Published in final edited form as:

ACS Chem Biol. 2017 May 19; 12(5): 1346–1352. doi:10.1021/acscchembio.6b01133.

AFFINITY SELECTION-MASS SPECTROMETRY IDENTIFIES A NOVEL ANTIBACTERIAL RNA POLYMERASE INHIBITOR

Scott S. Walker^{1,*}, David Degen^{2,*}, Elliott Nickbarg³, Donna Carr¹, Aileen Soriano¹, Mihir Mandal¹, Ronald E. Painter¹, Payal Sheth¹, Li Xiao¹, Xinwei Sher³, Nicholas Murgolo¹, Jing Su¹, David B. Olsen⁴, Richard H. Ebright^{2,**}, and Katherine Young^{1,**}

¹Merck & Co., Inc., Kenilworth, NJ 07033

²Department of Chemistry and Waksman Institute, Rutgers University, Piscataway, NJ 08854

³Merck & Co., Inc., Boston, MA 02115

⁴Merck & Co., Inc., Upper Gwynedd, PA 19454

Abstract

The growing prevalence of drug resistant bacteria is a significant global threat to human health. The antibacterial drug rifampin, which functions by inhibiting bacterial RNA polymerase (RNAP), is an important part of the antibacterial armamentarium. Here, in order to identify novel inhibitors of bacterial RNAP, we used affinity-selection mass spectrometry to screen a chemical library for compounds that bind to *Escherichia coli* RNAP. We identified a novel small molecule, MRL-436, that binds to RNAP, inhibits RNAP, and exhibits antibacterial activity. MRL-436 binds to RNAP through a binding site that differs from the rifampin binding site, inhibits rifampin-resistant RNAP derivatives, and exhibits antibacterial activity against rifampin-resistant strains. Isolation of mutants resistant to the antibacterial activity of MRL-436 yields a missense mutation in codon 622 of the *rpoC* gene encoding RNAP β' subunit or a null mutation in the *rpoZ* gene encoding RNAP ω subunit, confirming that RNAP is the functional cellular target for the antibacterial activity of MRL-436, and indicating that RNAP β' subunit residue 622 and RNAP ω subunit are required for the antibacterial activity of MRL-436. Similarity between the resistance determinant for MRL-436 and the resistance determinant for the cellular alarmone ppGpp suggests a possible similarity in binding site and/or induced conformational state for MRL-436 and ppGpp.

Graphical Abstract

^{**}Corresponding authors: ebright@waksman.rutgers.edu; katherine_young@merck.com.

^{*}These authors contributed equally to this work

INTRODUCTION

Life-threatening, difficult-to-treat, antibiotic-resistant bacterial infections have created an urgent need for identification and development of new antibacterial drugs (1). Bacterial RNA polymerase (RNAP), the enzyme responsible for bacterial RNA synthesis, is a proven, but relatively underexploited, target for antibacterial drugs (2). Two classes of approved antibacterial drugs function by inhibiting bacterial RNAP: rifamycins and lipiarmycins (3, 4). The rifamycin class of antibacterial drugs, which include rifampin (Rif), rifabutin, rifapentine, and rifaximin, inhibit bacterial RNAP by binding to a site adjacent to the RNAP active center and sterically blocking the extension of RNA beyond a length of 2–3 nt (5, 6). The rifamycins are first-line treatments for tuberculosis, biofilm infections of catheters and implanted medical devices, and certain gastrointestinal infections, however poor efficacy against Gram-negatives and the rapid emergence of resistance limits their broader use (4). Resistance to rifamycins typically involves mutations that alter the binding site for rifamycins on RNAP, interfering with the binding of rifamycins to RNAP. There is an urgent need for new antibacterial agents that target RNAP through binding sites that do not overlap the rifamycin binding site and that therefore do not share cross-resistance with Rif.

Here we report the identification of a novel antibacterial agent that inhibits RNAP through a binding site that does not overlap the rifamycin binding site and that does not share cross-resistance with rifamycins: MRL-436 (Figure 1).

RESULTS

Identification of MRL-436, a novel small molecule that binds to *E. coli* RNAP

Using AS-MS, we screened a proprietary collection of approximately 43,000 small molecules, identified in unpublished work as having growth-inhibitory activity against a sensitized *E. coli* strain (efflux-defective and outer membrane impaired), for the ability to bind to *E. coli* core enzyme RNAP. We identified approximately 200 primary hits with apparent affinity for RNAP and amongst those were 24 that reproducibly bound, while 14 of those compounds showed selective binding under our screening conditions (i.e, did not bind to yeast invertase). One example of a selective hit is MRL-436 (Figure 1), which then exhibited clear concentration-dependent binding to RNAP with an apparent equilibrium dissociation constant, K_d , of ~3 μ M (Figure 2A).

MRL-436 and Rif do not compete for binding to RNAP

Using AS-MS, we performed RNAP competition binding assays to assess whether MRL-436 and Rif compete for binding to RNAP. Increasing concentrations of MRL-436 were applied to a mixture containing fixed concentrations of RNAP and Rif (Figure 2A), and, in parallel, increasing concentrations of Rif were applied to a mixture containing fixed concentrations of RNAP and MRL-436 (Figure 2B). In each case, no competition was observed. We conclude that the binding site on RNAP for MRL-436 is different from, and does not overlap, the binding site for Rif on RNAP.

MRL-436 inhibits RNAP, including Rif-Resistant RNAP

Whereas the AS-MS results establish that MRL-436 binds to RNAP, they do not establish whether MRL-436 inhibits RNAP activity. To determine whether MRL-436 inhibits RNAP, we assessed effects of MRL-436 in transcription assays. The results indicate that MRL-436 exhibits clear concentration-dependent inhibition of RNAP ($IC_{50} = 3.0 \pm 0.6 \mu M$; Figure 2C). We conclude that MRL-436 inhibits RNAP. Transcription assays comparing effects of MRL-436 on wild-type RNAP and on the Rif-resistant RNAP derivatives [D516V] β -RNAP, [H526D] β -RNAP, and [S531L] β -RNAP (each of which contains an amino-acid substitution in the Rif binding site that interferes with Rif binding; selected for analysis as substitutions at the three residue sites are most frequently substituted in Rif-resistant clinical isolates) (7), show that MRL-436 is fully effective against the Rif-resistant RNAP derivatives (Table 1). We conclude that MRL-436 inhibits Rif-resistant RNAP, consistent with the conclusion above that the binding site on RNAP for MRL-436 does not overlap the binding site for Rif on RNAP.

MRL-436 exhibits antibacterial activity against Rif-resistant strains

The results in Table 2 indicate that MRL-436 exhibits antibacterial activity against uptake-proficient/efflux-deficient *E. coli* strains (MIC = 2 $\mu g/ml$ against *lpxC tolC* strain MB5746; MIC = 8 $\mu g/ml$ against *rfa tolC* strain D21f2tolC (8)). MRL-436 lacks activity against wild-type *E. coli* (MIC > 64 $\mu g/mL$). The results in Table 2 further indicate that MRL-436 is fully effective against Rif-resistant strains (MICs for Rif-resistant D21f2tolC derivatives are identical to MICs for the D21f2tolC isogenic parent strain; Rif-resistant strains from (9)), consistent with the conclusion above that the binding site on RNAP for MRL-436 does not overlap the binding site for Rif on RNAP.

MRL-436 inhibits RNA synthesis in bacterial cells

As a first approach to demonstrate that the RNAP-inhibitory activity of MRL-436 is responsible for the antibacterial activity of MRL-436, we assessed effects of MRL-436 on incorporation of [^{14}C]-thymidine into DNA, [^{14}C]-uridine into RNA, and [^{14}C]-L-amino acids into protein in *E. coli* strain D21f2/tolC. The results show that MRL-436 significantly inhibits RNA synthesis from the earliest time point following addition, significantly inhibits protein synthesis only at late time points after addition, and does not significantly inhibit DNA synthesis at any time point (Figure 3, left column). The pattern observed for MRL-436 matches the pattern observed for the reference RNAP inhibitor Rif under identical conditions (Figure 3, right column) and is as expected for an antibacterial agent with RNA-

synthesis-inhibition mode of action (i.e., rapid inhibition of RNA synthesis, later inhibition of RNA-dependent protein synthesis; (9–11) and references therein). We conclude that MRL-436 selectively inhibits RNA synthesis in bacterial cells, consistent with the hypothesis that the RNAP-inhibitory activity of MRL-436 is responsible for the antibacterial activity of MRL-436.

MRL-436-resistant mutants map to *rpoC* and *rpoZ* genes, encoding RNAP β' and RNAP ω subunits

As a second approach to demonstrate that the RNAP-inhibitory activity of MRL-436 is responsible for the antibacterial activity of MRL-436, we assessed whether MRL-436-resistant mutants contain mutations in RNAP genes. We isolated spontaneous MRL-436-resistant mutants of *E. coli* strain MB5746 by spreading cells on agar plates containing MRL-436 and picking colonies that arose (observed spontaneous resistance frequency = 8×10^{-7}). We then performed whole-genome sequencing and broth microdilution MIC assays on a sample of these resistant isolates. The sequencing results indicated that all of the sequenced spontaneous MRL-436-resistant mutants contained mutations in RNAP genes, and that no mutations were present outside of RNAP genes. One mutant contained a missense mutation in codon 622 of the *rpoC* gene, encoding RNAP β' subunit, and is inferred to result in the single-amino-acid substitution D622G in RNAP β' subunit (Table 3); the other five mutants contained frame shift mutations at codons 13, 20, 23, 24, or 35 in the *rpoZ* gene, encoding RNAP ω subunit, and are inferred to result in the loss of native residues 13–91, 21–91, 23–91, 24–91, 35–91 of RNAP ω subunit (and potentially to result in the complete loss of the non-essential RNAP ω subunit [see (12–14)]) (Table 3). The MIC results show that the spontaneous MRL-436-resistant mutants all possess high-level, >32-fold, resistance to MRL-436 (Table 3). We conclude that RNAP is the functional cellular target of MRL-436 and that residue 622 of RNAP β' subunit and C-terminal residues (and potentially all residues) of RNAP ω subunit are part of the MRL-436 functional determinant on RNAP.

Deletion of *rpoZ* confers MRL-436 resistance

To confirm that removal of the RNAP ω subunit suffices to confer resistance to MRL-436, we used λ -red mediated recombineering to delete the *rpoZ* gene from *E. coli* strain MB574. Broth microdilution MIC assays confirmed that complete deletion of *rpoZ* conferred high-level, >32-fold, resistance to MRL-436 (Table 3, bottom row). We conclude that the RNAP ω subunit is part of the MRL-436 functional determinant, and that removal of the subunit suffices to confer resistance to MRL-436.

Purified RNAP derivatives containing substitution in β' or lacking ω exhibit MRL-436-resistance

To confirm that the changes identified in MRL-436-resistant mutants confer resistance to RNAP in a purified system *in vitro*, we assayed the effects of MRL-436 on wild-type RNAP, and RNAP derivatives, one RNAP derivative containing the single-amino-acid substitution D622G in β' subunit ([D622G] β' -RNAP), and an RNAP derivative lacking ω subunit (ω -RNAP). The results show that both [D622G] β' -RNAP and ω -RNAP exhibit resistance to MRL-436 (Table 4). In contrast, neither of these RNAP derivatives exhibits resistance to Rif

(Table 4). We conclude that the changes identified in MRL-436-resistant mutants confer MRL-436-resistance to RNAP in a purified system *in vitro*.

DISCUSSION

Using an unbiased, cell-free, AS-MS method, we identified MRL-436, a novel small molecule that binds to and inhibits *E. coli* RNAP. Our results show that MRL-436 does not compete with the bacterial RNAP inhibitor Rif for binding to RNAP, and that MRL-436 does not exhibit cross-resistance with Rif. Our results further show that the single amino acid substitution β' -D622G or deletion of the RNAP ω subunit are sufficient to confer resistance to MRL-436, but do not confer resistance to Rif. Taken together, our results demonstrate that MRL-436 functions through a binding site on RNAP that differs from, and does not overlap, the binding site for Rif.

In the three-dimensional structure of *E. coli* RNAP, residue 622 of RNAP β' subunit is located close to RNAP ω subunit, and both are far from the binding site for Rif (Figure 4). We point out that β' residue 622 and ω previously have been shown to form part of a binding site on RNAP for the cellular alarmone ppGpp (15–17), and that substitution of β' residue 622 or deletion of the ω subunit can confer resistance to RNAP inhibition by ppGpp (13, 16–18). The striking similarity between the resistance determinant for MRL-436 and the resistance determinant for ppGpp immediately suggests that the binding site and/or induced RNAP conformational state for MRL-436 may be similar to a binding site and/or induced RNAP conformational state for ppGpp. It is attractive to speculate that the synthetic antibacterial compound MRL-436 and the natural regulator ppGpp may exploit the same binding site and/or the same induced RNAP conformational state to inhibit RNAP. However, this speculation must be regarded with caution in the absence of results directly defining the binding site of MRL-436, the mechanism of MRL-436, and the relationship between the binding site and mechanism of MRL-436 and those of ppGpp.

MATERIALS AND METHODS

RNAP

RNAP core enzyme, RNAP holoenzyme, [D516V] β -RNAP holoenzyme, [H526D] β -RNAP holoenzyme, and [S531L] β -RNAP holoenzyme for experiments in Table 1 in Figs. 2–3 and for determination of [D622G] β' -RNAP IC₅₀ ratios in Table 4 were prepared from *E. coli* strain XE54 (19) transformed with plasmid pRL706, pRL706-D516V, pRL706-H526D, pRL706-S531L which encodes C-terminally hexahistidine-tagged *E. coli* RNAP β subunit or β subunit derivatives (20), using procedures essentially as in (21). [D622G] β' -RNAP holoenzyme was prepared in the same manner from *E. coli* strain 397c ((22)) transformed with plasmid pRL663-D622G (generated by QuikChange site-directed mutagenesis [Agilent] of plasmid pRL663, which encodes C-terminally hexahistidine-tagged *E. coli* RNAP β' subunit (23)).

RNAP holoenzyme and ω -RNAP holoenzyme for determination of ω -RNAP IC₅₀ ratios in Table 4 were prepared as in (24), except that the reconstitution mixture (10 ml) contained 0.8 mg hexahistidine-tagged α (prepared under denaturing conditions), 3 mg β , 6 mg β' , 6

mg σ^{70} , and 1.2 mg ω (for RNAP holoenzyme; prepared essentially as in (25), but using plasmid pCDF ω (26) and omitting n-octyl-beta-D-glucopyranoside from sonication buffers) or 0 mg ω (for ω -RNAP holoenzyme, respectively); the dialysis steps used 1.5 l volumes of dialysis buffer and the metal-ion-affinity chromatography used 3 ml of Ni-NTA agarose (Qiagen). The sample was exchanged into ~36 ml 10 mM Tris (pH 7.9), 300 mM NaCl, 5% glycerol (v/v), 0.1 mM EDTA, and 5 mM DTT and concentrated to 150 μ l by using an Amicon Ultracel-30K centrifugal filter (EMD Millipore), was mixed with an equal volume of glycerol, and stored at -80°C .

Affinity selection-mass spectrometry (AS-MS)

AS-MS was performed using the automated ligand identification system (ALIS), a dual chromatography LC-MS system that incorporates a size-exclusion chromatography column to separate mixtures of unbound compounds from protein-bound compounds, and then separates and identifies protein-bound compounds using reversed-phase LC-MS. The system and methodologies used have been described (27–29). The ALIS hardware used here was modified from that used in previous work by incorporation of a custom-built four-switching-valve box to enable dual RPC column switching for increased throughput (a 40% increase compared to previous ALIS setups). In addition to this modification, mass spectrometric detection was accomplished using a high-resolution Exactive Orbitrap mass spectrometer (ThermoScientific) scanning from 150 to 800 m/z at 100,000 resolution with a mass accuracy of <5 ppm and a scan rate of 1 Hz.

RNAP binding assays using AS-MS

Compounds were screened for the ability to bind to *E. coli* RNAP core enzyme as follows: approximately 110 compound mixtures, each containing approximately 100–500 compounds at 20 μ M each in dimethylsulfoxide (DMSO) were prepared (~43,000 compounds in total). Mass encoding was used to enable each compound to be identified uniquely by its molecular mass (methods as in (30)). For each ligand binding reaction, 1 μ L of 40x compound mixtures was pre-diluted in 19 μ L of 50 mM Tris (pH,8.0), 350 mM NaCl, 5 mM DTT. Starting with a 12 μ M stock of RNAP core enzyme in a buffer containing 10 mM Tris (pH 8.0), 350 mM NaCl, 5 mM DTT, 0.1 mM EDTA and 5% (v/v) glycerol, RNAP was diluted with 50 mM Tris (pH = 8.0), 350 mM NaCl, 5 mM DTT to a concentration of 4 μ M, and 1 μ L of the diluted RNAP was mixed with an equal volume of buffer-solubilized ligand mixture in a well of a 96-well plate. As such, the final RNAP concentration was set at 2 μ M in 50 mM Tris (pH=8.0), 350 mM NaCl, 5 mM DTT, and 2.5% DMSO(v/v), and the final compound concentration was 0.5 μ M per component for all reactions. After incubation at room temperature for 30 min, the samples were cooled to 4°C and then analyzed by the ALIS. Sample injections of 2 μ L each were conducted on rapid size exclusion chromatography using a custom column packed with proprietary gel filtration media using a mobile phase of 0.7 M ammonium acetate (pH=8.0) buffer at a flow rate of 0.3 ml/min in order to separate protein-ligand complexes from unbound compounds. The resulting isolated protein-ligand complexes were then captured by an in-line valve system and delivered to a reverse phase chromatographic unit where they were subjected to pH=2.0 at 60°C to dissociate ligands for subsequent detection by electrospray ionization-based mass spectrometry. Library mixtures that showed positive signals were repeated, and then the

compound of interest was tested again as a single, pure compound against the RNAP target and also a negative control protein (yeast invertase, Sigma-Aldrich) to rule out non-specific binding. Those compounds that showed reproducible MS signals that were specific to the RNAP target were considered to be hits. To verify selectivity of Rif and MRL-436, each was counter-screened separately at 10 μ M against 5 μ M invertase and did not show an AS-MS signal.

RNAP competition binding assays using AS-MS

Competition binding assays were performed using AS-MS as implemented by ALIS (29, 31). Conditions were as described in the preceding section, except that the RNAP target final concentration was increased to 4 μ M., MRL-436 (or Rif, Sigma-Aldrich) was at 4 μ M, and Rif (or MRL-436) was varied from 0 to 80 μ M. Signals for each individual compound were normalized to allow for comparison. Curve fitting was done using GraphPad PRISM software.

RNAP-inhibition assays

Fluorescence-detected RNAP inhibition assays in Tables 1 and 4 were performed using a DNA fragment containing the *lacUV5* promoter as template and the profluorescent ATP analog γ -[2'-(2-benzothiazoyl)-6'-hydroxybenzothiazole]-ATP (BBT-ATP, Jena Biosciences) as substrate (methods as in (32)). Fluorescence-detected RNAP inhibition assays in Figure 2C were performed essentially as described in (33, 34). The assay used a proprietary circular single-stranded DNA as template (Kool NC-45 Universal RNAP Template, Epicentre). The synthesized RNA was quantified using a sensitive fluorescent dye, RiboGreen (ThermoFisher), which exhibits fluorescence enhancement upon binding to RNA. A volume of 0.2 μ L MRL-436 or Rif [10 concentrations from a 3-fold serial dilution in 100% DMSO(v/v)] was loaded into a 384 -well plate using an Echo liquid handler (Labcyte). 5 μ L of 20 nM recombinant RNAP in assay buffer [50 mM HEPES (pH=8.0), 50 mM KCl, 4 mM MgCl₂, 5% glycerol (v/v), 1 mM DTT, 0.1 mg/ml BSA, and 0.01% Triton X-100 (v/v)] was then added. After a 30 min incubation at room temperature, 5 μ L assay buffer solution containing 0.04 ng/ μ L DNA template and rNTPs (ATP, CTP, GTP, UTP; 600 μ M each; Epicentre) was added to start the reaction. After incubation for 2 h at 37°C, 70 μ L Quant-iT RiboGreen RNA Reagent [1:350 dilution in buffer containing 10 mM Tris-HCl (pH=7.5) and 1 mM EDTA] was added to stop the reaction and develop the signal. The plate was read using a PheraStar Plus plate reader (BMG). IC₅₀ values were determined by fitting the data to a 4-parameter dose response (inhibitor) equation using GraphPad PRISM software.

Broth microdilution minimum inhibitory concentration (MIC) measurements

Compound 2-fold serial dilutions were prepared in 100% DMSO (Sigma-Aldrich) and 2 μ L of each dilution was transferred to a 96-well plate with lid (Thermo Scientific). Inocula were prepared using the BBL Prompt Inoculation System (Becton Dickinson) as described by the manufacturer and a further dilution (1:1000) was made into cation adjusted Mueller-Hinton broth (CAMH broth, Becton-Dickinson). One hundred μ L of inoculum was transferred to each well of the 96-well plate containing diluted compound (above). Plates were covered

and incubated overnight (18 h) at 37°C. The MIC for the compound was determined as the minimum concentration required to completely inhibit visible growth of the cells.

Macromolecular-synthesis assays

Measurement of compound-dependent inhibition of cellular macromolecule synthesis was carried out as described in (9), except that stock concentrations for the 7 µl addition step were 260 kBq/ml [¹⁴C]-thymidine, 1100 kBq/ml [¹⁴C]-uridine, and 1100 kBq/ml [¹⁴C]-L-amino acid mix; DMSO was used as the solvent for MRL-436 and Rif; and the final concentrations of MRL-436 and Rif were 3.13 µg/ml and 0.39 µg/ml, respectively.

Isolation and sequencing of spontaneous resistant mutants

The MRL-436 agar-dilution MIC was determined prior to selection for resistance by first serially diluting the compound in 100% dimethylsulfoxide followed by the addition of the diluted compound to 4 ml of molten CAMH agar in each chamber of an 8-well dish (Thermo Scientific). After the agar solidified, 5 µL of a saturated culture of *E. coli* strain MB5746 [C600 *leu thr lac (thi) galK lpxC (envA1) tolC::tn10*] (35) grown in CAMH broth was dispensed on the agar surface and allowed to dry at room temperature. The plate was then incubated overnight at 37°C and the agar MIC was identified as the lowest dilution of compound that completely inhibited visible growth. To select for resistance, a 100 mm petri dish was filled with 25 ml CAMH agar containing 16 µg/ml MRL-436. The cells in 1 ml of an overnight culture of MB5746 (~10⁹ cfu/ml) were concentrated by centrifugation and spread onto the surface of the MRL-436 containing plate. After the surface dried at room temperature the plate was incubated at 37°C overnight. Colonies were counted and an approximate frequency of resistance was calculated. Genomic DNA from the parent strain (MB5746) and resistant isolates was obtained with a GeneJET genomic DNA purification kit according to the manufacturer's instructions (Fisher Scientific), and sequenced. Assembled whole genome mutant sequences were then compared to the parent strain and polymorphisms were identified as described (36–39).

Construction of *rpoZ* strain

The *rpoZ* deletion allele from the Keio collection (40) was amplified by PCR (primers: AGCAAATTGTTGGCAGACTGAACCTGA and CATCACGTGCAACGAGATACGCCTG) and was introduced into *E. coli* strain MB5746 as follows: *E. coli* strain MB5746 transformed with plasmid pKD46 (41) was used to inoculate SOB broth containing 0.2% arabinose (w/v) and 100 µg/ml, and grown overnight at 30°C. The overnight culture was then diluted 1:40 into 20 ml of the same media and grown at 30°C while shaking for ~3 h until OD₆₀₀ = 0.5. Cells were pelleted by centrifugation at 4000 rpm for 10 min, washed with 40 ml ice cold distilled H₂O, washed three times with 1 ml ice cold 10% glycerol (v/v), and centrifuged at 5000 g for 3 min. The pellet was resuspended in 100 µl of 10% glycerol (v/v) (1:200 of starting culture volume). For transformation, 5 µl of gel-purified PCR product was added to 50 µl cells, mixed, and immediately electroporated in a 0.2 cm gap cuvette at 2.5 kV, 25 µF, 200 Ω. Electroporated cells were recovered in 1 ml SOC 0.2% arabinose (w/v) at 37°C for 2 hours with shaking. Cells in amounts of 50 and 950 µl were then plated on LB agar + kanamycin (25 µg/ml) and incubated overnight at 37°C. Gene disruption was confirmed by PCR.

Acknowledgments

This work was supported, in part, by National Institutes of Health grant GM041376 to R.H.E. We thank J. Malinverni and C. Balibar for advice on this work and for reviewing the manuscript and providing helpful comments.

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Figure 1.
MRL-436.

Figure 2. MRL-436 binds to RNAP and does not compete with Rif for binding to RNAP
A) Normalized AS-MS response of Rif and MRL-436 to increasing concentrations of MRL-436 (0–80 μM ; Rif held constant at 4 μM ; stoichiometric binding; $K_d = 3 \mu\text{M}$). B) Normalized AS-MS response of Rif and MRL-436 to increasing concentrations of Rif (0–80 μM ; MRL-436 held constant at 4 μM ; stoichiometric binding; $K_d < 2 \mu\text{M}$). Data represent the average and standard error (SEM) of duplicate experiments. C) RNAP inhibition by MRL-436 ($\text{IC}_{50} = 3.0 \mu\text{M}$) and Rif ($\text{IC}_{50} = 0.02 \mu\text{M}$).

Figure 3. MRL-436 inhibits RNA synthesis in bacterial cells

Left column, data for MRL-436. Right column, data for Rif. Black, absence of inhibitor. Red or blue, presence of inhibitor. Asterisks, statistically significant differences (t-test; $p < 0.02$).

Figure 4. Locations of resistance determinant in three-dimensional structure of RNAP
Structure of bacterial RNAP (gray ribbons; violet sphere for active-center Mg^{2+} ; β' non-conserved region and σ omitted for clarity; PDB 1IW7), showing the sites of MRL-436-resistant substitutions and deletions (blue for β' residue 622 and cyan for the ω subunit), and Rif-resistant substitutions (red; (42–44)).

Table 1

MRL-436 inhibits RNAP, including Rif-resistant RNAPs

enzyme	IC50 (μM)	
	MRL-436	Rif
RNAP	2	0.03
[D516V]β-RNAP	4	70
[H526D]β-RNAP	3	>100
[S531L]β-RNAP	4	80

Table 2MRL-436 exhibits antibacterial activity, including antibacterial activity against Rif^R strains^a

organism	MIC (μg/ml)	
	MRL-436	Rif
<i>E. coli</i> MB5746 (<i>lpxC tolC</i>)	2	0.5
<i>E. coli</i> D21f2tolC (<i>rfa tolC</i>)	8	0.5
<i>E. coli</i> D21f2tolC Rif ^R -D516V (<i>rfa tolC rpoB-D516V</i>)	8	32
<i>E. coli</i> D21f2tolC Rif ^R -H526D (<i>rfa tolC rpoB-H526D</i>)	8	128
<i>E. coli</i> D21f2tolC Rif ^R -S531L (<i>rfa tolC rpoB-S531L</i>)	2	64

^aRif^R: rifampicin resistant *E. coli*

Table 3

MRL-436-resistant mutants: sequences and properties

amino acid substitution	number of isolates	resistance level (MIC/MIC _{wild-type}) ^a	
		MRL-436	Rif
<i>RpoC</i> (RNAP β′ subunit)			
D622G	1	>32	1
<i>RpoZ</i> (RNAP ω subunit)			
13–91 (frameshift)	1	>32	1
21–91 (frameshift)	1	>32	1
23–91 (frameshift)	1	>32	1
24–91 (frameshift)	1	>32	1
35–91 (frameshift)	1	>32	1
1–91 (deletion)	NA	>32	1

^aMRL-436 MIC_{wild-type} = 2 µg/ml

Table 4MRL-436-resistant RNAP derivatives: *in vitro* resistance to MRL-436

enzyme	IC50 ratio (IC50/IC50 _{wild-type}) ^a	
	MRL-436	Rif
RNAP (co-expressed)	1	1
[D622G]β'-RNAP	6 ^b	1
RNAP (reconstituted)	1	1
ω RNAP	6 ^b	0.5

^aMRL-436 and Rif IC50_{wild-type} (co-expressed) are as in Table 1; MRL-436 IC50_{wild-type} (reconstituted) = 3 μM; Rif IC50_{wild-type} (reconstituted) = 0.04 μM

^bstatistically significant differences between RNAP and the MRL-436-resistant RNAP derivative (t test; p<0.01)