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Structures of RNA polymerase-antibiotic complexes

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Abstract

Inhibition of bacterial RNA polymerase (RNAP) is an established strategy for antituberculosis therapy and broad-spectrum antibacterial therapy. Crystal structures of RNAP-inhibitor complexes are available for four classes of antibiotics: rifamycins, sorangicin, streptolydigin, and myxopyronin. The structures define three different targets, and three different mechanisms, for inhibition of bacterial RNAP: (1) rifamycins and sorangicin bind near the RNAP active center and block extension of RNA products; (2) streptolydigin interacts with a target that overlaps the RNAP active center and inhibits conformational cycling of the RNAP active center; and (3) myxopyronin interacts with a target remote from the RNAP active center and functions by interfering with opening of the RNAP active-center cleft to permit entry and unwinding of DNA and/or by interfering with interactions between RNAP and the DNA template strand. The structures enable construction of homology models of pathogen RNAP-antibiotic complexes, enable *in silico* screening for new antibacterial agents, and enable rational design of improved antibacterial agents.

Bacterial RNAP as an antibiotic target

Bacterial RNA polymerase (RNAP) is a proven target for broad-spectrum antibacterial therapy and for antituberculosis therapy [1–3]. RNAP is a suitable target for three reasons: (1) RNAP is an essential enzyme (permits efficacy); (2) bacterial RNAP-subunit sequences are highly conserved (permits broad-spectrum activity); (3) bacterial RNAP sequences and eukaryotic RNAP sequences are less highly conserved (permits therapeutic selectivity).

The rifamycin antibacterial agents—rifampin (also known as rifampicin), rifapentine, and rifabutin—function by binding to and inhibiting bacterial RNAP [1–3]. The rifamycins are in clinical use in treatment of Gram-positive and Gram-negative bacterial infections, are first-line antituberculosis agents, and are among the few antituberculosis agents that can kill non-replicating tuberculosis bacteria.

For all major bacterial pathogens, including the tuberculosis pathogen, strains resistant to rifamycins have arisen [1–3]. Resistance to rifamycins involves substitution of residues within

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the rifamycin-binding site on bacterial RNAP, i.e., substitutions that directly decrease rifamycin binding (Fig 1A). In view of the public-health threat posed by rifamycin-resistant bacterial infections, particularly rifamycin-resistant tuberculosis, there is an urgent need for new antibacterial agents that (1) inhibit bacterial RNAP (and thus have the same biochemical effects as rifamycins), but that (2) inhibit bacterial RNAP through binding sites or binding poses that are distinct from the rifamycin binding site and binding poses (and thus that do not share cross-resistance with rifamycins).

RNAP-rifamycin complexes: "Rif/Sor target"

Rifamycins are macrocyclic antibacterial agents of the ansamycin family [4,5]. They comprise a naphthyl moiety, an ansa ring, and, optionally, side chains at positions 3 and/or 4 of the naphthyl moiety. The most important rifamycins are rifampicin, rifapentine, and rifabutin, which contain, respectively, a methyl-piperazinyliminomethyl side chain at position 3, a cyclopentyl-piperazinyliminomethyl side chain at position 3, and a cyclic spiro-piperidyl side chain at positions 3 and 4. The rifamycins form the lynchpin of modern short-term chemotherapy for tuberculosis [1–5]. The introduction of rifamycins permitted a marked reduction in the treatment time for tuberculosis, from 18–24 months to 6–9 months. The ability of rifamycins to accelerate clearance of tuberculosis bacteria from tissues is thought to be due to their higher activity, compared to other anti-tuberculosis agents, against non-replicating tuberculosis bacteria ("persisters").

Structures have been determined of *Thermus aquaticus* RNAP core enzyme (subunit composition $\alpha^I/\alpha^{II}/\beta/\beta'/\omega$; competent for sequence-independent transcription initiation) in complex with rifampicin (3.2 Å resolution; [6]) and of *T. thermophilus* RNAP holoenzyme (subunit composition $\alpha^I/\alpha^{II}/\beta/\beta'/\omega/\sigma$; competent for sequence-specific transcription initiation) in complex with rifapentine and rifabutin (2.5 Å resolution; [7]). The rifamycin binding site is located within the RNAP active-center cleft adjacent to the RNAP active center ("Rif/Sor target"; Fig. 1). The site does not overlap determinants for interaction with DNA or for synthesis of RNA but does overlap determinants for interaction with the nascent RNA product [6,7]. There is essentially complete overlap, and essentially complete steric incompatibility, between the position of a bound rifamycin and the positions of nucleotides n-4, n-3, and n-2 of the nascent RNA product (where n is the 3' nucleotide of the nascent RNA product; [6,8; compare 9]). Consistent with the position of the site, and with the predictions of a simple steric-interference model, rifamycins do not inhibit formation of the catalytically competent RNAP-promoter open complex, and generally do not inhibit synthesis of RNA products up to 2 nt in length, but do inhibit synthesis of RNA products >2–3 nt in length [6,8,10]. It has been proposed that rifamycins, in addition to inhibiting transcription sterically, may also inhibit transcription allosterically by modulating affinity of the RNAP active center for Mg^{2+} [7], but evidence for this proposal has been shown to be unsound [8].

The rifamycins bind within a shallow concave depression formed by residues of the RNAP β subunit (Fig. 2A). The rifamycin naphthyl moiety (atoms C1–C10) contacts β residues 146, 511, 513, 529, 531, 533–534, 568, and 572 (Fig. 2A). The rifamycin ansa moiety (atoms C15–C29) contacts β residues 143, 510–512, 514, 516, 525–526, 564, and 761 (Fig. 2A). [Functional data for RNAP have been obtained primarily from experiments using *Escherichia coli* RNAP as the model system. Therefore, here and elsewhere in the text, residues are numbered as in *E. coli* RNAP.] The rifamycin side chain—the moiety that differs in rifampicin, rifapentine, and rifabutin—makes no significant interactions in the cases of rifampicin and rifapentine [3,7], but makes interactions with the RNAP σ subunit in the case of rifabutin [7]. The additional interactions in the case of rifabutin may account for the higher potency of rifabutin and for differences in the ability of rifabutin to inhibit synthesis of RNA products 3–4 nt in length [7,11]. Sites of substitutions that confer resistance to rifamycins map in, or immediately

adjacent to, the rifamycin binding site on RNAP (Fig. 1A; [1–7,12–16]). Substitutions at three sites— β D516, β H526, and β S531—confer high levels of rifamycin-resistance and little or no loss of fitness, and, accordingly, are especially frequently encountered in clinical isolates of rifamycin-resistant bacteria [16]. The β D516V, β H526D, β H526Y, and β S531L substitutions account for ~75% of clinical isolates of rifamycin-resistant *Mycobacterium tuberculosis* [16].

RNAP-sorangicin complexes: "Rif/Sor target"

Sorangicin (Sor) is a polyketide-derived macrocyclic-polyether produced by the *Myxobacterium Sorangium cellulosum* [17–19]. As with rifamycins, Sor inhibits transcription initiation but does not inhibit transcription elongation [11,18,20]. As with rifamycins, Sor inhibits transcription initiation at a step subsequent to formation of RNAP-promoter open complex, preventing extension of short RNA products (RNA products >2–4 nt in length; [11, 20]).

Rifamycins and Sor exhibit partial cross-resistance: some rifamycin-resistant mutants are cross-resistant to Sor, and all Sor-resistant mutants are cross-resistant to rifamycins (Fig. 1B; [11,20–22; E. Sineva and R.H.E., unpublished]). Substitutions conferring moderate- to high-level resistance to Sor are obtained at β positions 512, 513, 516, 522, 526, 563, and 574 (Fig. 1B; [11,20–22; E. Sineva and R.H.E., unpublished]). It is expected that the ~50% of rifampicin-resistant clinical isolates of *M. tuberculosis* that contain substitutions at other positions within β , including the ~40% that contain substitutions at β residue 531 ([16]), will be fully sensitive to Sor. As such, Sor has potential promise as an antituberculosis agent effective against a subset of rifamycin-resistant bacterial infections.

A structure has been determined of *T. aquaticus* RNAP core enzyme in complex with sorangicin (3.3 Å resolution; [20]). The structure shows that Sor binds to the same site on RNAP as do rifamycins and makes contact with the same residues as do rifamycins ("Rif/Sor target"; Fig. 2B; [20]). The observation that the resistance spectrum of Sor is significantly narrower than the resistance spectrum of rifamycins (Fig. 1B; [11,20–22; E. Sineva and R.H.E., unpublished]) appears to reflect the fact that Sor macrocycle is significantly more flexible than the rifamycin macrocycle (which contain a naphthyl fused ring system), and thus is able to exploit conformational flexibility ("wiggling") and re-orientation ("jiggling") to overcome some potential resistance substitutions [20]. Analogous correlations of narrowness of resistance spectra with flexibility have been observed in other systems [23].

RNAP-streptolydigin complexes: "bridge-helix site/trigger-loop target"

Streptolydigin (Stl) is a polyketide-derived tetramic-acid antibiotic produced by the Actinomycete *Streptomyces griseoflavus* [24,25]. Stl inhibits all major reactions of the RNAP active center, including nucleotide addition in transcription initiation, nucleotide addition in transcription elongation, and pyrophosphorolysis [26]. Rifamycins and Stl exhibit only minimal cross-resistance [11,22,27–30]; in our hands, cross-resistance is observed only for substitutions at a single position: β residue 571 (Fig. 1C; 30; Sineva and R.H.E., unpublished).

Structures have been determined of *T. thermophilus* RNAP holoenzyme in complex with Stl (2.4 Å and 3.0 Å resolution; [30,31]) and of a *T. thermophilus* transcription elongation complex (RNAP core plus DNA and RNA) in complex with the NTP analog AMPcPP and Stl (2.5 Å [32]). Stl binds to a site comprising two key structural elements of the RNAP active center—the "bridge helix" and the "trigger loop"—and to adjacent loops containing β residues 543–548 and 567–571 ("bridge-helix/trigger-loop target"; Fig. 3; [30–32]). The Stl tetramic-acid moiety contacts the bridge helix and trigger loop; the Stl streptolol moiety contacts the bridge helix and β residues 543–548 and 567–569 (Fig. 3).

Stl inhibits transcription by interfering with bridge-helix/trigger-loop conformational cycling that occurs during, and is critical for, nucleotide addition and pyrophosphorolysis [30–32; see 33–35]. Through direct interactions with the bridge helix and trigger loop, Stl traps the bridge helix in a straight (helical) conformational state and traps the trigger loop in an open (unfolded) conformational state [30–32]. As such, Stl appears to prevent both cycling of the bridge helix between straight and bent conformational states and cycling of the trigger loop between open and closed conformational states of the trigger loop.

RNAP-myxopyronin complexes: "switch-region target"

Myxopyronin (Myx) is a polyketide-derived α -pyrone antibiotic produced by the Myxobacterium *Myxococcus fulvus* Mxf50 [36–38]. Myx inhibits transcription initiation by preventing formation of a catalytically competent RNAP-promoter open complex [39,40]. DNA-footprinting results indicate that Myx traps an aberrant RNAP-promoter complex in which the upstream portion, but not the downstream portion, of the transcription-bubble region of the promoter is unwound [40]. Isolation, sequencing, and biochemical and biophysical characterization of Myx-resistant mutants indicates that Myx interacts with the RNAP "switch region," the hinge that mediates opening and closing of the RNAP "clamp" and thereby mediates opening and closing of the RNAP active-center cleft (Fig. 1D; [39,40; see 41–43]). Rifamycins and Myx exhibit absolutely no cross-resistance (Fig 1D; [22,39]).

Structures have been determined of *T. thermophilus* RNAP holoenzyme in complex with Myx A (3.0 Å resolution; [39]; Fig. 4A) and in complex with 8-desmethyl-Myx B (2.7 Å resolution [40,44]; Fig. 4B). The structures establish that Myx binds in the RNAP switch region, interacting with "switch 1" and "switch 2" [39,40]. Myx binds within a nearly completely enclosed, predominantly hydrophobic, binding pocket; the binding pocket is crescent-shaped, has dimensions of ~ 25 Å (measured along the curve of the crescent) $\times \sim 5$ Å $\times \sim 4$ Å, and has a volume of ~ 500 Å³ (Fig. 4; [39,40]). The enclosed, hydrophobic character of the Myx binding pocket, and the location of the Myx binding pocket in a hinge region that mediates opening and closing of an enzyme active-center cleft, are formally reminiscent of properties of the allosteric non-nucleoside drug binding pocket of HIV-1 reverse transcriptase [39; see 23,45]. The Myx α -pyrone ring contacts β' residues 343–345 and 1352 and β residue 1322 (Fig. 4). The Myx dienone sidechain (Myx atoms C15–C24) contacts β' residues 334–338, 1323–1328, and 1352, and β residue 1326 (Fig. 4). The Myx enecarbamate sidechain (Myx atoms C7–14) makes direct or water-mediated contacts with β' residues 343–344, 801–805, and 1348–1351, and β residues 1271–1279 and 1291 (Fig. 4). It is unclear whether the differences in the contact details of the two structures reflect the differences in the Myx derivatives or reflect fitting uncertainties in the structures.

The structures further establish that binding of Myx alters the conformation of a nine-residue segment of switch 2 (β' residues 336–344; [39,40]). This segment of switch 2 previously has been shown to adopt different conformations in open, partly closed, and fully closed conformational states of the RNAP active-center cleft [9,41–43,46–48]. This segment of switch 2 also previously has been shown to contact the DNA template strand in a transcription elongation complex [9,43].

Based on the above structural and functional data, it has been proposed that Myx inhibits transcription by interfering with opening of the RNAP active-center cleft to permit entry and unwinding of DNA ["hinge jamming" mechanism; [39)] and/or by interfering with interactions between RNAP and the unwound DNA template strand [40]. Unpublished biophysical results, involving use of fluorescence resonance energy transfer to measure effects of Myx on opening of the RNAP active-center cleft, provide support for the hinge-jamming mechanism (A. Chakraborty, D. Wang, Y. Korlann, S. Weiss, and R.H.E., unpublished data)].

Genetic results, involving analysis of cross-resistance patterns, and biochemical results show that two additional antibiotics function through the same switch-region target and same mechanism as Myx [40]: i.e., the structurally related α -pyrone antibiotic coralloporynin (Cor; [50,51] and the structurally unrelated macrocyclic-lactone antibiotic ripostatin (Rip; [52,53]).

Homology models of pathogen RNAP-antibiotic complexes

The available resolution crystal structures of bacterial RNAP were obtained using *Thermus* sp. RNAP, which is only distantly related to Gram-positive and Gram-negative bacterial pathogen RNAP [6,7,9,20,39,40,46–49]. In order to enable consideration of RNAP-antibiotic interactions for a bacterial pathogen RNAP, we have constructed and analyzed homology models of *M. tuberculosis* RNAP-antibiotic complexes (Fig. 5).

Our analysis predicts that there are three differences in RNAP-antibiotic contact residues between the *Thermus* sp. RNAP-Myx complex and the *M. tuberculosis* RNAP-Myx complex (Figs. 4A, [5]): (1) a valine residue that makes van der Waals interactions with the Myx enecarbamate sidechain is replaced by a cysteine residue (β V1037 in *T. thermophilus*; β C1073 in *M. tuberculosis*); (2) a glutamic acid residue that makes a H-bond with the Myx enecarbamate—a H-bond requiring protonation of the glutamic acid carboxylate [39]—is replaced by a glutamine residue (β E1041 in *T. thermophilus*; β Q1077 in *M. tuberculosis*); and (3) a histidine residue that makes van der Waals interactions with the terminal atom of the Myx enecarbamate is replaced by an aspartic acid residue (β' H1103 in *T. thermophilus*; β' D882 in *M. tuberculosis*).

The predicted presence of a cysteine residue within the *M. tuberculosis* RNAP binding site for Myx (lower yellow circles in Fig. 5) suggests an avenue for structure-based design of Myx analogs with specifically increased potency against *M. tuberculosis* RNAP: namely, to incorporate into the Myx enecarbamate sidechain reactive functionality the ability to form a reversible or irreversible covalent bond with the cysteine residue, potentially yielding high-potency, low-off-rate, reversible covalent inhibitors or high-potency, irreversible covalent inhibitors.

The predicted presence of an interfacial water molecule within both *Thermus* sp. RNAP and the *M. tuberculosis* RNAP binding sites for Myx (Fig 4; upper yellow circles in Fig. 5), suggests an avenue for structure-based design of Myx analogs with generally increased potency against a broad spectrum of bacterial RNAP: namely, to incorporate into the Myx enecarbamate sidechain functionality the ability to mimic the interfacial water molecule, thereby negating the requirement for presumably unfavorable recruitment and immobilization of a water molecule.

The homology models of *M. tuberculosis* RNAP-antibiotic complexes also enable virtual, *in silico*, screening for novel small-molecule inhibitors of *M. tuberculosis* RNAP. Virtual screening efforts are in progress.

Conclusions

Structural and functional data define three different targets, and three different mechanisms, for inhibition of bacterial RNAP (Fig. 1). The targets do not overlap, or only minimally overlap, and thus inhibitors that function through one target exhibit no, or only minimal, cross-resistance with inhibitors that function through other targets (Fig. 1). Crystal structures and homology models of target-inhibitor complexes enable structure-based optimization of inhibitors and virtual screening for new inhibitors (Figs. 2–5). One of the targets, the "Rif/Sor target," has been exploited to date in antituberculosis and broad-spectrum antibacterial therapy. The classical inhibitors that function through this target, the rifamycins, have been the subject of

drug-development efforts spanning five decades and arguably have limited potential for further optimization [4,5]. We suggest that alternative inhibitors that function through this target but exhibit narrower resistance spectra, such as Sor, warrant attention as lead compounds for new therapeutic agents effective against subsets of rifamycin-resistant bacterial infections.

The other two targets, the "bridge-helix/trigger-loop target" and the "switch-region target," have not been exploited to date in antituberculosis and broad-spectrum antibacterial therapy. These targets, especially the switch-region target, offer significant promise. For the switch-region target, three different inhibitors have been identified: the α -pyrones Myx and Cor, and the macrocyclic lactone Rip [39,40]. We note that Myx exhibits no cross-resistance with rifamycins [22,39], exhibits high antibacterial potency [36,38,39], exhibits low toxicity [36], and is synthetically tractable [38,44]. We suggest that Myx warrants close attention as a lead compound for new therapeutic agents effective against rifamycin-resistant bacterial infections.

Methods

The homology model was constructed starting from the crystal structure of the *T. thermophilus* RNAP-Myx complex [39] using Modeller9v4 [54]. Multiple-sequence alignments were prepared using sequences of RNAP subunits from *Thermus-Deinococcus*-clade, Gram-positive, and Gram-negative bacterial species. Structure-sequence alignments were edited to place deletions and insertions in loop regions and to create compact folded domains. A 24-residue species-specific sequence insert in *M. tuberculosis* RNAP β subunit was not modeled (β residues 944 to 968). Modeller was run for multiple cycles, with hydrogens and heteroatoms included and with symmetry imposed between α^I and α^{II} subunits. The model with the lowest DOPE score [55] is presented.

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promoter open complex. The results indicate that Myx traps an RNAP-promoter complex in which the upstream portion, but not the downstream portion, of the transcription-bubble region of the promoter is unwound. The authors propose that Myx inhibits RNAP by interfering with interactions between RNAP and the downstream portion of the transcription-bubble region of the promoter.

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Figure 1. Targets of small-molecule inhibitors of RNAP

(A) Target of rifamycins (Rif; [6,7;12–16]). (B) Target of sorangicin (Sor; [11,20; E. Sineva and R.H.E., unpublished]). (C) Target of streptolydigin (Stl; [27–32]). (D) Target of myxopyronin (Myx; [39,40]). Each panel shows two orthogonal views of RNAP. Red, sites of substitutions conferring resistance to the specified inhibitor; blue, sites of substitutions conferring resistance to rifamycins; violet sphere, active-center Mg^{2+} .

Figure 2. Structural basis of transcription inhibition by rifamycins and sorangicin: "Rif/Sor target."

(A) Crystal structure of *T. aquaticus* RNAP-rifampin complex (PDB 1YNN; [6]). (B) Crystal structure of *T. aquaticus* RNAP-sorangicin complex (PDB 1YNJ; [20]). Left subpanels: RNAP-inhibitor contacts (stereoview). Gray, RNAP backbone (ribbon representation) and RNAP sidechain carbon atoms (stick representation); green, rifampin carbon atoms; red, oxygen atoms; blue, nitrogen atoms. Dashed lines, H-bonds. Right subpanels: schematic summary of RNAP-inhibitor contacts. Red dashed lines, H-bonds. Blue arcs, van der Waals interactions. RNAP residues are numbered both as in the crystal structure of *Thermus sp.* RNAP and, in parentheses, as in *E. coli* RNAP.

Streptolydigin

Figure 3. Structural basis of transcription inhibition by streptolydigin: "bridge-helix/trigger-loop target."

Crystal structure of *T. thermophilus* RNAP-streptolydigin complex (PDB 1ZYR [30]; see also [31,32]). Left and right subpanels and residue numbering as in Figure 2.

Figure 4. Structural basis of transcription inhibition by myxopyronin: "switch-region target." (A) Crystal structure of complex of *T. thermophilus* RNAP and myxopyronin A (PDB 3DXJ [39]). (B) Crystal structure of complex of *T. thermophilus* RNAP and 8-desmethyl-myxopyronin B (PDB 3EQL [40]). As compared to myxopyronin A, 8-desmethyl-myxopyronin B lacks a methyl group at the start of its enecarbamate sidechain (right sidechain) and contains one additional carbon atom at the end of its dienone sidechain (left sidechain). Left and right subpanels and residue numbering as in Figure 2. "W," in panel (A), interfacial water molecule.

Figure 5. Homology model of the *M. tuberculosis* RNAP-Myx complex
Left and right subpanels as in Figure 2. Bold labels, RNAP residues that differ in the homology model of the *M. tuberculosis* complex vs. in the crystal structure of the *T. thermophilus* complex. “W,” interfacial water molecule present in the homology model of the *M. tuberculosis* complex and in the crystal structure of the *T. thermophilus* complex. RNAP residues are numbered both as in *M. tuberculosis* RNAP and, in parentheses, as in *E. coli* RNAP. Structural features that potentially can be exploited for design and synthesis of more potent inhibitors of *M. tuberculosis* RNAP are highlighted in yellow: i.e., the binding-site cysteine and the binding-site interfacial water molecule.