated in MHB and BHIB and grown overnight to a late log phase (around 12 hours) in a shaking incubator at 37°C. The culture was centrifuged at 4000rpm for 10 minutes at 4°C and the cells were resuspended in sterile normal saline.
(0.9% NaCl, v/v). The cells were washed twice similarly by centrifuging and resuspending in saline. The final inoculums were prepared to 1 OD580 (optical density at 580nm spectrophotometer reading), which were equal to a known number of bacteria (from previous expt.), and then diluted further to 5x10^6 cfu/ml. A volume of 0.1ml of the inoculums was injected into one thigh of each mouse.

The mice were rendered neutropenic by injecting cyclophosphamide at 150gm/kg and 100mg/kg by intraperitoneal (IP) route on four (Day –4) and one day (Day –1) before the day of infection (Day 0). On Day 0, the mice were injected with 0.1ml of the inoculums, as described above, into one of the thighs of each mouse. Vehicle and SKI-356313 were administered by intraperitoneal injection, but vancomycin was injected by subcutaneous injection 2 and 8 hours after infection. The mice were euthanized humanely after 24 hours post-infection by carbon dioxide inhalation and the infected thighs were excised aseptically. The muscles from the thighs were dissected and collected in a round-bottomed tube containing 3ml sterile saline. The tissues were homogenized by BrinkmannPolytron PT300 homogenizer at 22–24K rpm and the resulting homogenates were serially ten-fold diluted (six times) in sterile saline. One hundred microlitres (100μL) of each dilution was cultured on MHA or BHIA plates in duplicates and the plates were incubated at 37° C for 24–48 hours. The colonies were counted and the colony forming units for each thigh (CFU/3ml) were determined and log_{10} of the counts were calculated.
SUPPLEMENTARY RESULTS

MGM 6025 and MGM 6063 reporter strains provide real time readout of antibiotic action for the three major cellular biosynthetic processes. GFP-tagged α-subunit DNA polymerase III appears as either one or two foci within the cell, due to the presence of two replication forks on the circular bacterial chromosome (Supplemental Figure 2A). Ciprofloxacin at 2X MIC (6 μM) resulted in a loss of GFP-tagged α-subunit DNA polymerase III foci and diffusion of GFP signal at time points as early as 20 minutes post drug treatment (Supplemental Figure 2B). Despite this early loss of DNA polymerase foci, arrest of cell growth did not occur until 13 hours post initiation of drug exposure (Supplemental Movie 1), in agreement with previously reported evidence that quinolone-mediated inhibition of DNA replication does not directly correlate with rapid cell killing (17). In contrast to ciprofloxacin treatment, treatment with rifampin at 2.5X MIC (60 μM) did not result in a rapid loss of GFP-tagged α-subunit DNA polymerase III foci (Supplemental Figure 2C). mCitrine-tagged RNAP is visible in M. smegmatis in a pattern that outlines the bacterial nucleoid and mCherry-tagged ribosomes localize in a pattern surrounding the bacterial nucleoid (Supplemental Figure 3A). Treatment of MGM 6063 with rifampin at 5X the M. smegmatis MIC (120 μM) resulted in a condensation of the mCitrine-tagged RNAP signal and dissipation of mCherry-tagged ribosome signal. Growth arrest occurred after four hours of rifampin exposure, followed by loss of both mCitrine-tagged RNAP and mCherry-tagged ribosomes fluorescence over time (Supplemental Figure 3B and Supplementary Movie 2). Chloramphenicol at 5X the M. smegmatis MIC (93 μM),
resulted in a condensation of the mCitrine-tagged RNAP signal, dissipation of mCherry-tagged ribosome signal and eventual loss of both mCitrine and mCherry fluorescence over time (Supplemental Figure 4B and Supplementary Movie 3). These results indicate that these reporter strains provide a real time readout of antibiotic action for the three major biosynthetic processes of the bacterial cell and allow direct observation of the timing of growth arrest with antibiotic treatment.
**Supplementary Table 1.** MICs (μM) of SKI-356313 and synthesized derivatives for 14 bacterial strains and *Candida albicans*

| Resistance profile | M. smegmatis mc²155 | S. aureus ATCC 29213 | S. aureus ATCC 3591 | S. aureus BA4-39 | S. pneumoniae ATCC 700674 | S. pneumoniae ATCC 6301 | E. faecalis ATCC 29212 | E. faecalis ATCC 700221 | E. faecium CT-26 | E. coli ECM1994 | E. coli ATCC 25922 | E. coli ATCC 13883 | K. pneumoniae ATCC 51503 | K. pneumoniae ATCC 61603 | K. pneumoniae ATCC 9027 | P. aeruginosa ATCC 14063 | C. albicans ATCC 14053 |
|--------------------|----------------------|----------------------|----------------------|------------------|--------------------------|--------------------------|----------------------|----------------------|-----------------|-----------------|-----------------|-----------------|-------------------|-----------------|------------------|-----------------|------------------|-----------------|
| SKI-356313          | 0.095                | 0.59                 | 0.59                 | 0.28             | 0.28                      | 0.59                     | 0.14                 | 1.18                 | 2.37            | 2.37            | 4.75            | 2.37            | 2.37               | 2.37            |
| SKI-2               | ND                   | 22.6                 | 11.3                 | 11.3             | 5.6                       | 5.6                       | 5.6                  | 5.6                  | >177            | >177            | >177            | >177            | >177               | >177            | 45.3             | 90.6            | 181             | 181             |
| SKI-4               | ND                   | 2.82                 | 2.82                 | 2.82             | 0.16                      | 1.41                      | 1.41                 | 0.70                 | 0.33            | >180            | >180            | >180            | >180               | >180            |
| SKI-6               | ND                   | 22.2                 | 22.2                 | 88.8             | >177                      | >177                      | 177                  | 177                  | 11.1            | >177            | >177            | >177            | >177               | >177            |
| SKI-7               | ND                   | 5.34                 | 5.34                 | 5.34             | 10.6                      | 85.5                      | 42.7                 | 42.7                 | 0.66            | >171            | >171            | >171            | >171               | >171            | 85.5             | 173             | 173             |
| SKI-8               | ND                   | 43.4                 | 21.7                 | 43.4             | 21.7                      | 86.9                      | 10.8                 | 10.8                 | 5.4             | >173            | >173            | >173            | >173               | >173            |
| SKI-10              | ND                   | 1.16                 | 1.16                 | 1.16             | 0.27                      | 0.27                      | 0.58                 | 0.27                 | 0.58            | 4.64            | 9.28            | 18.5            | 37.1               | 18.5            |
| SKI-11              | ND                   | 18.2                 | 9.1                  | 9.1              | 36.5                      | 9.1                       | 2.28                 | 2.28                 | 36.5            | >146            | >146            | >146            | >146               | >146            |
| SKI-12              | ND                   | 2.32                 | 2.32                 | 2.32             | 0.27                      | 0.27                      | 0.58                 | 0.27                 | 0.58            | 9.3             | 9.3             | 18.6            | 18.6               | 74.4            |
| SKI-20              | ND                   | >144                 | >144                 | >144             | 4.5                       | 4                         | 72                   | 36                   | 18              | >144            | >144            | >144            | >144               | >144            |
| SKI-22              | ND                   | >126                 | >126                 | >126             | >126                      | >126                      | >126                 | >126                 | >126            | >126            | >126            | >126            | >126               | >126            |
| SKI-23              | ND                   | >108                 | >108                 | >108             | >108                      | >108                      | >108                 | >108                 | >108            | >108            | >108            | >108            | >108               | >108            |
| SKI-24              | ND                   | >117                 | >117                 | >117             | >117                      | >117                      | >117                 | >117                 | >117            | >117            | >117            | >117            | >117               | >117            |
| SKI-26              | ND                   | >96                  | >96                  | >96              | >96                       | >96                       | >96                  | >96                  | >96             | >96             | >96             | >96             | >96                | >96             |
| CPLX                | ND                   | 0.75                 | 0.36                 | 24.1             | 1.5                       | 6                         | 3                    | >193                 | >193            | >0.024          | <0.36           | <0.36           | <0.36              | 0.75            |
| IMP                 | ND                   | 0.05                 | >213                 | 3.3              | 0.2                       | 0.2                       | 3.3                  | >213                 | >213            | 1.67            | 0.83            | 3.3             | 0.835               | 6.6             |

*MSSA, methicillin-sensitive S. aureus; MRSA, methicillin-resistant S. aureus; MDR, multi-drug resistant S. aureus; PSSP, penicillin-susceptible S. pneumoniae; PRSP, penicillin-resistant S. pneumoniae; VRE, vancomycin-resistant enterococcus. ND, not determined; CPLX, ciprofloxacin; IMP, imipenem.*
### Supplementary Table 2. Single Nucleotide Polymorphisms (SNP) identified by whole genome sequencing of SKI-356313-resistant MMS mutants.

| Strain | MIC fold increase | Gene   | SNP     | Gene annotation                      |
|--------|-------------------|--------|---------|--------------------------------------|
| MMS 42 | 5X                | Msmeg 1526 | A134V   | Cutinase                             |
|        |                   | dnaE2  | R1085H  | Error-prone DNA-polymerase           |
|        |                   | glgC   | D274N   | Glucose-1-phosphate adenyltransferase|
|        |                   | hicB   | P12T    | HicB antitoxin family protein        |
| MMS 76 | 2X                | hicB   | P12T    | HicB antitoxin family protein        |
| MMS 82 | 2X                | hicB   | P12T    | HicB antitoxin family protein        |
| MMS 87 | 4X                | rfbB   | G330D   | dTDP-glucose 4,6-dehydrogenase       |
|        |                   | dapE   | A132V   | Succinyl-diaminopimelate desuccinylase|
|        |                   | hicB   | P12T    | HicB antitoxin family protein        |
| Strains       | Genotype          | Description                                      | Reference   |
|--------------|-------------------|-------------------------------------------------|-------------|
| mc²155       | Wild-type *M.* smegmatis | Lab stock                                       |             |
| EF2          | *M. tuberculosis* Erdman | Lab stock                                       |             |
| MGM381       | mc²155 pRv1883c-lacZ | *lacZ* reporter strain for HTS                  | This work   |
| MGM 5000     | mc²155/pmsg419-mc²155 genomic fragment library | Tet-On *M. smegmatis* genomic fragment overexpression library | This work   |
| MGM 5001     | mc²155/pmsg419-EF2 genomic fragment library | Tet-On *M. tuberculosis* genomic fragment overexpression library | This work   |
| MGM 5003     | mc²155/pKH03      | GroEL Msmeeg 5185 overexpression                | This work   |
| MGM 5004     | mc²155/pKH04      | GroEL hicB overexpression                       | This work   |
| MGM 5005     | mc²155/pKH05      | GroEL tet(V) overexpression                     | This work   |
| MGM 5006     | mc²155/pKH07      | GroEL Msmeeg 3815 overexpression                | This work   |
| MGM 5007     | mc²155/pKH08      | GroEL Msmeeg 1953 overexpression                | This work   |
| MGM 5008     | mc²155/pKH09      | mc²155 mutagenized with 0.2% MMS                | This work   |
| MGM 5009     | mc²155/pKH10      | mc²155 mutagenized with 0.4% MMS                | This work   |
| MGM 5010     | mc²155 HicBP12T   | HicB P12T in wild-type background               | This work   |
| MGM 5011     | mc²155/pKH11      | *lacZ* reporter for wild-type *hicB* promoter   | This work   |
| MGM 5012     | mc²155/pKH12      | *lacZ* reporter for P12T *hicB* promoter        | This work   |
| MGM 5013     | mc²155/pKH13      | *lacZ* reporter for wild-type tet(V) promoter   | This work   |
| MGM 5014     | mc²155/pKH14      | *lacZ* reporter for P12T tet(V) reporter        | This work   |
| MGM 5018     | mc²155 ΔhicB      | *hicB* deletion                                 | This work   |
| MGM 5019     | MGM 5018/pKH11    | *lacZ* reporter for wild-type *hicB* promoter in ΔhicB | This work   |
| MGM 5020     | MGM 5018/pKH12    | *lacZ* reporter for P12T *hicB* promoter in ΔhicB | This work   |
| MGM 5021     | MGM 5018/pKH13    | *lacZ* reporter for wild-type tet(V) promoter in ΔhicB | This work   |
| Strain        | Vector                                      | Description                                      | Source  |
|--------------|---------------------------------------------|--------------------------------------------------|---------|
| MGM 5022     | MGM 5018/pKH14                              | lacZ reporter for P12T tet(V) reporter in ΔhicB | This work |
| Mc21659      | mc2155/pJV75amber, sacB                     | Mycobacteriophage recombineering strain          | Lab stock |
| MGM 6025     | mc2155/pAJF 357                            | GFP-tagged DNA polymerase III α-subunit          | This work |
| MGM 6026     | mc2155/pAJF 441                            | mCitrine-tagged RNAP β‘-subunit                  | This work |
| MGM 6063     | MGM 6026/pAJF 486                          | mCitrine-tagged RNAP β‘-subunit, mCherry-tagged rplA | This work |
**Supplementary Table 4. Plasmids**

| Plasmids     | Description                                      | Reference         |
|--------------|--------------------------------------------------|-------------------|
| pmsg419      | Tet-On controlled expression vector,^a^ HA tag, hyg^R^ | This work         |
| pMV261       | GroEL promoter, kan^R^                           |                   |
| pKH03        | pMV261Kan/Msmeg 5185                            | This work         |
| pKH04        | pMV261Kan/hicB                                   | This work         |
| pKH05        | pMV261Kan/tet(V)                                 | This work         |
| pKH07        | pMV261Kan/Msmeg 3815                            | This work         |
| pKH08        | pMV261Kan/Msmeg 1953                            | This work         |
| pRGM18       | Che9c gp 61, inducible promoter, hyg amber, kan^R^, sacB | This work and ^b^ |
| pAJF067      | sacB, galK, hyg^R^                              |                   |
| pMV206Hyg    | hyg^R^                                           | This work         |
| pHMG 147     | Promoterless lacZ, RBS, hyg^R^                  | This work         |
| pKH11        | pHMG147, wild-type promoter region in hicB direction | This work         |
| pKH12        | pHMG147, HicBP12T promoter region in hicB direction | This work         |
| pKH13        | pHMG147, wild-type promoter region in tet(V) direction | This work         |
| pKH14        | pHMG147, HicBP12T promoter region in tet(V) direction | This work         |
| pAJF263      | Linker-mCitrine-StrepTagII, hyg^R^               | This work         |
| pAJF315      | Linker-msfGFP, hyg^R^                           | This work         |
| pAJF357      | 3'Msmeg 3178-msfGFP hyg^R^                      | This work         |
| pAJF441      | 3'rpoC-mCitrine-StrepTagII hyg^R^                | This work         |
| pAJF384      | mCherry, ori (pACYC), strep^R^                   | This work         |
| pAJF486      | 3'rplA-mCherry, ori (pACYC), strep^R^            | This work         |

^a^(12); ^b^(9); ^c^(18)
### Supplementary Table 5. Oligonucleotides

| Oligonucleotides          | Reference          |
|---------------------------|--------------------|
| pmsg419up                 | GAGATTCGCCCGCCCGAAATG This work |
| pmsg419sequp              | TCCAACCTGGCGTTCGAAGG This work |
| tetRBSHAClaIS             | CAGAAAAGGGCCATATGTACGGGTACGACGTGCCGACTACGCGAT This work |
| tetRBSHAClaL              | CGATGGCGGTAGCGTACGGGTACGACGTGCCGACTACGCGAT This work |
| oKH9                      | GCCCGGGTTATGACTTCCAGCGATGCCAGT This work |
| oKH10                     | TGAATTCTACTGCTGCTCAGCGACACACATGACACTGAGG This work |
| oKH11                     | TTGGCCATTATGTCTATGTTTGCGATACCTAAG This work |
| oKH12                     | TGAATTCTAGTACTGGCGATACCTAAG This work |
| oKH13                     | TTGGCCATTATGGCAATACGTACCTAAG This work |
| oKH14                     | TGAATTCTAGTACTGGCGATACCTAAG This work |
| oKH18                     | TCAATCTACTACGGCATGACCTAAG This work |
| oKH19                     | TGAATGCTACTGAGTCATGACCTAAG This work |
| oHK35                     | TCAGCTGAATTTGGCGAGGGCGCCTGCAAGCAG This work |
| oKH38                     | TGAATGCTACTGAGTCATGACCTAAG This work |
| JCV198                    | CCGCTGTGACACAAGAATCCCTGGTCGACCGTATTGATTCGGATGATTCCTACGCGAGCCTGCGGAACGACCAGGAATTCTGGGAGCCGCTGGC (9) |
| oKH66                     | ATTCATGGACTCGCGCCCGCCGAGTTGGTGAGCTACGCAACATGACCTAAG This work |
| oKH67                     | TCGTGCGCAGCTCCAGCCGACACCGCAGGTTGGTGAGCTACGCAACATGACCTAAG This work |
| oKH68                     | TGACCGCGGATCTGCTGCTG This work |
| oKH69                     | TGCAATGCGGCTGCAATGCTG This work |
| oKH70                     | TTAATGCGCAGCTGCAATGCTG This work |
| oKH71                     | TTACTACGCTAGCTGCAATGCTG This work |
| oKH72                     | TGCATGCCGCTGCTGCTG This work |
| AJF467                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| AJF468                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| AJF630                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| AJF631                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| oAF573                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| oAF574                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| oAF530                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| oAF531                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| oAF532                    | GATATTACGAAACCAGCGGAGATTGCAGC This work |
| Strain   | Sequence                           | Source       |
|----------|------------------------------------|--------------|
| oAF533   | GCAAATAATGTCTAAAACGGCAAAAGCACC    | This work    |
| oAF534   | CGGTGCTTTTGCCGTTTTAGACATTATTTGC   | This work    |
| oAF535   | GACGAGCTGTACAAGTAAGCTTCTCCCAAG    | This work    |
|          | GAC                                              |              |
Supplementary Figure 1. Contribution of the P12T mutation to hicB and tet(V) promoter activity. Promoter activity was measured by C2FDG fluorescence detection of beta-galactosidase fusions in A) wild-type or B) ΔhicB backgrounds in M. smegmatis. SP, stationary phase.
Supplementary Figure 2. The effect of ciprofloxacin and rifampin treatment on GFP-tagged DNA polymerase III α subunit. Growth of GFP-tagged DNA polymerase III α subunit *M. smegmatis* strain MGM 6025 on A) no drug growth conditions B) ciprofloxacin (6μM) or C) rifampin (60μM) visualized by time-lapse microscopy.
Supplementary Figure 3. The effect of rifampin treatment on mCitrine-tagged RNAP and mCherry-tagged ribosomes. Growth of mCitrine-tagged RNAP and mCherry-tagged ribosomes in *M. smegmatis* strain MGM 6063 on A) no drug growth conditions or B) rifampin (120 μM) visualized by time-lapse microscopy.
Supplementary Figure 4. The effect of chloramphenicol treatment on mCitrine-tagged RNAP and mCherry-tagged ribosomes. Growth of mCitrine-tagged RNAP and mCherry-tagged ribosomes in *M. smegmatis* strain MGM 6063 on A) no drug growth conditions or B) chloramphenicol (93 μM) visualized by time-lapse microscopy.
Supplementary Figure 5. Fluorescent protein expression strains, MGM 6025 and MGM 6063, express stable fusion proteins at expected sizes and have wild-type growth rates. (A) Immunoblot of lysates made from wild-type (lane 1), DNA polymerase III α-subunit-msfGFP expression strain (MGM 6025, lane 2), and RNAP-β'-mCitrine RplA-mCherry expression strain (MGM 6063, lane 3). Immunoblot probed for GFP (DNA polymerase III α-subunit-msfGFP, 156.8kDa, and RNAP-β'-mCitrine, 175.3 kDa, top panel), RFP (RplA-mCherry, 52.6 kDa, middle panel), and CarD as loading control (bottom panel). (B) Growth curves for wild-type (circles) DNA polymerase III α-subunit-msfGFP expression strain (MGM 6025, squares), and RNAP-β'-mCitrine RplA-mCherry expression strain (MGM 6063, triangles). Cultures grown at 37°C in LB media to an OD_{600} 0.4 were diluted back to an OD_{600} of 0.1 for start of assay. Each curve is the average of three independent cultures.
Supplementary Figure 6. Microscopic observation of SKI-356313 cellular localization pattern. Treatment of wild-type *M. smegmatis* with DMSO (control), SKI-356313, or derivatives of SKI-356313 at a subinhibitory concentration (0.048μM). SKI-356313 fluorescence was visualized on the CFP channel. H, Hoechst 33342.
Supplementary Figure 7. Fluorescence intercalator displacement assay to measure the ability of SKI-356313 and derivatives to displace intercalated EtBr from double stranded 63 base pair oligonucleotides. Control wells contained EtBr and DNA only, and represent 100% fluorescence. EtBr displacement from double stranded oligonucleotides by A) SKI-356313 B) SKI-7 C) SKI-22 and D) SKI-23 is measured by a decrease in RFU. Hoechst 33342 was used as a positive control for EtBr displacement. *P values were calculated by student’s t-test for measurement of statistical significance of RFU decrease compared to control wells. *, **, *** = P value ≤ 0.05, 0.005, 0.0005, respectively. Bars plotted represent averages of three experimental replicates. Each graph is representative of biological triplicate experiments.
**Supplementary Movie 1.** Ciprofloxacin treatment of MGM 6025 results in loss of DNA polymerase III α-subunit foci. Loss of GFP-tagged DNA polymerase III α-subunit foci occurs within 20 minutes post addition of MGM 6025 onto an agar pad containing ciprofloxacin at 2X MIC (6 μM). Cell growth arrest occurs after approximately seven hours of ciprofloxacin treatment. Left panel is the GFP channel and the right panel is the DIC channel.

**Supplementary Movie 2.** Rifampin treatment of MGM 6063 results in aberrant RNAP and ribosome fluorescence and localization. Treatment of MGM 6063 with rifampin at 5X the *M. smegmatis* MIC (120 μM) results in a condensation of the mCitrine-tagged RNAP signal (center panel) and dissipation of mCherry-tagged ribosome signal (left panel). The DIC channel is the right panel.

**Supplementary Movie 3.** Chloramphenicol treatment of MGM 6063 results in aberrant RNAP and ribosome fluorescence and localization. Treatment of MGM 6063 with chloramphenicol at 5X the *M. smegmatis* MIC (93 μM), results in a condensation of the mCitrine-tagged RNAP signal (center panel), dissipation of mCherry-tagged ribosome signal (left panel) and eventual loss of both mCitrine and mCherry fluorescence over time. The DIC channel is the right panel.

**Supplementary Movie 4.** SKI-356313 treatment of MGM 6025 results in loss of GFP-tagged DNA polymerase III α-subunit foci. Treatment of the GFP-tagged α-subunit DNA polymerase III strain with SKI-356313 at 5X the MIC (0.475μM) resulted in a loss of GFP-tagged DNA polymerase α-subunit foci over time, with the majority of foci disappearing by six hours and 20 minutes of SKI-356313 treatment and cell growth arrest occurring by 7 hours post initiation of SKI-356313 exposure. Left panel is the GFP channel and the right panel is the DIC channel.

**Supplementary Movie 5.** SKI-356313 treatment of MGM 6063 has no effect on mCitrine-tagged RNAP or mCherry-tagged ribosome fluorescence localization. Upon treatment of MGM 6063 with SKI-356313 at 5X the MIC (0.475μM), the citrine-tagged RNAP (center panel) and mCherry-tagged ribosomes (left panel) remain visible in their pre-treatment patterns. The DIC channel is the right panel.
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