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X-ray crystal structures of the *Escherichia coli* RNA polymerase in complex with Benzoxazinorifamycins

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Abstract

Rifampin, a semi-synthetic rifamycin, is the cornerstone of current tuberculosis treatment. Among many semi-synthetic rifamycins, benzoxazinorifamycins have great potential for TB treatment due to their superior affinity for wild-type and rifampin-resistant *Mycobacterium tuberculosis* RNA polymerases, and their reduced hepatic Cyp450 induction activity. In this study, we have determined the crystal structures of the *Escherichia coli* RNA polymerase complexes with two benzoxazinorifamycins. The ansa-naphthalene moieties of the benzoxazinorifamycins bind in a deep pocket of the β subunit, blocking the path of the RNA transcript. The C3'-tail of benzoxazinorifamycin fits a cavity between the β subunit and σ factor. We propose that, in addition to blocking RNA exit, the benzoxazinorifamycin C3'-tail changes the σ region3.2 loop position, which influences the template DNA at the active site thereby reducing the efficiency of transcription initiation. This study supports expansion of structure–activity relationships of benzoxazinorifamycins inhibition of RNA polymerase toward uncovering superior analogues with development potential.

Keywords

Escherichia coli RNA polymerase; Benzoxazinorifamycins; X-ray crystallography; Tuberculosis

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Author Contributions: V.M. and K.S.M. crystallized *E. coli* RNAP complex with inhibitors and determined their X-ray crystal structures. P.D.K. provided coordinates for the inhibitors and assisted with the structure refinements. I.N.N. and N.T.S. performed the enzyme purification and IC₅₀ studies, supervised by G.A.G. K.S.M., H.D.H.S. and G.A.G. wrote the manuscript, and all authors discussed the results and commented on the manuscript.

Supporting Information Available: Log IC₅₀s and standard errors of the fits for the benzoxazinorifamycins with *E. coli* wild-type (WT) and mutant RNAPs, and dose-response plots for each benzoxazinorifamycin with *E. coli* (WT) and mutant RNAPs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Tuberculosis (TB) is one of the most significant global challenges to human health. In 2010, there were an estimated 8.8 million new cases and 1.1 million deaths from TB among HIV-negative people (an additional 0.35 million deaths from HIV-associated TB). There were also an estimated 650,000 cases of multi-drug resistant TB (MDR-TB)¹. Treatments for TB are essentially 40-years old and very few new drugs have been introduced into clinical practice in that time frame. TB treatment involves a 6–9 month, multi-drug regimen that targets the causative agent, *Mycobacterium tuberculosis* (MTB). Many aspects of TB treatment are problematic; however, the increasing prevalence of drug and multi-drug resistant strains of MTB and the negative drug-drug interactions are arguably the most severe^{2, 3}.

Rifampin (**RMP**), a semisynthetic derivative of the rifamycin natural product, has been used as one of the first line drugs for the treatment of tuberculosis for over four decades⁴. Although many Rif^R strains of MTB, with mutations in the Rif-binding site of the target RNA polymerase (RNAP), have been identified in clinical isolates, mutations of only three specific residues; D435V, H445Y and S450L (MTB *rpoB* numbering; D516V, H526Y and S531L in *E. coli rpoB* numbering), account for ~84 % of MTB Rif^R strains^{5, 6}. One of the key drawbacks of **RMP** involves its incredibly effective interaction with the human pregnane X receptor (hPXR), which cause the induction of hepatic Cyp450 and other proteins involved in xenobiotic metabolism⁷. This is particularly critical for persons co-infected with HIV and MTB, as certain HIV drugs (*e.g.*, protease inhibitors) are metabolized by Cyp450.

Among the many synthetic rifamycin derivatives that have been made, rifalazil (a benzoxazinorifamycin, **bxRIF2a**, Figure 1) is the most advanced due to its reported superior affinity toward wild-type and some Rif^R mutants of the MTB RNA polymerase^{8–11}. Rifalazil also exhibits dramatically reduced Cyp450 induction activity in rat and dog¹² and essentially no hPXR activation *in vitro* below 100 μ M (MIC₉₀ < 4 nM)¹³.

The crystal structure of the *Thermus aquaticus* RNAP core enzyme and RMP complex identified the RMP binding site on the β subunit and elucidated the interactions between the ansa-naphthalene core of RMP and β subunit amino acids¹⁴. This structure is consistent with rifamycin resistant mutations reducing the affinity of RNAP for the rifamycins and explains why RMP blocks the synthesis of RNA products longer than 3 nt. Subsequent crystal structures of the *Thermus thermophilus* RNAP holoenzymes in complex with rifapentin and rifabutin revealed interactions between their C-3/C-4 tails and the σ factor region 3.2 loop (σ finger) that highlights additional chemical space in the RNAP rifamycin-binding pocket and suggests a novel mechanism contributing to inhibition of transcription¹⁵.

Recently, we have determined the first X-ray crystal structure of *E. coli* RNAP σ^{70} holoenzyme¹⁶. Since the sequences and antibiotic sensitivities of *E. coli* and the MTB RNAPs are more similar than those of MTB and *Thermus* RNAPs¹⁴, the *E. coli* RNAP is a better model to study RNAP-antibiotic interactions by X-ray crystallography and for later application of derived information towards TB drug discovery. We have also synthesized a novel subclass of benzoxazinorifamycins (bxRIFs) and established that these analogues generally display superior affinity toward wild-type and Rif^R mutants of the MTB RNAP relative to rifalazil¹³. In the present study, we have determined the crystal structures of *E. coli* RNAP σ^{70} holoenzyme complexes with two selected bxRIF derivatives and determined the IC₅₀ values for those derivatives with the *E. coli* RNAP to establish a structural basis for further structure-activity relationship studies of bxRIF derivatives against RNAP.

RESULTS AND DISCUSSION

Structure determination of *E. coli* RNAP holoenzyme in complex with RIF and bxRIF2b/bxRIF2c

Crystals of *E. coli* RNAP σ^{70} holoenzyme in complex with **RMP**, **bxRIF2b** or **bxRIF2c** (Figures 1 and 2) were prepared by soaking 0.1 mM inhibitors into the preformed RNAP crystals¹⁶, and X-ray crystal structures of these complexes were determined at 3.85, 3.96 and 3.69 Å resolutions, respectively (Table 1).

Crystals contain two 440 kDa RNAP holoenzyme molecules, designated RNAP_A and RNAP_B, per asymmetric unit. Although these two RNAPs have almost identical structures, the electron density maps of the σ finger and the inhibitor binding on the RNAP_A are more visible than ones from the RNAP_B. This trend holds for all three complexes (RNAP-**RMP**, RNAP-**bxRIF2b** and RNAP-**bxRIF2c**) determined in this study. Therefore we used only RNAP_A for the structure analysis in this study.

There is no conformational change in the RNAP structure induced by binding of the inhibitors. Electron-density maps (Figure 2) showed unambiguous density for the ansa-naphthalene core of all inhibitors that bind in a pocket of the β subunit within the DNA/RNA channel (Figures 2 and 3), which holds the extending RNA from -3 to -6 nt during the transcription elongation. The ansa-naphthalene core faces amino acid regions where Rif^R mutations have been identified, and the ansa-naphthalene core and β subunit interaction is essentially identical to the *Thermus* RNAP-**RMP** complexes¹⁴ with the significant exception of the interactions involving the β subunit fork loop 2 (described later).

Previous crystal structures of *Thermus* RNAP holoenzymes in complex with rifabutin and rifapentin revealed a loss of the catalytic Mg²⁺ at the RNAP active site¹⁵. Although we have crystallized RNAP and soaked inhibitors into RNAP crystals without explicitly adding Mg²⁺, the RNAP active site exhibited electron density corresponding to the catalytic Mg²⁺, indicating that neither **RMP**, **bxRIF2b** nor **bxRIF2c** displaces the high affinity catalytic Mg²⁺ from the active site of *E. coli* RNAP under our experimental conditions.

E. coli RNAP and RMP/bxRIF interactions

Strong electron density maps were attainable not only from the ansa-naphthalene core but also from the C3-tail of **RMP** (Figure 2A left) for the RNAP-**RMP** complex. The C3-tail points toward the active site but appears to make no interaction with any RNAP residues. The closest distance between one of the methylenes of the RMP N-methylpiperazinyl side chain (designated by an asterisk in Figure 1) and the active site Mg²⁺ is 14 Å.

In addition to the ansa-naphthalene core, strong electron densities for the benzoxazino moieties were observed for the RNAP-**bxRIF2b** and RNAP-**bxRIF2c** complexes (Figure 2A middle and right). We fit the **bxRIF2b** and **bxRIF2c** compounds into structure models based upon the computational modeling in our previous study¹³. The closest distance between the **bxRIF2b/bxRIF2c** (C5' atom of the benzoxazino moiety) and the active site Mg²⁺ is ~17 Å. Electron densities for the C3'-tails of both **bxRIF2b** and **bxRIF2c** are traceable and are located in a gap between the β subunit fork loop 2 (residues 533–548, Arg residue 540) and σ finger (residues 513–515) (Figures 2 and 3). Arg540 of fork loop 2 and acidic residues (D513, D514 and E515) of the σ finger are located adjacent to the bxRIF C3'-tails. These residues have hydrophobic interactions with the C3'-tails of the **bxRIF2b** and **bxRIF2c** compounds. Modification of the C3'-tail to optimize these interactions may further enhance binding affinity.

In this study, we have also observed a novel interaction between the fork loop 2 and the naphthalene ring that was not reported in the previous structures of *Thermus* RNAPs in complex with **RMP**, rifabutin or rifapentin (likely due to a modeling error of the fork loop 2 conformation). This modeling error was first made in the *T. aquaticus* RNAP core enzyme structure and it has been corrected only in recent crystal structures of the *E. coli* holoenzyme¹⁶ and the *T. thermophilus* RNAP--promoter DNA complex¹⁷.

Although Arg540 of the fork loop 2 is proximal to the ansa-naphthalene core of RMP/bxRIF, there have been no reports of the mutation of this residue in the TB drug resistance mutation database¹⁸. The fork loop 2 faces a front edge of the downstream double-stranded DNA and plays an important role in the DNA unwinding during transcription. Therefore, it seems likely that mutation of Arg540 may cause a larger defect in RNAP "fitness" relative to other *ipoB* mutations such that the organism cannot survive such a mutation even though it might impart rifamycin resistance. The fork loop 2 of RNAP could be a new target for developing improved rifamycin derivatives that may be less susceptible to the development of resistance.

Alternative mechanism of transcription inhibition by bxRIF

The σ finger plays an important role in transcription initiation^{19, 20} by direct interaction with the -3 and -4 bases of the template DNA strand¹⁷. This interaction induces the single-stranded template DNA into an A-form helical conformation to facilitate the binding of initiating NTPs at a very early stage of transcription. In the RNAP complexes with **bxRIF2b** and **bxRIF2c**, the C3'-tails are next to the σ finger, but the position of the σ finger is unchanged relative to RNAP in the absence of inhibitor. To determine if the C3'-tail can be accommodated between the fork loop 2 and σ finger in the transcription initiation complex, we modeled **bxRIF2c** into a recently determined *Thermus* RNAP transcription initiation complex structure¹⁷ by overlaying amino acid residues of the **RMP**-binding clusters of *Thermus* and *E. coli* RNAPs. The main DNA binding channel of the *Thermus* RNAP transcription initiation complex is in a more closed conformation relative to the *E. coli* RNAP, and its σ finger is ~5 Å closer to the active site and overlaps with the C3'-tail of **bxRIF2c** (Figure 3). The sequences and structures around the σ fingers are highly conserved in the *E. coli* and *Thermus* RNAPs. It seems likely that the σ finger of *E. coli* RNAP also moves toward the active site in the transcription initiation complex and supports the template DNA positioning at the active site. If this occurs, then the presence of **bxRIF2c** would cause the σ finger to disengage from its position, which may consequently alter the position of the template DNA and inhibit the formation of the first phosphodiester bond (Figure 3). This structure-based prediction is consistent with previous observations that some rifamycin derivatives inhibit the formation of the first phosphodiester bond^{15, 21}. Experiments to probe this potentially important interaction are in progress.

In vitro inhibition of wild-type and RIF-resistant mutant *E. coli* RNAPs

The inhibition constants (IC₅₀) of the wild-type and Rif-resistant (Rif^R) mutants of the *E. coli* RNAP for **bxRIF2a–d** were determined via dose-response studies as previously described, with each compound tested in duplicate at the specified concentrations²². The data were plotted (log of the bxRIF concentration vs % activity, example plot of wild-type *E. coli* RNAP and **bxRIF2b** in Figure 4) and then fit by nonlinear regression. The log IC₅₀ values and their standard errors (of the fit) are reported in S-Table 1 of Supporting information. These errors roughly translate into a ~15% error in the IC₅₀ values. The IC₅₀ values are listed in Table 2, along with the data for **bxRIF2a–d** against MTB wild-type and Rif^R mutant RNAP for comparison¹³. **bxRIF2b–d** inhibit the wild-type *E. coli* RNAP in the 10⁻⁹ molar (nM) range, similar to rifampin (**RMP**) and rifalazil (**bxRIF2a**). The IC₅₀ values for **bxRIF2a–d** against the *E. coli* Rif^R mutant RNAPs were much higher, generally in the

10–100 μM range. However, **bxRIF2b–d** exhibited significantly enhanced (as much as 100-fold) inhibition of the Rif^R mutant RNAPs D516V and S531L relative to **RMP** and rifalazil. The Rif^R mutant H526Y was particularly resistant to all rifamycins, exhibiting only 50% or lower inhibition of activity by **bxRIF2b–d** even at mM concentrations.

The interactions that we have observed of the naphthalene rings of the bxRIFs with the β subunit fork loop 2 (R540) are consistent with the increased efficacies of **bxRIF2c–d** with the D516V and S531L mutants relative to rifalazil. The low activity of all rifamycins with the H526Y is difficult to understand at present. It seems possible that this mutant may largely perturb the orientation between the ansa-naphthalene core and its binding pocket such that the C3' tail does not make the interactions with the RNAP that are observed for the wild-type RNAP. The generation and characterization of β subunit R540 mutants to probe the interaction of our bxRIFs' with the fork loop 2 is in progress.

These studies have provided a structural framework in which to interpret the enhanced activities of our benzoxazinorifamycins. These results also confirm that our initial approach of extending the bxRIFs to interact with the σ factor is indeed occurring. The structures reported herein have also identified a potentially novel Rif-RNAP interaction involving the β subunit R540 residue that may provide a promising target for elaboration of our bxRIF scaffold. All of this is helping to inform our optimization of the bxRIF scaffold to produce an enhanced anti-tuberculosis agent.

EXPERIMENTAL SECTION

Benzoxazinorifamycins

The benzoxazinorifamycins rifalazil (**bxRIF2a**) and analogues (**bxRIF2b–d**) used in this study were synthesized and analytically characterized as previously described¹³. Their purity was determined to be 95% by hplc.

Expression and purification of wild-type and RIF-resistant mutant *E. coli* RNAPs

The wild-type and RIF-resistant mutant *E. coli* RNAPs for *in vitro* transcription assays were prepared as previously described with minor alterations²². The enzyme lysate was incubated with 2 mL Ni-NTA His-Bind Resin with gentle shaking for 12 h and then the mixture was applied to the column.

In Vitro Transcriptional Activity of *E. coli* RNAPs and Dose Response Curves

Dose response studies with rifalazil (**bxRIF2a**) and analogues (**bxRIF2b–d**) were performed via rolling circle transcription assay as described previously²² to determine the IC₅₀ values. Figure 4 shows an example dose response curve for wild-type *E. coli* RNAP and **bxRIF2b**. Each of the compounds was tested in duplicate (n = 2). The concentration range used for benzoxazinorifamycins (**bxRIF2b–d**) with the wild-type *E. coli* RNAP was 1.56–100 nM, for **bxRIF2a** the range was 3.12–200 nM. The concentration ranges used for the bxRIFs with *E. coli* RNAP (D516V) were as follows: for **bxRIF2a**, 31.2–2000 μM ; for **bxRIF2b**, 0.82–800 μM ; for **bxRIF2c–d**, 0.82–200 μM . The concentration ranges used for the bxRIFs with *E. coli* RNAP (H526Y) were as follows: for **bxRIF2b**, 8.2–2000 μM ; for **bxRIF2a** and **bxRIF2c–d**, 31.2–2000 μM . The concentration ranges used for the bxRIFs with *E. coli* RNAP (S531L) were as follows: for **bxRIF2a**, 31.2–2000 μM ; for **bxRIF2b**, 0.82–200 μM ; for **bxRIF2c**, 2.46–600 μM , and for **bxRIF2d**, 6.55–1600 μM . The final concentration of the wild-type *E. coli* RNAP was 10 nM, whereas the final concentrations of the mutant RNAPs were 100 nM in the reactions.

The core RNAP was incubated with the test compound for 10 min at 37 °C in a 25 μ L reaction volume of 1 $\times$ RNAP reaction buffer (40 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM MgCl₂, 0.01% Triton X-100), DTT (8 mM) and RNase inhibitor (1.12 U/ μ L). The DNA nanocircle template (NC45, 80 nM) was added next and the reaction was initiated by the addition of NTP solution (500 μ M each NTP). The IC₅₀ values were determined via nonlinear regression to a modified four-parameter logistic equation as described previously²². The log IC₅₀ values and their standard errors are reported in STable 1 of Supporting information.

X-ray crystal structure determinations of the *E. coli* RNAP• σ ⁷⁰ holoenzyme and RMP or bxRIF complexes

The method for preparation of *E. coli* RNAP σ ⁷⁰ holoenzyme for the X-ray crystallography study was as described previously¹⁶. RNAP crystals were obtained by using hanging drop vapor diffusion by mixing equal volume of protein solution (~20 mg/ml) and crystallization solution [0.1 M HEPES-HCl (pH 7.0), 0.2 M calcium acetate, ~15 % PEG400, 10 mM DTT] and incubating at 22 °C over the same crystallization solution. For preparing the RNAP•RMP and RNAP•bxRIF complex crystals, the crystals were soaked in crystallization solution containing 30 % PEG400 and 0.1 mM of **RMP** or bxRIF overnight at 22 °C and then flash frozen by liquid nitrogen.

The crystallographic datasets were collected at the Macromolecular Diffraction at the Cornell High Energy Synchrotron Source (MacCHESS) A1 beamline (Cornell University, Ithaca, NY) and the data were processed by HKL2000²³. The *E. coli* RNAP structure¹⁶ was used as an initial model for the rigid body and positional refinement with non-crystallographic symmetry and secondary structure restraints using the programs Phenix²⁴. The resulting maps allowed **RMP** or bxRIF that were not present in the initial search models to be constructed using Coot²⁵. Final coordinates and structure factors were submitted to the PDB with ID listed in Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

RNAP	RNA polymerase
RMP	Rifampin
bxRIF	benzoxazinorifamycin
MTB	<i>Mycobacterium tuberculosis</i>
MDR-MTB	multidrug-resistant strains of MTB
σ finger	σ factor region 3.2 loop
hepatic cytochrome P450	Cyp450

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Figure 1. Structures of rifampin (**RMP**), rifalazil (**bxRIF2a**), and benzoxazinorifamycins (**bxRIF2b–2d**).

Figure 2.

Electron density maps of RMP/bxRIF and molecular details of RNAP•RMP/bxRIF interactions

A) 2Fo-Fc maps of RMP (left), **bxRIF2b** (center) and **bxRIF2c** (right) (cyan color meshes, sigma cutoff = 3). The C3-tail and C3'-tail are highlighted by yellow.

B) Molecular details of the RNAP•RMP/bxRIF interactions. Fork loop 2 (green, residues 533–548, Arg540), positions of Rif resistant mutants (D516, H526 and S531) labeled, beta subunit residues from 1240 to the C-terminus have been removed for clarity, Sigma finger (orange, residues 513–516).

Figure 3.

Model of transcription initiation complex in the presence of **bxRIF2c**

A) Overlay between *E. coli* RNAP-**bxRIF2c** complex and *T. thermophilus* transcription initiation complex.

B) Possible mechanism of C3'-tail dependent inhibition of transcription initiation.

Figure 4.

Dose response curves for wild-type *E. coli* RNAP and **bxRIF2b**

The average (n=2) of each data point was plotted as a function of log [**bxRIF2b**]. The individual data points were fit by nonlinear regression to determine the log (IC₅₀) values (see Supporting information), which were converted to the IC₅₀ values shown in Table 2.

Table 1

Data collection and refinement statistics

Complex	RMP	bxRIF2b	bxRIF2c
PDB code	4KMU	4KN4	4KN7
Data collection			
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Cell dimensions			
<i>a</i> (Å)	184.520	184.675	185.830
<i>b</i> (Å)	203.873	203.975	205.579
<i>c</i> (Å)	307.871	307.910	308.701
Resolution (Å)	30 – 3.75	30 – 3.96	30 – 3.69
Total reflections	437,171	468,938	497,535
Unique reflections	103,271	95,501	118,179
Redundancy	4.2 (2.0) *	4.9 (3.9) *	4.2 (2.6) *
Completeness (%)	90.6 (59.3) *	94.5 (76.7) *	93.1 (87.2) *
<i>I</i> / σ	5.6 (1.4) *	6.2 (1.6) *	7.6 (1.4) *
<i>R</i> _{sym}	0.167 (0.660) *	0.164 (0.757) *	0.115 (0.691) *
Refinement			
Resolution (Å)	30 – 3.85	30 – 3.96	30 – 3.69
<i>R</i> _{work}	0.264	0.251	0.252
<i>R</i> _{free}	0.321	0.314	0.309
R.m.s deviations			
Bond length (Å)	0.002	0.002	0.002
Bond angles (°)	0.65	0.61	0.56

Data sets were collected at MacCHESS-A1, Ithaca, NY

* Highest resolution shells are shown in parenthesis

Table 2

In Vitro RNAP IC₅₀ Values (μM)^a for Rifampin (**RMP**) Rifalazil (**bxRIF2a**) and Analogues (**bxRIF 2b–d**)

RNAP	RMP	bxRIF2a	bxRIF2b	bxRIF2c	bxRIF2d
<i>E. coli</i> WT	0.012 ^c	<0.01	0.057	0.024	0.021
<i>E. coli</i> D516V	233 ^c	204	35	2	41
<i>E. coli</i> H526Y	1130 ^c	299	1148	2000 ^b	1581
<i>E. coli</i> S531L	171 ^c	63	64	92	13
Data for <i>MTB</i> RNAPs					
MTB WT	0.017 ^c	<0.01 ^d	<0.01 ^d	<0.01 ^d	<0.01 ^d
MTB D435V	465 ^c	541 ^d	20 ^d	9 ^d	13 ^d
MTB H445Y	610 ^c	172 ^d	171 ^d	437 ^d	574 ^d
MTB S450L	109 ^c	117 ^d	16 ^d	18 ^d	122 ^d

^a IC₅₀ is the concentration of benzoxazinorifamycin resulting in 50% inhibition of transcription. The log IC₅₀ values and their standard errors are reported in the Supporting informations. Those errors roughly translate into a 15% error in the IC₅₀ values reported here.

^b 50% inhibition of transcription was observed at 2000 μM inhibitor.

^c From Gill, et al. (2011) Tuberculosis 91, 361–369.

^d From Gill, et al. (2012) Journal of Medicinal Chemistry 55(8), 3814–3826.