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Novel Imidazoline Antimicrobial Scaffold That Inhibits DNA Replication with Activity against Mycobacteria and Drug Resistant Gram-Positive Cocci

Kendra K. Harris,[†,‡] Allison Fay,[§] Han-Guang Yan,[§] Pratima Kunwar,[⊥,#] Nicholas D. Socci,[▽] Narender Pottabathini,[◆] Ramakrishna R. Juventhala,[◆] Hakim Djaballah,[○] and Michael S. Glickman*[,†,§,∥]

[†]Program in Immunology and Microbial Pathogenesis, Weill Cornell Graduate School of Medical Sciences, New York, New York 10021, United States

[‡]Weill Cornell, Rockefeller, Sloan Kettering Tri-Institutional MD-PhD Program, New York, New York 10065, United States

[§]Immunology Program and [∥]Division of Infectious Diseases, Memorial Sloan Kettering Cancer Center, New York, New York 10065, United States

[⊥]Viral Vaccine Program, Seattle Biomedical Research Institute, Seattle, Washington 98109, United States

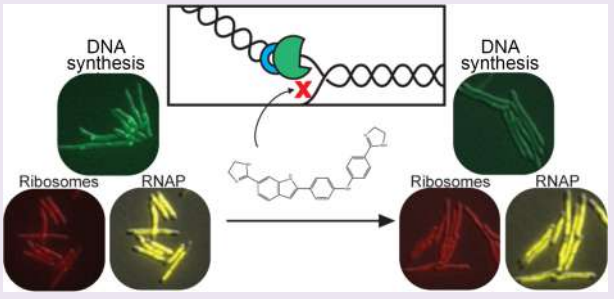
[#]Department of Global Health, University of Washington School of Medicine, Seattle, Washington 98109, United States

[▽]Bioinformatics Core and [○]HTS Core Facility, Memorial Sloan Kettering Cancer Center, New York, New York 10065, United States

[◆]Discovery Services Division, GVK Biosciences Pvt. Ltd, Plot 28A, IDA Nacharam, Hyderabad 500076, India

Supporting Information

ABSTRACT: Bacterial antimicrobial resistance is an escalating public health threat, yet the current antimicrobial pipeline remains alarmingly depleted, making the development of new antimicrobials an urgent need. Here, we identify a novel, potent, imidazoline antimicrobial compound, SKI-356313, with bactericidal activity against *Mycobacterium tuberculosis* and Gram-positive cocci, including vancomycin-resistant *Enterococcus faecium* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). SKI-356313 is active in murine models of *Streptococcus pneumoniae* and MRSA infection and is potently bactericidal for both replicating and nonreplicating *M. tuberculosis*. Using a combination of genetics, whole genome sequencing, and a novel target ID approach using real time imaging of core macromolecular biosynthesis, we show that SKI-356313 inhibits DNA replication and displaces the replisome from the bacterial nucleoid. These results identify a new antimicrobial scaffold with a novel mechanism of action and potential therapeutic utility against nonreplicating *M. tuberculosis* and antibiotic resistant Gram-positive cocci.



Antimicrobial resistance in pathogenic bacteria is an escalating public health threat.[1,2] The rise in antimicrobial resistance encompasses nosocomial pyogenic bacteria such as vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter*, *Klebsiella*, *Neisseria gonorrheae*,[3] and *Mycobacterium tuberculosis*.[4] In some cases, antimicrobial resistance has resulted in such an extreme limitation in therapeutic options that previously curable infectious diseases have been rendered virtually untreatable.[5,6]

The development of new antimicrobials is a particularly urgent need. Over the past two decades, the number of new antibiotics approved for marketing in the United States has dramatically decreased, and since 2009, only two new systemic antibiotics have been approved by the Food and Drug Administration (FDA).[2] The discovery and development of new tuberculosis (TB) drugs is of particular importance to global health. In 2012 an estimated 8.6 million people developed TB and an estimated 1.3 million people died from the disease.[7] Additionally, the incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB is a growing public health crisis worldwide. The majority of first- and second-line TB drugs target the processes of cell wall metabolism, DNA replication, or protein synthesis within the cell.[8] There are several new drugs with potent antimycobacterial activity that are in various stages of clinical development.[9] The optimal properties sought in a new TB drug candidate are extensive and include the ability to shorten the existing TB drug regimen, target latent TB infection (LTBI), and, ideally, have a novel mechanism of action, enabling its use in the treatment of MDR- and XDR-TB.

The process of antimicrobial discovery encompasses several challenges, including determining the most promising screening

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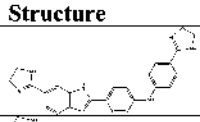
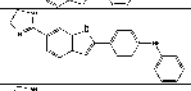
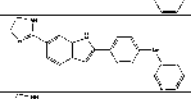
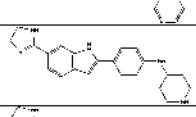
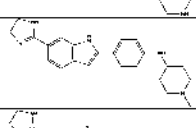
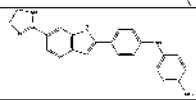
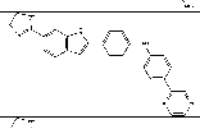
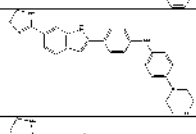
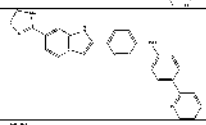
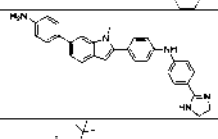
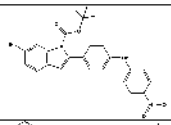
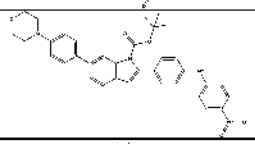
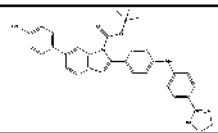
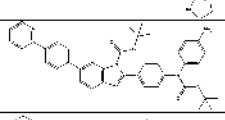
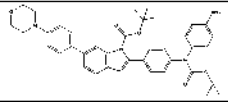
Table 1. Kirby–Bauer Disk Diffusion Assay and MICs of SKI-356313 and Derivatives

ID	Structure	Disk Diffusion Zone of Growth Inhibition (mm)		*M. tb* MIC (μM)
		M. smegmatis	*M. tuberculosis*	
SKI-356313		19.6	13	0.19
SKI-2		14.3	19	50
SKI-4		27.6	26	6.25
SKI-6		16.3	10	25
SKI-7		30	33	3.125
SKI-8		24	19	1.56
SKI-10		14.3	19	6.25
SKI-11		11	17	6.25
SKI-12		12	15	6.25
SKI-20		8	8	25
SKI-22		0	9	>50
SKI-23		0	0	>50
SKI-24		7	10	>50
SKI-25		0	7	>50
SKI-26		0	0	>50

approach for identification of novel drug targets and novel chemical scaffolds. Target-based screening approaches have traditionally had low success rates and are resource-intensive and financially demanding.[10] These limitations paved the way for the present preference for phenotypic whole cell screens, which have the advantage of identifying hits with antimicrobial activity under physiologically relevant conditions.[10,11] However, little to no information regarding the cellular target or mechanism of action of a particular hit is initially available. Even so, the whole cell screen approach has yielded the first

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new FDA approved TB drug in more than four decades.[12,13] Here, we describe the results of a high throughput whole cell screen for antimicrobials conducted in *Mycobacterium smegmatis*, a nonpathogenic rapid growing mycobacterium. We have identified a novel imidazoline-containing scaffold with activity against replicating and nonreplicating *M. tuberculosis* and drug-resistant Gram-positive cocci, which acts through inhibition of DNA replication.

RESULTS AND DISCUSSION

Live Cell-Based Reporter Screen for Antimycobacterials. We conducted a high throughput screen of 324 187 compounds, screened at 10 μM in 1% DMSO (v/v) in 1536-well microtiter plates against *M. smegmatis*. The screen employed a β-galactosidase reporter gene in wild-type *M. smegmatis*, using the β-galactosidase substrate 5-acetylamino fluorescein diβ-D-galactopyranoside (C2FDG), which is active in live cells and produces a fluorescence signal upon cleavage by the β-galactosidase enzyme.[14] This screening approach allowed sensitive detection of growth arrest in high throughput without cell lysis.

The MSKCC corporate chemical library at the time of the screen contained 324 187 small molecules obtained from ChemBridge Research Laboratories, Biofocus, AnalytiCon Discovery, SPECS, NCI, ChemDiv, and Magellan Bioscience.[15] Compounds scoring a percent inhibition of 50% or greater were reported as positive. A total of 757 positive compounds were identified yielding an initial hit rate of 0.23%. 319 positives were commercially available for confirmatory studies. 91 resupplied compounds confirmed activity with 30 hits exhibiting IC50 values $\leq$10 μM; among them was SKI-356313 with a confirmed IC50 value of 2.54 μM. Several of the confirmed hits were further evaluated by Kirby-Bauer disk diffusion testing on *M. smegmatis* and selected other Gram-positive and Gram-negative bacteria. This report will focus on confirmed hit SKI-356313 (4-(4,5-dihydro-1H-imidazol-2-yl)-N-(4-(6-(4,5-dihydro-1H-imidazol-2-yl)-1H-indol-2-yl)-phenyl)aniline), an imidazoline containing compound, the structure of which is shown in Table 1.

SKI-356313 Is Bactericidal for *M. tuberculosis* and Drug-Resistant Gram-Positives. SKI-356313 was resynthesized (Supporting Information Scheme 1) to confirm that the active chemical entity corresponds to the chemical structure given in Table 1. Chemically verified SKI-356313 is a potent antimycobacterial, as measured by disk diffusion killing assays and broth dilution minimal inhibitory concentration (MIC) testing. The MIC of SKI-356313 is 0.19 μM and 0.095 μM for *M. tuberculosis* and *M. smegmatis*, respectively (Table 1, Supporting Information Table 1). In time kill experiments treatment of log-phase *M. tuberculosis* at 1.9 μM resulted in a 2.4 log reduction in viable bacteria after 4 days, indicating SKI-356313 has bactericidal activity *in vitro* (Figure 1A), an activity that was confirmed in *M. smegmatis* (Figure 1B). To determine the antimicrobial spectrum of SKI-356313, we next tested SKI-356313 against a panel of Gram-positive and Gram-negative bacteria.

SKI-356313 had potent growth inhibitory activity against all Gram-positive cocci tested, including vancomycin-resistant *E. faecium* (VRE, 0.14 μM), penicillin-resistant *S. pneumoniae* (PRSP, 0.28 μM), and methicillin-resistant *S. aureus* (MRSA, 0.59 μM) (Supporting Information Table 1). SKI-356313 is significantly less active against Gram-negative bacilli (Supporting Information Table 1).

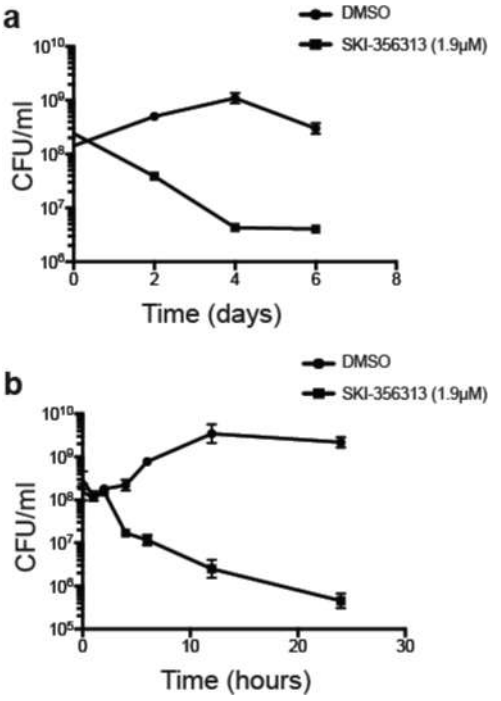

Figure 1. SKI-356313 has bactericidal activity against actively growing mycobacteria. Mean Colony Forming Units (CFU) per mL from triplicate experiments are plotted on a logarithmic *y*-axis and time of drug treatment is on the *x*-axis. (A) *M. tuberculosis* and (B) *M. smegmatis*.

Structure Activity Relationships of Imidazoline Derivatives. We examined the structure activity relationship of SKI-356313 through a limited focused library of 14 derivatives, based on the 2-phenyl-1H- indole core scaffold substituted at positions 1- and 6- on the indole and position 3- on the phenyl as an avenue in the search of more potent compounds (Table 1). Substitutions at positions 1 and 6 on the indole lead to a differential loss of activity with MIC values greater than 25 μM, whereas mono substitutions at position 3- on the phenyl are relatively well tolerated with disk diffusion zone of growth inhibitory activity better than SKI-356313 for both *M. tuberculosis* and *M. smegmatis* (Table 1). Several derivatives were found to enhance selectivity for Gram-positive cocci (SKI-4 and SKI-10) but had minimal activity against *Klebsiella*, *Pseudomonas*, and *Candida* (Supporting Information Table 1).

SKI-356313 Kills Nonreplicating Persistent *M. tuberculosis*. The bactericidal effectiveness of many antibiotics is proportional to the bacterial growth rate, and as a result, nonreplicating bacteria display phenotypic tolerance to many drugs.[16−19] To determine whether SKI-356313 is active against nonreplicating *M. tuberculosis*, we tested this compound in the Wayne model of anaerobiosis induced nonreplicating persistence that has been used to screen drugs for the ability to kill nonreplicating *M. tuberculosis*.[20] The experimental design of the Wayne model testing is given in Figure 2A and the nonreplicating state of *M. tuberculosis* cultures was verified (Figure 2B). Isoniazid resulted in a one-log decrease in viable bacteria in actively growing oxygenated cultures, whereas metronidazole had no activity (Figure 2C−E), consistent with prior reports.[20] SKI-356313 resulted in a 3.9 log decrease in oxygenated cultures (Figure 2F), consistent with the bactericidal activity for *M. tuberculosis*. In nonreplicating deoxygenated cultures, isoniazid treatment resulted in a

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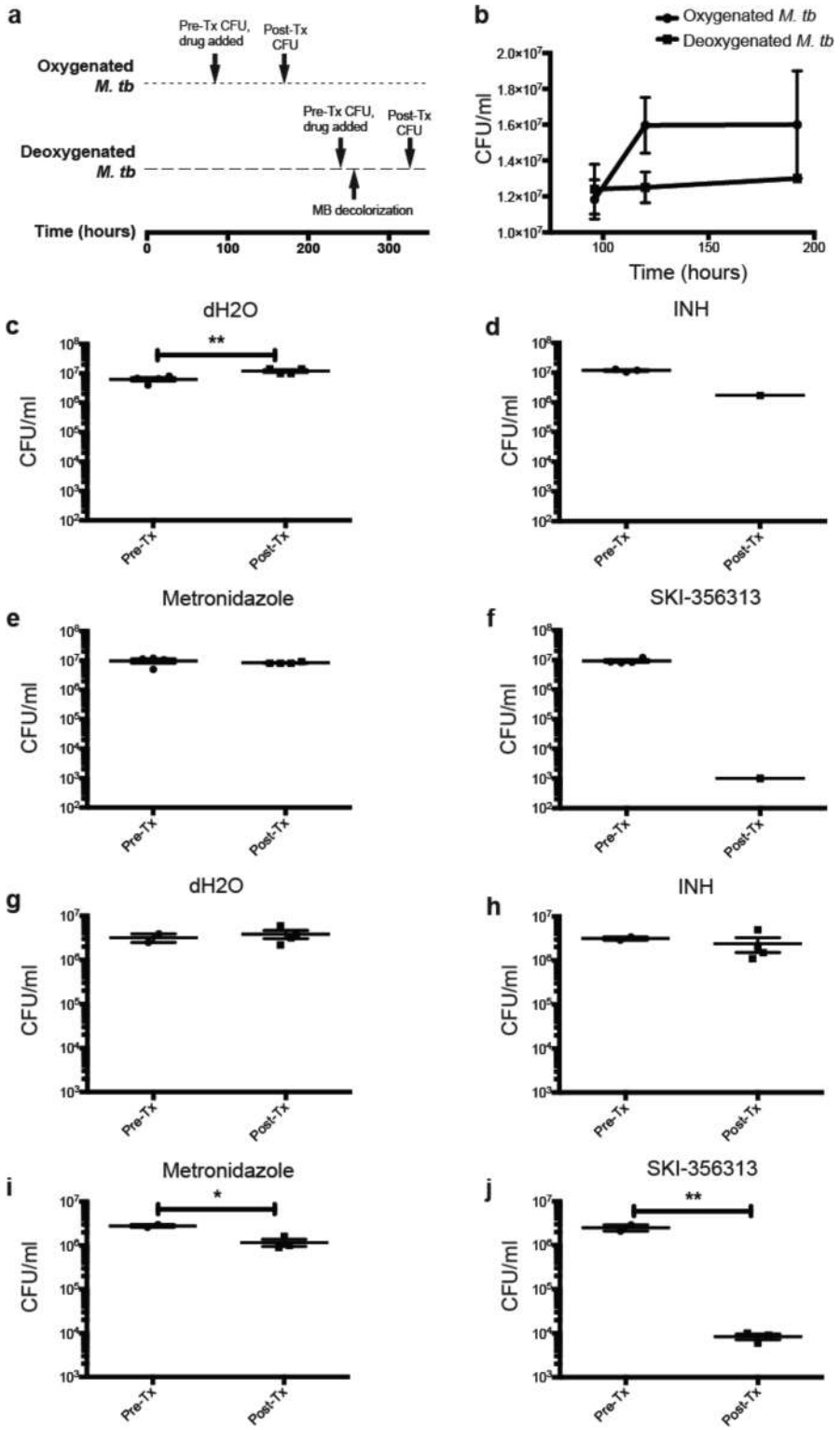

Figure 2. SKI-356313 antimycobacterial activity in the Wayne model of *M. tuberculosis* nonreplicating persistence. (A) Schematic of drug treatment regimens of *M. tuberculosis* cultures under oxygenated and deoxygenated conditions. Samples for pretreatment CFU determination from oxygenated cultures were taken at 96 h; post-treatment samples were taken at 192 h. Samples for pretreatment CFU determination from deoxygenated cultures were taken at 240 h; post-treatment samples were taken at 336 h. MB decolorization was observed beginning at 250 h. (B) Measurement of viable CFU over time of oxygenated and deoxygenated cultures without drug treatment. (C–F) CFU/mL of oxygenated cultures pre- and post-treatment treated with vehicle (C), INH (D), metronidazole (E), or SKI-356313 (F). (G–J) CFU/mL of deoxygenated cultures pre- and post-treatment with vehicle (G), INH (H), metronidazole (I), or SKI-356313 (J). *p* values were calculated by Student's *t*-test. * *p* value ≤ 0.05, ** *p* value ≤ 0.005. Graphs are representative of duplicate experiments.

nonstatistically significant decrease in CFU, whereas metronidazole resulted in a half-log decrease in CFU, consistent with prior reports (Figure 2G-J).[20] SKI-356313 resulted in a 2.5-log decrease in CFU in nonreplicating, deoxygenated cultures

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(Figure 2J). These data indicate that SKI-356313 potently kills nonreplicating *M. tuberculosis*.

SKI-356313 Has Bactericidal Activity against MRSA and *S. pneumoniae* in Mice. To test whether SKI-356313 is active in a mouse model of infection, we used a neutropenic mouse thigh inoculation model with MRSA and *S. pneumoniae*. The model tests the effect of two doses of antimicrobial on bacterial titer in infected thigh muscle. Treatment with SKI-356313 at both 10 mg/kg and 20 mg/kg resulted in a 1.8- and 2.2-log reduction in *S. pneumoniae* titers, respectively, compared to vancomycin treatment, which resulted in a 4.2-log reduction in *S. pneumoniae* titers (Figure 3A). Treatment with two doses

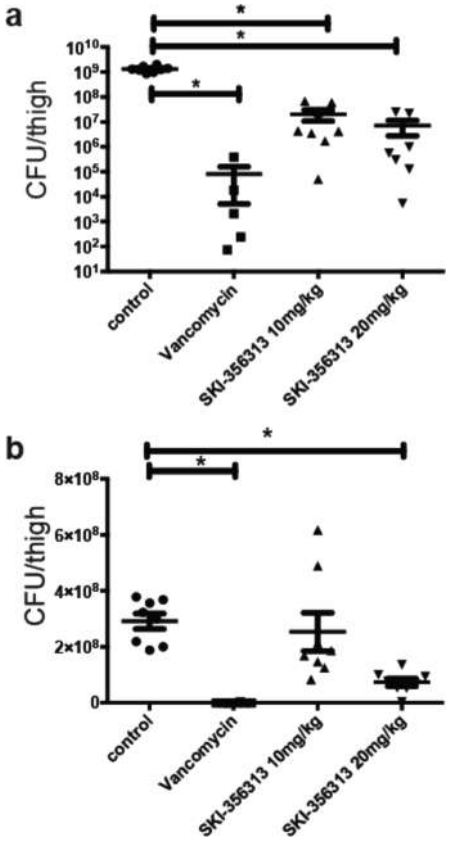

Figure 3. SKI-356313 activity in the neutropenic mouse thigh inoculation model. (A) *S. pneumonia* or (B) MRSA. *p* values were calculated by Student's *t*-test. * *p* value ≤ 0.0001.

of SKI-356313 at 20 mg/kg reduced MRSA titers in mouse thigh by 0.56 logs, a statistically significant reduction compared to untreated mice (Figure 3B). Vancomycin treatment resulted in a 2.6 log reduction in MRSA titers in mouse thighs (Figure 3B).

Overexpression of *Rv1634* and *whiB7* Confer Resistance to SKI-356313. To understand the mechanism of action of SKI-356313, we searched for determinants of resistance using both selection and target overexpression.[13,21,22] For the target overexpression approach, we constructed libraries of randomly fragmented chromosomal DNA from either *M. smegmatis* or *M. tuberculosis* in which the genomic fragments are expressed from a tetracycline inducible (Tet-On) promoter[23] (Figure 4A). Utilizing these libraries, we selected for resistance on anhydrotetracycline (ATc)-containing agar media with SKI-356313 at 2× (0.19 μM) the *M. smegmatis* MIC. We determined the chromosomal sequences from 50 independent *M. smegmatis* colonies that displayed SKI-356313 resistance. Selection for resistance with SKI-356313 at 4× (0.38 μM) yielded no resistant colonies. Three genomic loci were highly represented among all sequenced resistant clones (Figure 4B). 32 of 50 sequenced fragments contained the open reading frame of *Rv1634* as the only intact ORF represented in all of the sequenced clones. *Rv1634* is a drug efflux pump and has been shown in *M. smegmatis* to confer resistance to fluoroquinolones when overexpressed.[24,25] *M. smegmatis* overexpressing *Rv1634* displayed a 4-fold increase in MIC to SKI-356313. Six resistant clones contained genomic fragments from *M. tuberculosis* encompassing the genes *Rv3197*, *whiB7*, and *uvrD2*, with *whiB7* being the only gene shared among all six inserts (Figure 4B). The *whiB7* gene is annotated as a transcriptional regulatory protein involved in intrinsic antibiotic resistance.[26,27] Overexpression of the *M. smegmatis* homologue to *whiB7*, Msmeg1953, yielded a 2-fold increase in MIC to SKI-356313 (Figure 4C), confirming that WhiB7 overexpression is sufficient to mediate resistance to SKI-356313.

Overexpression of the HicB Antitoxin Protein Confers Resistance to SKI-356313. Nine resistant clones contained the genes Msmeg5185, Msmeg5186, and *tet(V)*, with Msmeg5186 as the only intact ORF present in all of the sequenced clones that conferred resistance. Overexpression of Msmeg5185, a predicted enoyl CoA isomerase, yielded no resistance to SKI-356313 (data not shown). Overexpression of Msmeg5186, encoding a predicted HicB antitoxin family protein and hitherto referred to as HicB, conferred a 2-fold increase in SKI-356313 MIC (Figure 4C). The HicAB toxin–antitoxin system (TAS) has been characterized via comparative genomics as a type II TAS.[28] Overexpression of *tet(V)*, which has been previously characterized as a tetracycline efflux membrane transport protein, conferred an 8-fold increase in MIC to SKI-356313 (Figure 4C).[29]

***hicB* Promoter Mutation Confers Resistance to SKI-356313 and Ciprofloxacin.** Selection for spontaneous resistance to SKI-356313 in both *M. smegmatis* and *S. pneumoniae* at concentrations as low as 2-fold above the MIC failed to yield resistant mutants, indicating a frequency of resistance of less than 1×10^{-10}. Selection of a methylmethanesulfonate (MMS) mutagenized *M. smegmatis* library for SKI-356313 resistance yielded five resistant mutants (designated MMS 22, 42, 76, 82, and 87) with varying levels of resistance to SKI-356313 (Figure 5A). Whole genome sequencing of MMS 42, 76, 82, and 87 identified a small number of single nucleotide polymorphisms (SNPs) that were present in resistant strains but not present in the parental wild-type *M. smegmatis* strain (Supporting Information Table 2). A single mutation was shared between all four sequenced resistant strains: a P12T mutation in the same *M. smegmatis hicB* gene that was identified in our overexpression approach. Each resistant mutant strain also harbored nonoverlapping mutations, indicating that these strains are not siblings. Introduction of the HicB P12T SNP into the wild-type *M. smegmatis* chromosome (MGM 5010) was sufficient to phenocopy the resistance phenotype of MMS 42, yielding a 4× increase in the SKI-356313 MIC (Figure 5B). Additionally, deletion of *hicB* from wild-type *M. smegmatis* (MGM 5018) had no effect on viability but caused increased sensitivity to SKI-356313, reducing the MIC by half (Figure 5B).

There are two potential translational initiation codons annotated for the HicB gene. The 5′ start would place the HicB P12T SNP within the *hicB* protein (encoding a P12T

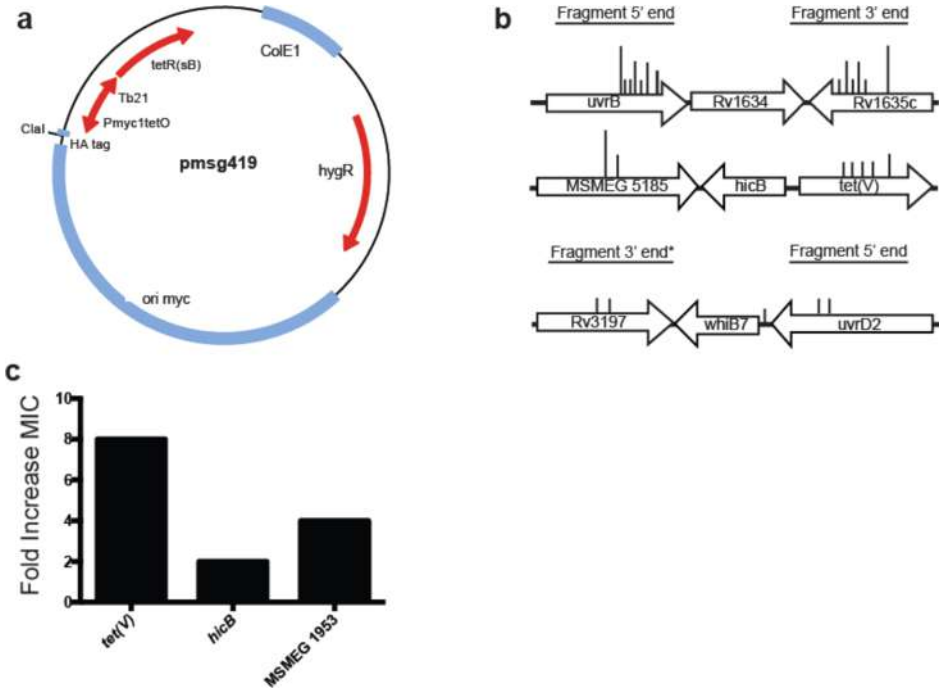

Figure 4. Overexpression of HicB confers resistance to SKI-356313. (A) The pmsg419 vector contained either *M. smegmatis* genomic fragments or *M. tuberculosis* genomic fragments under a tetracycline-inducible promoter and hygromycin selection (strains MGM 5000 and MGM 5001, respectively). ColE1, *E. coli* origin of replication; hygR, hygromycin resistance cassette; ori myc, mycobacteria origin of replication; HA tag, hemagglutinin tag; Pmyc1tetO, mycobacterial promoter with Tet operator; Tb21, constitutive promoter; tetR(sB), tetracycline repressor. (B) Schematics of genomic fragments sequenced from SKI-356313 resistant clones. Longer lines indicate multiple clones of a particular start or end position. (C) The indicated ORFs (*M. smegmatis tet(V)*, *hicB*, and *whiB7* (Msmeg 1953) were expressed from the constitutive GroEL promoter and the SKI-356313 MIC of each strain expressed as a ratio to wild-type *M. smegmatis* laboratory strain containing pMV261 empty vector.

amino acid substitution), whereas the more 3′ start codon is 39 nucleotides downstream of the HicB P12T SNP, thereby placing the mutation within the *hicB/tet(V)* intergenic region. To test whether the P12T mutation altered *hicB* or *tet(V)* promoter activity, we constructed transcriptional fusions that coupled either the *tet(V)* or the *hicB* promoters to *β*-Galactosidase production. *β*-Galactosidase assays on *M. smegmatis* strains containing these fusions revealed that the P12T mutation increased *hicB* promoter activity 10-fold in early log phase, and 50-fold in stationary phase, compared to the wild-type *hicB* promoter (Supporting Information Figure 1A). In contrast, the P12T mutation had minimal effects on the *tet(V)* promoter. In contrast to other Type II TAS that autoregulate the TAS operon by binding their own promoter,[28,30] *tet(V)* and *hicB* promoter activity in a Δ*hicB* background was nearly identical to the wild-type background (Supporting Information Figure 1B), indicating that HicB does not autoregulate its own expression. These data indicate that the P12T SNP is likely acting as a promoter mutation, resulting in increased transcription of *hicB* in the MMS resistant mutants. These data are consistent with our findings that overexpression of *hicB* also confers resistance to SKI-356313 (Figure 4C).

MMS 22, 42, 76, 82, and 87 were not resistant to kanamycin, rifampin, and isoniazid (Figure 5C, D, E), indicating that the mechanism of resistance to SKI-356313 did not confer cross-resistance to these antimicrobials. However, the HicB P12T strain was resistant to the fluoroquinolone ciprofloxacin, with an increase in MIC of 2-fold. Additionally, the Δ*hicB* strain had increased sensitivity to ciprofloxacin, reducing the MIC by half (Figure 5F). The common mechanism of resistance between SKI-356313 and quinolones led to the hypothesis that, although SKI-356313 does not chemically resemble quinolones, it may be acting via inhibiting DNA transactions.

SKI-356313 Inhibits DNA Replication. Ciprofloxacin exerts its bactericidal activity through inhibition of DNA gyrase, resulting in inhibition of DNA replication and an accumulation of dsDNA breaks.[31] To test whether SKI-356313 similarly inhibits DNA replication, we measured incorporation of [5,6-^{3}H]-uracil into alkali stable nucleic acid in logarithmically growing *M. smegmatis* cultures according to previously published protocols.[32−34] *M. smegmatis* treated with SKI-356313 showed a dose-dependent decrease in incorporation of radiolabeled substrate into DNA, but not RNA, indicating that SKI-356313 inhibits DNA synthesis (Figure 6A−D). *M. smegmatis* treated with rifampin showed the expected inhibition of RNA synthesis, and *M. smegmatis* treated with ciprofloxacin showed the expected magnitude of inhibition DNA synthesis, consistent with prior reports.[35,36] These results indicate that the bactericidal activity of SKI-356313 may be a result of inhibition of DNA replication.

Cytologic Evidence for Inhibition of DNA Synthesis by SKI-356313. To corroborate our biochemical finding that SKI-356313 inhibits DNA replication, we investigated the effect of SKI-356313 on DNA replication, RNA synthesis, and protein synthesis by live cell time-lapse microscopy utilizing two reporter strains that allow direct visualization of these three core biosynthetic processes: MGM 6025, an *M. smegmatis* strain containing a functional GFP-tagged DNA polymerase III *α*-subunit, and MGM 6063, an *M. smegmatis* strain with both mCitrine-tagged RNA polymerase *β*′-subunit(RNAP) and mCherry-tagged ribosomes (through an *rplA* fusion). Both of these strains grew identically to wild type and were not

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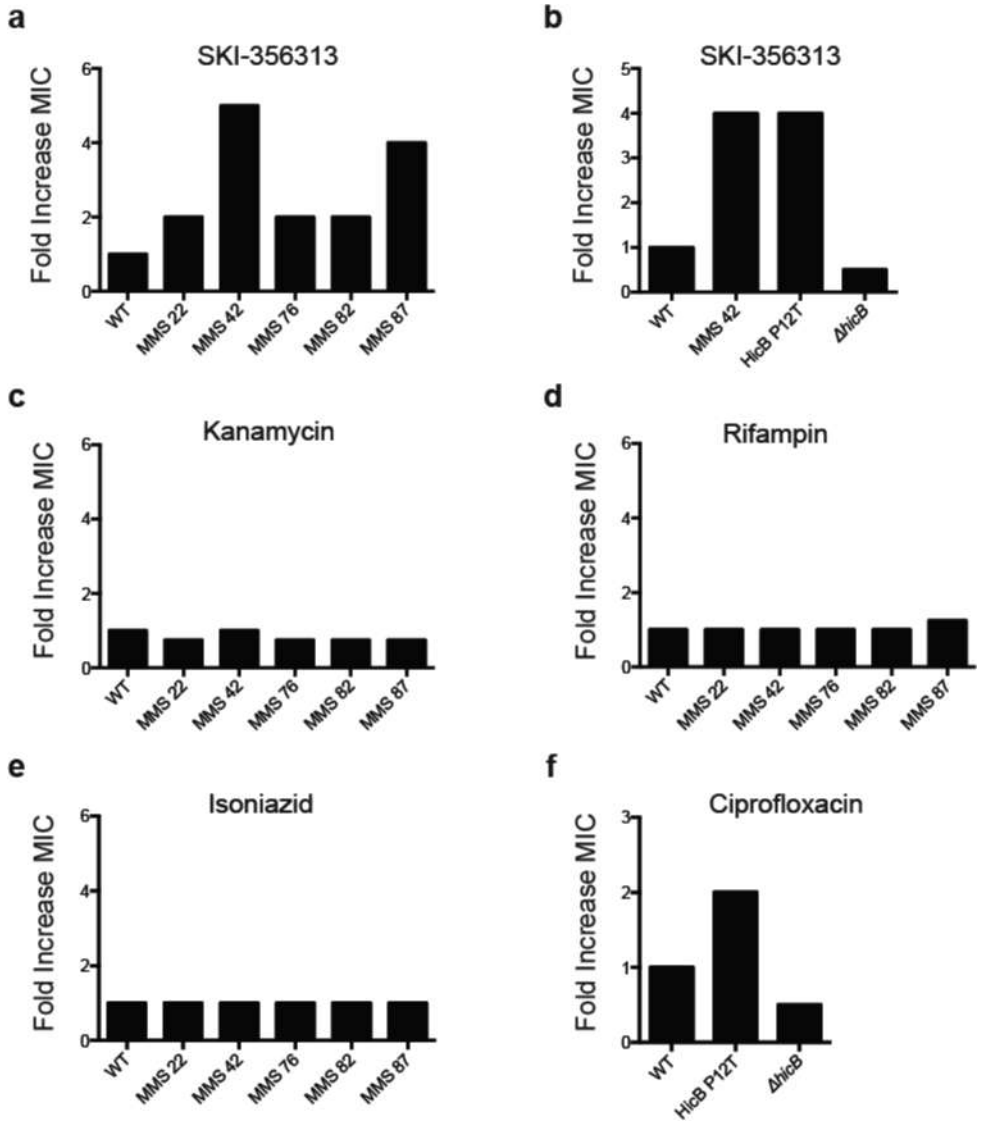

Figure 5. Effect of the HicBP12T mutation on SKI-356313 and ciprofloxacin resistance. (A) SKI-356313 resistance in 5 strains selected from MMS mutagenized *M. smegmatis* libraries. Wild type MIC is set to 1. (B) SKI-356313 MIC of WT *M. smegmatis* (MIC set to 1), the MMS42 mutant, an isogenic HicBP12T strain, and *M. smegmatis* Δ*hicB*. (C−E) *M. smegmatis* mutants resistant to SKI-356313 are not resistant to kanamycin, rifampin or isoniazid. (F) HicB affects ciprofloxacin sensitivity. MICs were determined on LB plates containing the indicated drugs. The MIC of each strain is expressed as a ratio to wild-type *M. smegmatis*.

hypersusceptible to ciprofloxacin, rifampin, or chloramphemicol (Supporting Information Figure 5 and data not shown). GFP-tagged α-subunit DNA polymerase III appears as either one or two foci within the cell (Figure 7A), whereas 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 (Figure 7A).

To characterize the behavior of these strains when treated with antibiotics with known mechanisms of action, we observed these reporter strains via time-lapse microscopy with antibiotics known to inhibit DNA replication, transcription, or translation (ciprofloxacin, rifampin, and chloramphenicol, respectively). Ciprofloxacin at 2× 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 min post drug treatment (Supporting Information Figure 2B and Movie 1). Treatment of MGM 6063 with rifampin at 5× the *M. smegmatis* MIC (120 μM) resulted in a condensation of the mCitrine-tagged RNAP signal and dissipation of mCherry-tagged ribosome signal (Supporting Information Figure 3B and Movie 2). Chloramphenicol at 5× 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 (Supporting Information Figure 4B and Movie 3). Importantly, rifampin treatment did not alter the number or intensity of DNA polymerase foci, indicating that inhibition of gene expression does not inhibit this reporter in the time frame of our experiments (Supporting Information Figure 2C). 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.

Treatment of MGM 6025 with SKI-356313 at 5× the MIC (0.475 μM) resulted in a loss of GFP-tagged DNA polymerase α-subunit foci over time, with the majority of foci disappearing

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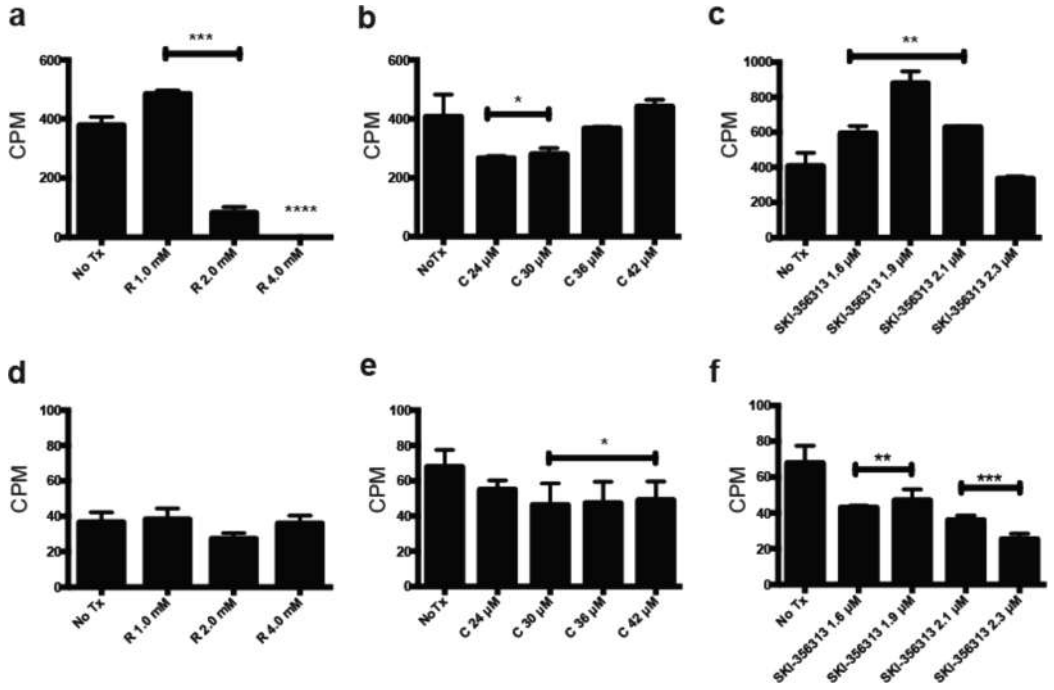

Figure 6. SKI-356313 inhibits DNA synthesis. (A–C) The effects on [5,6-^{3}H]-uracil incorporation into RNA in (A) rifampin, (B) ciprofloxacin, or (C) SKI-356313 treated triplicate cultures. (D–F) The effects on [5,6-^{3}H]-uracil incorporation into DNA in (D) rifampin, (E) ciprofloxacin, or (F) SKI-356313 triplicate treated cultures. *p* values were calculated by Student's *t*-test. * *p* value ≤ 0.05, ** *p* value ≤ 0.005, *** *p* value ≤ 0.0005, **** *p* value ≤ 0.0001. Graphs are representative of triplicate experiments.

by 380 min of SKI-356313 treatment, which coincided with growth arrest (Figure 7B, C, and Supporting Information Movie 4). However, SKI-356313 treatment did not affect RNAP or ribosome localization (Figure 7B and Supporting Information Movie 5). Quantification of the number of GFP-tagged α-subunit DNA polymerase III foci in cells treated with SKI-356313, ciprofloxacin, and rifampin revealed that cells treated with ciprofloxacin or SKI-356313 had a significantly reduced number of DNA polymerase foci compared to cells treated with rifampin, suggesting the phenotype of loss of the DNA polymerase α-subunit fluorescence is not a general feature of cell death or inhibition of gene expression but correlates to drugs that inhibit DNA replication (Figure 7C).

SKI-356313 Co-localizes with the Bacterial Nucleoid. Microscopic observation of wild-type *M. smegmatis* cultures treated with a subinhibitory concentration of SKI-356313 revealed a nucleoid-like pattern of fluorescence visible via the cyan fluorescent protein (CFP) channel that was distinguishable from the diffuse cyan autofluorescence of *M. smegmatis* cells treated with DMSO (Supporting Information Figure 6A–D). The fluorescence pattern observed in SKI-356313-treated *M. smegmatis* was similar to the nucleoid staining seen with Hoechst 33342. In *M. smegmatis* treated with the inactive compounds SKI-22 and SKI-23, no nucleoid staining was observed (Supporting Information Figure 6E–H). *M. smegmatis* treated with a subinhibitory concentration of SKI-7 revealed an intermediate nucleoid-like pattern of fluorescence that was less sharp and less punctate compared to the pattern of SKI-356313-treated *M. smegmatis* (Supporting Information Figure 6I and J). SKI 356313 has a maximum absorbance at 380 nm and negligible emission, indicating SKI-356313 is not a fluorescent molecule in solution and suggesting the fluorescence observed in SKI-356313-treated *M. smegmatis* is a result of SKI-356313 DNA binding.

SKI-356313 Displaces the DNA Intercalator Ethidium Bromide from dsDNA *In Vitro*. We next tested whether SKI-356313 and its active and inactive derivatives were capable of directly binding DNA utilizing a fluorescence intercalator displacement (FID) assay, which measures the loss of fluorescence that accompanies displacement of ethidium bromide (EtBr) from DNA.[37] SKI-356313 displaced EtBr from DNA in a dose-dependent manner at concentrations approaching or above the $MIC_{M.tb}$, but not below the $MIC_{M.tb}$ (Supporting Information Figure 7A). SKI-7 also displaced EtBr at concentrations approaching or above the $MIC_{M.tb}$, but not below (Supporting Information Figure 7B). Compounds SKI-22 and SKI-23, both of which are inactive against mycobacteria, showed no activity in the FID assay up to concentrations of 50.4 μM (Supporting Information Figure 7C and D). These data suggest a correlation between the DNA binding of SKI-356313 and its chemical derivatives and the whole cell activity of these compounds.

Implications. Herein, we described the identification of a novel antimicrobial compound with nanomolar-range activity for *M. tuberculosis* and drug-resistant Gram-positive cocci, activity *in vivo*, and very low rates of resistance. It is notable that SKI-356313 has potent growth inhibitory properties against nonreplicating *M. tuberculosis* in the Wayne model, a property that holds potential for the development of a novel, shorter TB drug regimen through the targeting of persistent bacteria. Surprisingly, the potent activity of SKI-356313 against nonreplicating *M. tuberculosis* is accompanied not by inhibition of a cellular process thought required for the nonreplicating state but by inhibition of DNA replication. This finding suggests that inhibitor development against core macromolecular processes may be a fruitful avenue of antimicrobial development for both replicating and nonreplicating *M. tuberculosis*. There is support for this idea in the prior findings

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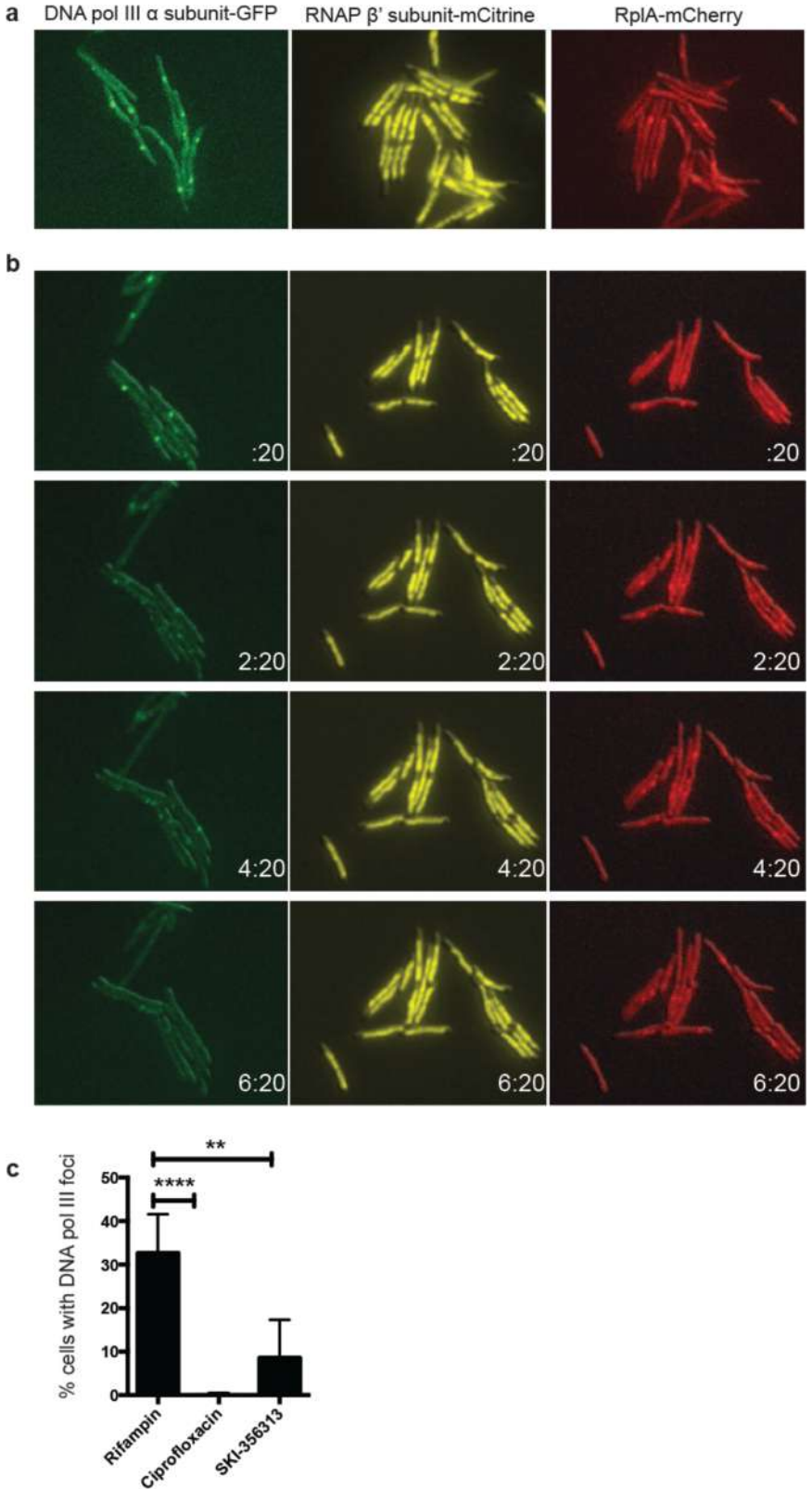

Figure 7. Direct observation of inhibition of DNA replication by SKI-356313. (A) MGM 6025 with GFP-tagged DNA polymerase III α-subunit and (B) MGM 6063 with mCitrine-tagged RNAP and mCherry-tagged ribosomes grown in the presence of SKI-356313 at 5X *M. smegmatis* MIC (0.475 μM) and observed via time lapse microscopy. All strains were added onto drug-containing agar pads and imaging was initiated within 20 min of strain exposure to drug. Time shown is hours:mins of imaging. (C) Percent of MGM 6025 cells with DNA foci after 6 h and 40 min treatment with either rifampin (2.5× MIC), ciprofloxacin (2× MIC), or SKI-356313 (5× MIC). Averages of five microscope fields for each treatment condition are reported. p values were calculated by student's t test. ** p value $\leq$ 0.005, **** p value $\leq$ 0.0001.

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that quinolones and rifampin, which inhibit DNA and RNA synthesis, respectively, are active against replicating and nonreplicating *M. tuberculosis*.[38]

Our data strongly supports the model that SKI-356313 inhibits DNA replication and that this effect is the likely mechanism of its antimicrobial action. SKI-356313 inhibition of DNA replication is supported by the observation that treatment of *M. smegmatis* with SKI-356313 resulted in biochemical inhibition of DNA, but not RNA synthesis, as well as inhibition of DNA replication observed directly using time-lapse microscopy of the DNA replication reporter strain MGM 6025. We were unable to isolate spontaneously resistant mutants to SKI-356313, but overexpression of *hicB*, either through plasmid overexpression or the *hicB* P12T promoter mutation, confers resistance to both SKI-356313 and ciprofloxacin. The mechanism by which *hicB* overexpression confers resistance to agents that inhibit DNA replication will require further study, but this finding further supports the idea that the mechanism by which SKI-356313 acts is through DNA replication. Our data further supports a model in which direct DNA binding by SKI-356313 inhibits DNA replication. Additionally, notwithstanding this DNA binding, our preliminary results indicate the SKI-356313 is Ames test negative and nontoxic to mice in short-term administration.

Additional mechanisms of action are also possible for SKI-356313. Parkesh et al. report an imidazoline compound of a similar chemical structure to SKI-356313 is capable of binding hairpin RNA forming from pathologic triplet nucleotide repeats.[39] Additionally, the SKI-356313 chemical structure has also been characterized in a separate screen as an inhibitor of the anthrax lethal factor,[40] suggesting that protease inhibition could be an additional mechanism of action, although no antimicrobial activity was demonstrated.

Our data support the use of the MGM 6025 and MGM 6063 reporter strains for the investigation of the ability of novel antimicrobial candidates to target the major cell processes of DNA replication, transcription, and translation by facilitating tracking of changes in GFP-tagged DNA polymerase III α-subunit, mCitrine-tagged RNAP, and mCherry-tagged ribosomes in live cells during antimicrobial treatment. MGM 6025 and MGM 6063 can be cultured in 96 well plates and thus have the potential to be used in a medium-throughput survey of the mechanism of action of novel antimicrobial compounds via time-lapse microscopy.

Future studies of this promising antimicrobial will focus on the detailed mechanism of action, the existence of additional molecular targets, and the activity and safety in longer term infection models.

METHODS

High Throughput Screen. A high-throughput screen of 324 187 compounds was conducted against whole-cell *Mycobacterium smegmatis* carrying a β-galactosidase reporter construct. In brief, 8 μL of cells grown in Luria Broth (LB) media containing 50 μg/mL hygromycin were dispensed using a FlexDrop IV (PerkinElmer) into compound preplated 1536-well black plates. For quality control, each assay plate contained at a final concentration 1% DMSO (v/v) vehicle only as high control wells in columns 25−28 in row A-P and 1% trifluoroacetic acid (TFA) in 1% DMSO (v/v) enabling 100% kill of *M. smegmatis* as low control wells in columns 25−28 in row Q-AF. Assay plates were incubated in a dedicated Steri-Cult automated incubator (Thermo Scientific) at 37 °C for 16 h; after which, 1 μL of the C2FDG substrate is added to achieve a final concentration of 16 μM. Assay plates were further incubated at 37 °C for 3 h and the β-galactosidase activity was read on the LEADseeker Multimodality Imaging System (GE Healthcare). Full description of the assay development and optimization will be published elsewhere.

Whole Genome Sequencing to Detect Resistance Mutations. The parental wild-type *M. smegmatis* strain and five MMS mutant strains were sequenced via the Ion Torrent Personal Genome Machine (PGM) sequencing platform. A simple pipeline to processes the Ion Torrent PGM sequencing runs for bacterial whole genome data and can be found in Supporting Information.

Time-Lapse Microscopy. For time-lapse microscopy on agar pad slides, cells were added to a 1.5% (w/v) low melting point agarose LB pad with or without addition of the indicated drug (SKI-356313, rifampin, etc.). For pad preparation, LB agarose was heated to 65 °C and poured into a 17 × 28 mm geneframe (Thermoscientific, AB-0578) adhered to a 25 × 75 mm glass slide. A second slide was pressed down on top and the setup was allowed to cool at room temperature (RT) for 10 min. The top slide was removed and the pad was cut and removed so that a 3−4 mm strip remained near the center. 2−3 μL of *M. smegmatis* culture was added to the pad and a No. 1.5 24 × 40 mm coverglass was sealed to the geneframe. Slides were allowed to warm to 37 °C in the microscope stage top incubator and six to eight fields were selected for time lapse microscopy imaging. All movies were started within 20 min of addition of *M. smegmatis* onto drug pads.

ASSOCIATED CONTENT

Supporting Information

Supplementary methods, chemical synthesis schemes, and time-lapse microscopy movies. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Email: glickmam@mskcc.org.

Notes

The authors declare the following competing financial interest(s): M.G. and H.D. are inventors on patent application No. PCT/US2013/069639.

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