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Bacterial Transcription as a Target for Antibacterial Drug Development

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SUMMARY

Transcription, the first step of gene expression, is carried out by the enzyme RNA polymerase (RNAP) and is regulated through interaction with a series of protein transcription factors. RNAP and its associated transcription factors are highly conserved across the bacterial domain and represent excellent targets for broad-spectrum antibacterial agent discovery. Despite the numerous antibiotics on the market, there are only two series currently approved that target transcription. The determination of the three-dimensional structures of RNAP and transcription complexes at high resolution over the last 15 years has led to renewed interest in targeting this essential process for antibiotic development by utilizing rational structure-based approaches. In this review, we describe the inhibition of the bacterial transcription process with respect to structural studies of RNAP, highlight recent progress toward the discovery of novel transcription inhibitors, and suggest additional potential antibacterial targets for rational drug design.

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INTRODUCTION

Bacteria are prokaryotic microorganisms representing one of the three domains of life (1). While most bacteria occupy an incredibly diverse range of ecological niches, a small proportion can cause disease (2, 3). Due to the rate at which these pathogens are acquiring resistance to antibiotics, the very real possibility that we will not be able to effectively treat many infections is fast becoming reality (4, 5). A recent report projects that if there is no concerted effort to discover and develop new antibiotics, by 2050 there will be >10,000,000 deaths per year associated with antibiotic-resistant infections, with an associated cost to the global economy of ~$1 trillion (6).

Prior to the clinical development of sulfonamides and penicillin in the 1930s and 1940s, bacterial infections were the primary cause of death for children and working-age adults. This is rare now, and few people remain who can still recall life before access to effective antibiotics. Since the 1960s, antibiotic research and development by major pharmaceutical companies have dropped precipitously (7), and there has been complacency within the medical profession as well as the general public based on the assumption that we can effectively control and treat all microbial infections. The blasé overuse of these precious drugs has contributed to the rate at which antibiotic resistance has developed within hospital environments as well as the community.

Antibiotic resistance represents a serious and growing problem in the treatment of bacterial infections (8). The dramatic slowdown in development of new antibacterial agents has coincided with an alarming increase in the number of resistant, multiresistant, and even totally antibiotic-resistant infections. The new compounds released to market are almost all derivatives of existing classes, and consequently cross-resistance is often preexisting within the microbiome (9). Resistance is rapidly acquired by spontaneous or induced mutation and horizontal gene transfer from resistant species. Decreased membrane permeability, increased efflux capacity, enzymatic inactivation, and direct mutation of the binding sites of drug targets are the major causes of antibiotic resistance (10–14). Consequently, structure-function-based studies on known and potential targets essential to bacterial viability would help in optimizing current antibiotics and for the rational design of new antibacterial agents. Most clinically approved antibiotics target bacterial cell wall growth/integrity, translation, and DNA replication/segregation, while transcription appears to be an underutilized target.

Transcription is the process by which RNA is synthesized from its template DNA by the enzyme RNA polymerase (RNAP) (15). So far there are only two antibiotics targeting bacterial RNAP on the market: the rifamycin series (16) and fidaxomicin/lipiarmicin (17). A transcription factor Rho inhibitor, bicyclomycin, has also been commercialized, mainly for use as a growth promoter in animal feedstock (18). Nevertheless, bacterial transcription represents an excellent target for novel antibacterial development for the following reasons: (i) transcription is an essential process for cell viability; (ii) bacterial RNAP and its associated transcription factors are highly conserved, permitting the potential development of broad-spectrum antitranscriptional antibiotics (19); (iii) eukaryotic RNAP is not similar to its bacterial homolog at the sequence level (barring the active site), which suggests low potential cytotoxicity (20); (iv) numerous high-resolution structures are available, enabling structure-based drug design (21); and (v) the essential RNAP-associated transcription factors are not conserved between bacterial and eukaryotic cells, providing the opportunity for development of compounds targeting RNAP-transcription factor interactions (22).

RNAP STRUCTURE AND FUNCTION

Overview of the Transcription Cycle

There are three main sequential steps in the transcription cycle: promoter binding/initiation, RNA chain elongation, and termination. For transcription initiation to occur, the bacterial RNAP core must associate with an initiation protein factor, σ, forming a holoenzyme, which is competent for specific binding to the promoter regions on DNA (23). After promoter recognition, ~14 bp of DNA is melted upstream of the transcription start site to form an open promoter complex (24). The template strand, including the transcription start site, is directed toward the active site of RNAP, where RNA synthesis is initiated (21). Several rounds of abortive initiation are likely to occur, generating short transcripts 2 to 9 nucleotides (nt) in length (25).

Once about 12 nucleotides of RNA have been synthesized, σ factor is released, while RNAP escapes from the promoter and undergoes a significant conformational change resulting in the formation of a stable transcription elongation complex (EC) (21, 26). Actively transcribing EC is stably associated with template DNA and RNA, adding one nucleotide (NMP) at a time to the growing transcript. It is highly processive, transcribing at 30 to 100 nt/s along the DNA template *in vivo* (27). Once an intrinsic or factor-dependent transcription termination signal is reached, RNAP core is released from the template DNA, allowing initiation of another round of transcription (28, 29).

Bacterial RNAP

In bacteria there is only one RNAP responsible for the transcription of all classes of RNA (30), and the ~350-kDa bacterial RNAP core enzyme (subunit composition, $\alpha_2\beta\beta'\omega$) consists of five subunits: two large subunits (β and β′), two α subunits, and an accessory subunit (ε) (Fig. 1A) (31–34). In the firmicutes, there is a sixth subunit, ε, of unknown function (33). Due to recent advances in structural biology, high-resolution structures of RNAP have been solved in different forms (see reference 35 for a comprehensive review of available structures), including core RNAP (36), holoenzyme (37, 38), and initiation (39, 40) and elongation (41) complexes.

Bacterial RNAP core enzyme has an overall "crab claw" structure, being 150 Å long, 110 Å wide, and 115 Å tall (36). The two α subunits lie at the back of the enzyme, and the two large subunits β and β′ interact extensively, each forming one "pincer" of the claw (36). A number of important structural features of bacterial RNAP can be identified from the core and holoenzyme homology models (Fig. 1). The primary channel formed by the cleft between β and β′ is about 27 Å wide, which is adequate to accommodate a double-stranded DNA (dsDNA) template, allowing RNA synthesis to occur (36). The switch region is located at the top of the RNAP clamp (Fig. 1C) and which mediates opening and closing of the RNAP clamp to allow DNA loading at the active site (15). The downstream face of the active center is formed by the bridge helix (BH) (also

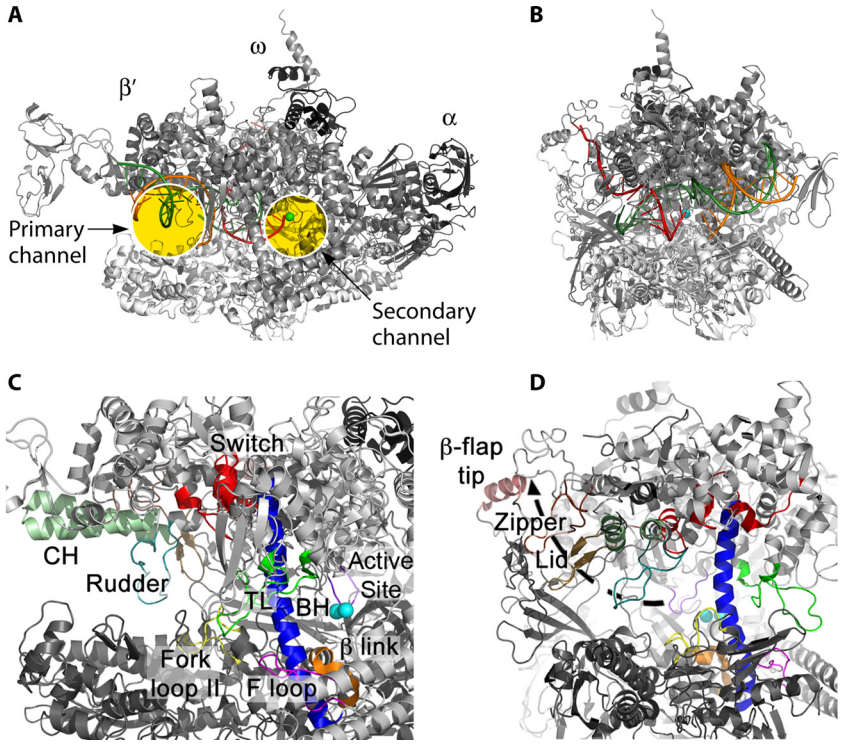

FIG 1 RNAP structure and functional motifs. In all panels the structure of the *Thermus thermophilus* RNAP elongation complex was used. (A and B) Side and front views, respectively. α subunit, dark gray; β subunit, light gray; β′ subunit, medium gray; ω subunit, black. The DNA primary channel and secondary channel for NTP entry are highlighted with yellow circles. Template-strand DNA, green; nontemplate-strand DNA, orange; RNA, red. The active-site magnesium ion is shown as a cyan sphere. (C and D) Enlarged views from panels A and B, respectively, with functional regions colored and labeled. CH region, pale green; rudder, cyan; switch, red; active-site residues (NADFDGD), purple; β link, orange; F loop, magenta, fork loop II, yellow; bridge helix (BH), blue; trigger loop (TL), green; lid, bronze; zipper, brown; β flap tip, salmon. The approximate path of RNA through the RNA exit channel is indicated by the arrow in panel D. Structure images were prepared using PDB files 1IW7 and 2O5I in PyMol v1.7.4 (Schrödinger, LLC).

referred to as the F helix) and the trigger loop (TL) (or G loop). The BH extends directly across the active channel and splits it into two separate channels, the downstream DNA (active) channel and the secondary channel (Fig. 1A) (36, 42). The 10- to 12-Å-wide secondary channel is not large enough for double-stranded nucleic acids and is proposed to be involved in nucleoside triphosphate (NTP) entry (36). On the upstream side of RNAP, the β G region (β-flap) and the rest of the upstream boundary of the active site together form an RNA exit channel, through which the newly synthesized RNA transcript is threaded (Fig. 1B) (43). The α-helical motif at the top of the β-flap, called the β-flap tip, is functionally important, as it is the binding site for σ region 4 and the essential elongation factor NusA (Fig. 1D) (44). Although there are many contacts between core RNAP and σ factor, a solvent-exposed clamp-helix (CH) region of the RNAP β′ subunit (Fig. 1C) represents the major site for σ interaction (38).

Human RNAP

In the nuclei of eukaryotic cells there are three distinct multisubunit RNAPs: RNAP I transcribes rRNA precursors, RNAP II is responsible for the synthesis of mRNA and certain small nuclear RNAs, and RNAP III performs the synthesis of 5S rRNA and tRNA (45). The overall architecture of RNAP is similar and the sequence of the active site highly conserved in all living organisms, so drugs targeting this region may have cross activity (30, 46, 47). Indeed, α-amanitin, an anticancer drug that traps the bridge helix and trigger loop in RNAP II, preventing nucleotide addition, is also a bactericidal agent (48, 49). Consequently, cytotoxicity could be a considerable issue when adopting a strategy of drug design based on the conserved active-site region.

COMPOUNDS THAT INHIBIT TRANSCRIPTION

Considerable effort has been spent in the discovery of inhibitors of bacterial transcription since the isolation of rifampin (RIF) (17), and several reviews, listed in Table 1, have summarized the research results from different perspectives. Representative structures for compounds with specific activities outlined in this review are shown in Fig. 2 and will be referred to in the appropriate sections below. These molecules bind to a diverse range of sites on RNAP and inhibit transcription through binding to sites in and around the primary channel, as well as targeting the secondary channel and switch regions. The activity or interaction with RNAP of transcription factors is also a source of established (bicyclomycin) (Fig. 2) or emerging lead compounds.

Since the mid-20th century, most of the inhibitors of bacterial transcription have been isolated from microorganisms, except for synthetic squaramides, ureidothiophene, CBR703, and the SB series of compounds (Fig. 2) (50, 51). So far, however, only rifamycins and fidaxomicin/lipiarmycin (Fig. 2) have been approved for clinical use, and no new transcription inhibitor compounds are in clinical trials. The high rate of acquisition of bacterial resistance

TABLE 1 Past reviews on inhibitors of bacterial transcription

Author(s)	Yr	Content summary (reference)
Darst	2004	Described four inhibitors, i.e., two classic molecules, rifampin and streptolydigin, and two new inhibitors, microcin J25 and CBR703; their binding sites on bacterial RNAP structures were also examined (229)
Villain-Guillot et al.	2007	Summarized all of the inhibitors known to date; this complete review illustrated the chemical structures of the inhibitors and their binding sites on RNAP (230)
Mariani and Maffioli	2009	A comprehensive review of all the inhibitors known to date; the progress of chemical modification of inhibitors and their activity against bacteria, as well as clinical applications, were detailed (50)
Ho et al.	2009	Focused on the structure of RNAP and inhibitor complexes; four cocrystal structures of *Thermus aquaticus* RNAP with rifampin and sorangicin and of *Thermus thermophilus* RNAP with streptolydigin, and myxopyronin were presented; the authors also constructed a homology model of *Mycobacterium tuberculosis* RNAP in complex with myxopyronin (231)
Srivastava et al.	2011	A comprehensive review of the inhibitors myxopyronin, corallopyronin, ripostatin, and lipiarmycin, which target the switch region; the sequence of this region is highly conserved in bacteria but not in eukaryotes, providing a new avenue for the rational development of a potent new class of broad-spectrum antibiotics (136)

prevented the further development of many compounds, while myxopyronins were sequestered by serum albumin, preventing them from being free to access their target, CBR703 was proven to be unattractive due to cytotoxicity, and the SB series were found to have a nonspecific mode of action (52–54).

In the following sections, we describe binding sites and modes of action of known inhibitors and envision some potential targets for structure-based drug design.

PRIMARY CHANNEL INHIBITORS

The primary channel is a large cleft formed by the two large subunits βand β′ and is highly conserved among RNAPs (31, 55). The cleft has an overall positive charge, while the surface of RNAP is mostly negatively charged. Specifically, β regions H and I interact with β′ region D, which positions the absolutely conserved -NAD FDGD- β′ D motif at the base of the cleft (Fig. 1C). The Mg^{2+} ion is chelated by the aspartate (D) residues from the NADFDGD motif at the base of the channel, catalyzes nucleotide addition to the growing RNA transcript, and may also be involved in DNA melting (36, 56).

During elongation, a transcription bubble is formed when 10 to 12 bp of downstream duplex DNA yet to be transcribed enters the active channel (21). The duplex DNA is then separated within the active channel, and the newly synthesized RNA forms a 9- to 10-bp hybrid with the template DNA strand, with the 3′ end of the RNA positioned within the active site near the center of the enzyme (Fig. 1B). The nontemplate DNA strand is rewound with the template DNA on the upstream side, and the length of the RNA-DNA hybrid and DNA bubble stays approximately constant as transcription proceeds (57). The four-step nucleotide addition cycle starts with the binding of an NTP complementary to the template DNA at the insertion site (i+1) in the active center. Initially, the TL is in its unfolded conformation (Fig. 1C and D), and the NTP enters via the secondary channel to form a preinsertion complex. Upon folding of the TL (see below), an insertion complex forms. Catalyzed by the active-site-bound Mg^{2+} ions, the 3′-OH group of the growing RNA chain attacks the α-phosphate of the incoming NTP, resulting in one nucleotide addition to the 3′ end of the RNA transcript and release of pyrophosphate (PP_i). The newly added RNA nucleotide is then translocated to the product site (i site), exposing the next template base in the i+1 site for the incoming NTP (39, 58, 59). The newly synthesized RNA transcript moves away from the active site through the RNA exit channel formed by the β subunit flap domain, as well as the β′ lid and zipper domains (Fig. 1C and D).

Rifamycins

Rifamycins (Fig. 2) were the first group of antimicrobials targeting RNAP, discovered in the last century from metabolites of *Amycolatopsis mediterranei* (60). The first drug of this class was rifamycin SV, which was used only intravenously and topically in clinics (61). Rifampin (RIF), synthesized from rifamycin SV, is orally effective and has broad-spectrum activity against Gram-positive, Gram-negative, and especially mycobacterial pathogens (62). As it is the key element in combinatorial antitubercular chemotherapy, rigorous studies on the chemical derivation of RIF led to the development of two analogues, rifabutin (RBT) and rifapentine (RPT), possessing improved pharmacological characteristics (63, 64). Rifaximin was also derived from RIF and was approved for the treatment of gastrointestinal disorders and hepatic encephalopathy (64, 65).

Cocrystal structures of different rifampins with *Thermus aquaticus* RNAP (66) and *Thermus thermophilus* holoenzyme (67) indicated that they bind within the cleft close to the active center of RNAP, which sterically hinders growth of the RNA product rather than DNA binding or RNA synthesis sites (Fig. 3A and B). Biochemical experiments also showed that RIF did not inhibit the formation of the RNAP-promoter open complex but could stall the formation of RNA products that are >3 nt (66–68). Structurally, the rifamycin naphthyl group interacts with *Escherichia coli* β subunit residues 146, 511, 513, 529, 531, 533, 534, 568, and 572, and a recent cocrystal structure of *E. coli* RNAP holoenzyme and RIF showed that it also interacts with β fork loop II, especially residue 540 (69), while the remaining functional groups bind to residues 143, 510, 511, 512, 514, 516, 525, 526, 564, and 761 (Fig. 3B). Artsimovitch et al. demonstrated that RIF and RPT bind to RNAP in a similar fashion despite their different side chains, whereas RBT can additionally interact with the σ subunit (67) (Fig. 3C).

The antimicrobial activity of RIF proved that the region adjacent to the active center of bacterial RNAP was a valid target for discovery of drugs acting against transcription. However, the ease with which resistance to RIF is gained by mutation at multiple sites illustrates that there are significant problems with this site for the development of antimicrobials with long therapeutic lives. While alteration of the RIF binding site does have implications for cell

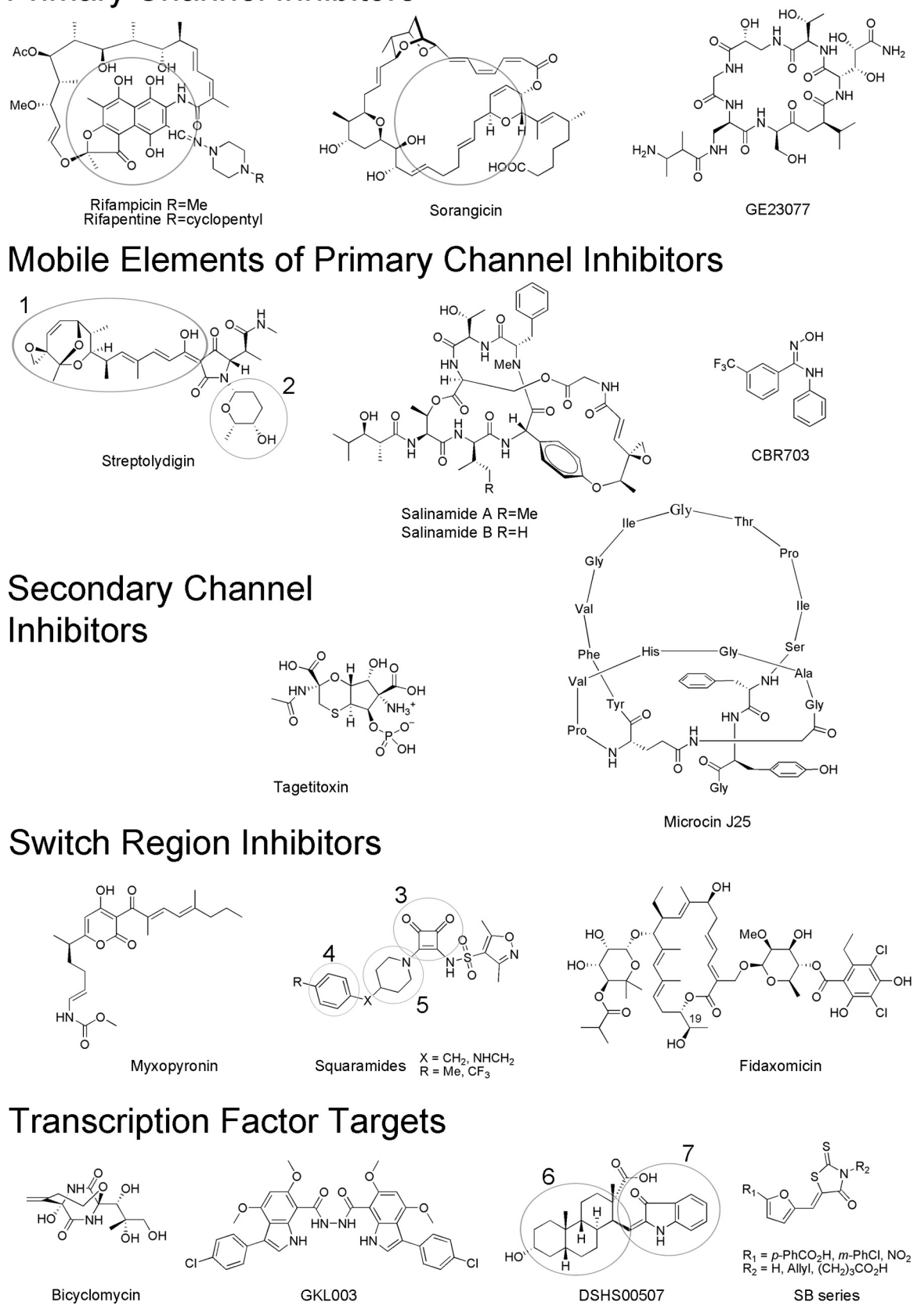

FIG 2 Compounds that inhibit bacterial transcription. The compounds featured in this review are shown as chemical structures, illustrating the diversity in size and complexity of RNAP inhibitors. The compounds are arranged with respect to their targets. The naphthyl group of rifampin in the primary-channel inhibitors is circled, with the equivalent region of sorangicin also circled. Numbered circles: streptolol (1) and monosaccharide (2) moieties of streptolydigin, squarate ring (3), benzylamine ring (4) and piperidine (5) groups of squaramides, and steroid-like carboxylic acid (6) and indolone (7) groups of DSHS0057. See the text for further details.

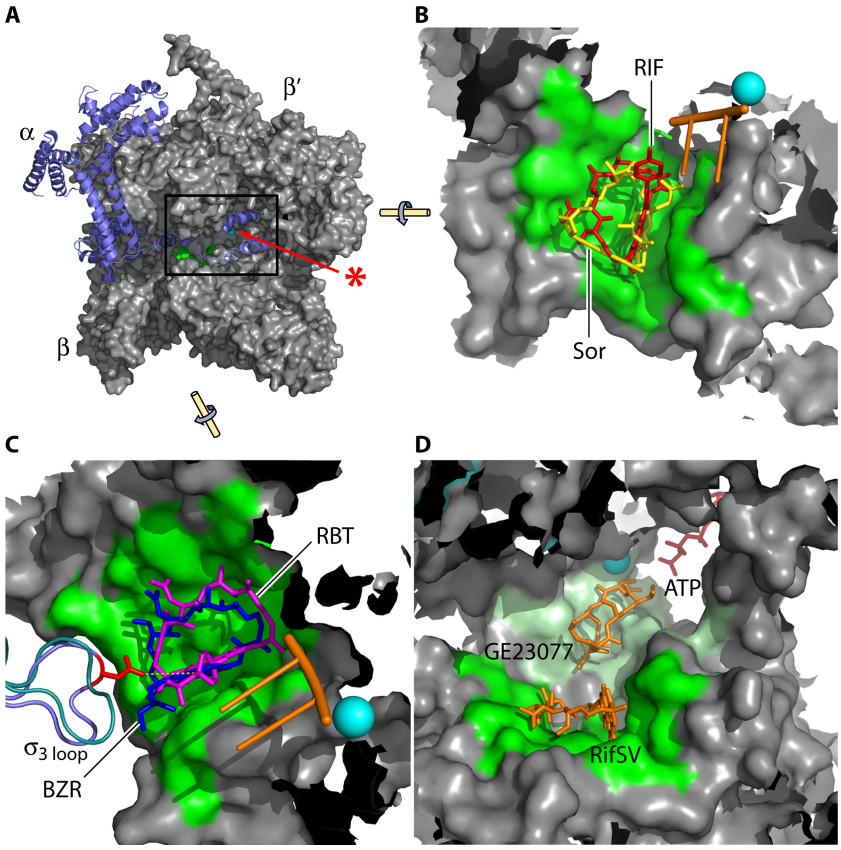

FIG 3 Antibiotics that bind close to the active site. (A) Space-filled structure of *T. thermophilus* RNAP holoenzyme with core subunits (α, β, β′, and ω) in gray and initiation factor σ shown as a slate blue cartoon. The catalytic Mg^{2+} ion is shown as a cyan sphere, and its location is indicated by the red arrow and star. The rifamycin/sorangicin binding site is shown in green, and the area used in panels B and C is boxed. The approximate rotations for regions shown in panels B and C are indicated with arrows. In all panels the catalytic Mg^{2+} ion is shown as a cyan sphere, and in panels B and C an initiating RNA dinucleotide (orange) is shown adjacent to the active site. (B) Rifampin (RIF) (red) and sorangicin (SOR) (yellow) have overlapping binding sites. (C) Rifabutin (RBT) (magenta) has improved binding to holoenzyme through interaction with D513 (red stick) located on a region of the σ factor called the σ_3 loop. When benzoxazinorifamycin 2b (BZR) (blue) is bound, the C-3′ tail causes a distortion in the σ_3 loop (teal), preventing it interacting with the template strand of DNA. (D) GE23077 (orange) binds in the i and i+1 sites, inhibiting RNA synthesis. The binding site is shown in pale green. ATP entering RNAP via the secondary channel is shown in red. Rifamycin SV (RifSV) (orange) binds near to GE23077, and the two molecules can be covalently linked to form a compound with activity against rifampin-resistant RNAP. Structure images were prepared using PDB files 1IW7, 1YNJ, 1YNN, 2A68, 2O5J, 4G7O, 4KN4, and 4OIR in PyMol v1.7.4 (Schrödinger, LLC).

fitness, a whole-genome sequencing study of RIF-resistant *Mycobacterium tuberculosis* strains showed that alterations of residues α 187 and β′ 434, 483, 485, 491, and 698 were compensatory (70). A separate study with *Staphylococcus aureus* also showed that alterations at β residues 471 and 481 gave intermediate levels of resistance to RIF with no loss of cell fitness (71). Efforts to minimize RIF drug resistance include combinatorial therapy (with isoniazid, ethambutol, and pyrazinamide for tuberculosis), short-term use in clinical practice, and the development of new delivery mechanisms such as nanoparticles (72) and metal conjugation (73). Furthermore, chemical derivatives have also been developed, such as the benzoxazinorifamycins, which demonstrate superior affinity to RIF-resistant *M. tuberculosis* strains and reduced adverse side effects caused by cytochrome P450 induction (69, 74–76). Cocrystal structures showed that the interactions of benzoxazinorifamycins with the RNAP β fork loop II and the σ_3 loop may occur and may explain the improved biological activity (67, 69). Careful inspection of the structure of *E. coli* RNAP in complex with benzoxazinorifamycins suggests that the C-3′ tail may prevent σ_3 loop interaction with the −3 and −4 bases of the template DNA, which is required for binding the initiating NTP at the i+1 site (69). Residue D513 of the σ_3 loop is able to interact with the ansa ring of RBT (Fig. 3C, dotted line) (67), whereas benzoxazinorifamycin 2b (BZR) causes a distortion of the σ_3 loop that prevents template DNA interaction (Fig. 3C, teal ribbon) (69). The improved properties of the benzoxazinorifamycins has led to one derivative, Rifalazil, entering, but failing, phase II trials for the treatment of *Chlamydia* infection (77, 78).

Sorangicin

Sorangicin is a macrocyclic antibiotic isolated from *Sorangium cellulosum* (79). A cocrystal structure of sorangicin with *T. aquaticus* RNAP demonstrated that this molecule bound to exactly the same site as RIF adjacent to the active center and shared similar interactions with the corresponding residues (Fig. 3B) (80). Thus, the same mechanism of action, biological activity, and cross-resistance with RIF mutants can be expected (81).

However, despite being structurally related to rifamycins,

sorangicin comprises a more flexible skeleton than RIF, which contains a naphthyl moiety (equivalent regions are circled in Fig. 2), suggesting that the former might be able to adapt better than RIF to conformational change and mutations in RNAP (80). Studies on RIF and sorangicin against resistant mutant *M. tuberculosis* strains revealed that a narrower range of mutant strains were resistant to sorangicin than to RIF and proved that RIF was more sensitive to the change of shape of the binding pocket due to its rigid structure, while sorangicin displayed more conformational flexibility, as demonstrated by molecular dynamics simulations (80, 82).

Extensive studies have been performed on the structure-activity relationship of rifampin and sorangicin, which could help us to understand how the region around the active site of RNAP could be exploited as a valid drug target (50, 66, 67). Using information based on the RIF-RNAP cocrystal structure, five amino acids were identified, corresponding to *T. thermophilus* RNAP β subunit residues Q390, F394, R405, Q567, and Q633, and used in fragment-based drug design. Two compounds containing four functional groups designed for binding to residues Q390, F394, R405, and Q567 displayed good *in vitro* activities of 70 and 62 μM, respectively (83). However, R405 is not conserved in *E. coli* (T525), and these compounds were subsequently also shown to inhibit *E. coli* β-galactosidase but not other *E. coli* or mammalian enzymes (84).

GE23077

GE23077 (Fig. 2) was isolated from an *Actinomadura* sp. in 2004 and is a relatively new macrocyclic heptapeptide antibiotic (85). The cocrystal structure of *T. thermophilus* RNAP with GE23077 has recently been published, which illustrated that the compound bound to the i and i+1 sites of the active center, adjacent to the catalytic Mg^{2+} ion and close to the RIF binding site (Fig. 3D) (86). More precisely, it was demonstrated using saturation mutagenesis that *E. coli* β subunit residues P564, E565, G566, N568, R678, M681, N684, M685, Q688, K1065, K1073, and H1237 and β′ residues D462, T786, and A787 were involved in binding with GE23077.

GE23077 alone is not a particularly good antibiotic, as it has poor membrane permeativity due to its hydrophilic nature and shows good antibacterial activity only when used against Δ*tolC* strains or in combination with a membrane-perturbing agent (85, 87). Chemical derivation does not result in a great improvement of antibiotic activity (88). However, the target-dependent resistance spectrum for GE23077 was much smaller than those for RIF and other RNAP inhibitors, with resistant substitutions being obtained at only four residues (E565, G566, M681, and N684) in the β subunit (86), suggesting that the i and i+1 sites at the active center might be a good target for drug discovery.

By combining GE23077 and rifamycin SV, a bipartite molecule which bound to adjacent sites near the active site was created (Fig. 3D) (86). This compound demonstrated excellent activity against GE23077 or RIF-resistant RNAP, showing that the RIF binding pocket and GE23077 binding i and i+1 sites could be considered for use as a combined target for drug design.

MOBILE ELEMENTS OF THE PRIMARY CHANNEL

The activity of RNAP is highly regulated during all stages of the transcription cycle, and a great proportion of the regulation is mediated via mobile structural elements adjacent to the active site. The conserved 37-amino-acid (aa) bridge helix (BH) in the β′ subunit of RNAP spans the DNA binding cleft downstream of the catalytic center and makes tight contacts with the mobile β′ trigger loop (TL) (Fig. 1C and 4A and B). The TL and BH are involved in the regulation of substrate loading and nucleotide addition by switching the DNA binding cleft between open and closed conformations. The open conformation (Fig. 4A) allows the translocation of the 3′ end of the RNA transcript and release of the pyrophosphate by-product (89). Bending of the BH toward the DNA/RNA hybrid has been proposed to facilitate RNA translocation (90, 91). The closed conformation (Fig. 4B) involves refolding of the TL into a three-helix bundle with the BH into the catalytic conformation (59). The three-helix bundle aligns the NTP substrate with the active site, as well as shifting the position of the 3′ end of the RNA and catalytic Mg^{2+} ions (92). Residues in the amino-terminal extension of the BH, the F-loop, and the β-link (Fig. 1C) contact the TL directly and stabilize its refolding (93).

Streptolydigin

Streptolydigin (STL) was isolated from *Streptomyces lydicus* cultures in the last century, even earlier than the discovery of rifamycins (94–96). This molecule is a derivative of tetramic acid containing an acyl side chain (streptolol) and a monosaccharide (Fig. 2, circles 1 and 2, respectively), and it displayed broad-spectrum antibacterial activity by inhibiting bacterial transcription initiation, elongation, and pyrophosphorolysis (97). Extensive studies on STL have been performed since its isolation, and it was demonstrated that STL could stabilize the translocation state by preventing the conformational changes in the BH and TL during the nucleotide addition cycle, thus decreasing the rate of nucleotide addition (98, 99).

The cocrystal structure of STL with *T. thermophilus* RNAP showed that STL bound to the region adjacent to the active site, which is functionally involved in the nucleotide addition cycle (59, 98, 99). More specifically, the streptolol side chain of STL interacts with the BH and the "STL pocket," which is formed by *E. coli* β residues 538 to 552 and 557 to 576 (98), while the tetramic acid moiety binds to the TL, causing its displacement (Fig. 4C, dashed arrow). In fact, deletion of the TL can increase the binding affinity of *T. thermophilus* RNAP to STL (98). The sugar moiety substituted on the *N*-tetramic acid was not thought to interact directly with RNAP, but the cocrystal structure with an RNAP-DNA complex revealed that it forms a hydrogen bond with the downstream DNA and hydrophobic interactions with the DNA and TL (59). Analysis of the same cocrystal structure revealed that the downstream DNA also interacts with the streptolol side chain and the tetramic acid of STL.

Although STL was shown to bind multiple sites within RNAP, most of the interactions were hydrophobic van der Waals forces (59, 98, 99), except for the interaction of the streptolol ring with β fork loop II residue R548 and the interactions of the acetamide substitution of tetramic acid with β′ BH residues K789 and N792 (59). It was demonstrated that *E. coli* RNAP with a β subunit R548A substitution was resistant to STL, and enzyme with an N792D substitution in the β′ subunit was 75 times more sensitive than the wild type to STL due to enhancement of the polar interaction with acetamide (98).

Bacterial resistance to STL was first observed many years ago, and a saturation mutagenesis study isolated 72 independent resistant mutations on 26 residues, suggesting that it may not be a good antibiotic for further development, despite its specificity for bac-

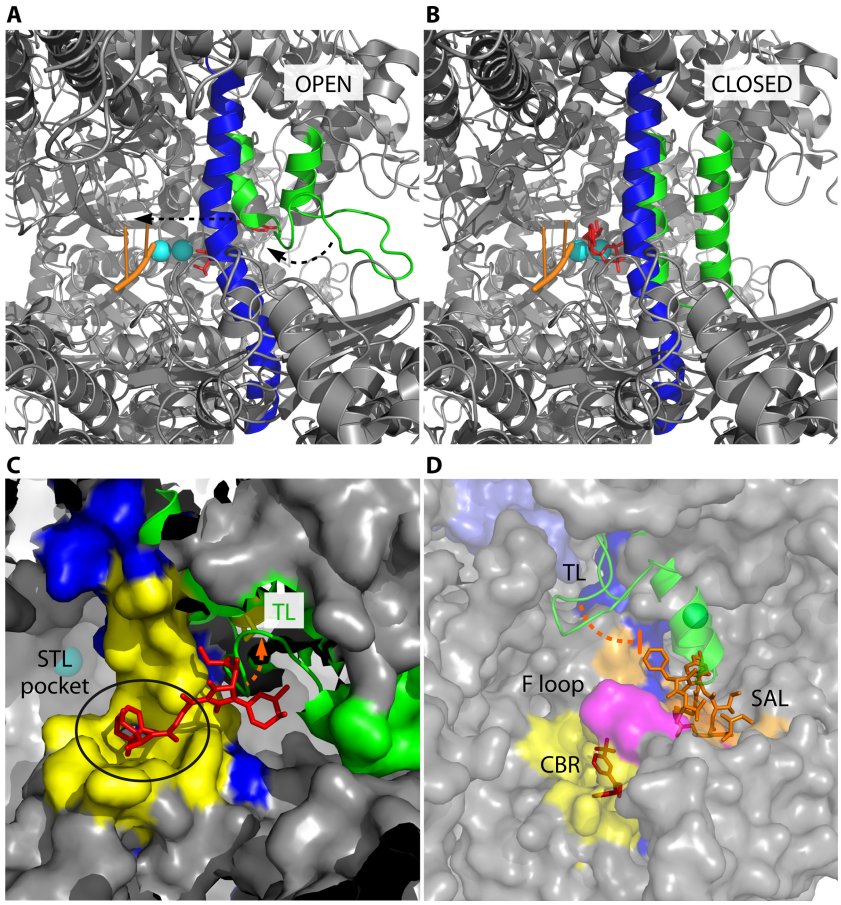

FIG 4 Inhibitors that target the bridge helix and trigger loop. (A and B) Bridge helix (BH) (blue) and trigger loop (TL) (green) in alternative conformations. The BH and TL colors are conserved in all panels in this figure, and active-site Mg^{2+} ions are shown as cyan spheres. Bending of the BH in panel A, indicated by the dashed straight arrow, aids translocation of the transcript to help incorporation of the incoming NTP, shown in red behind the BH. The TL is in the "open" conformation, allowing NTP entry into the active site. In panel B, the BH is straight and the TL has formed extended α helices behind the BH, forming a "closed" conformation that helps position the incoming NTP (red) in the active site. The movement of the TL from an open to a closed conformation is indicated with the curved dashed arrow in panel A. (C) Streptolydigin (STL) bound in a space-filled model of RNAP. Amino acids involved in interaction with STL are colored yellow. The STL pocket formed by the BH and β fork loop II is circled. The TL in the open conformation (shown as a cartoon) clashes with the tetramic moiety of STL, providing an understanding of how its deletion can stabilize STL binding. (D) Salinamide (SAL) bound within the secondary channel adjacent to the TL and CBR703 bound adjacent to the F loop (magenta). The binding site for SAL is shown in orange and that for CBR703 in yellow. RNAP surface and cartoon elements are shown in semitransparent form so that CBR703 within its binding site can be seen. Conformational change of the TL from the open to the closed form (transparent cartoons) will be sterically blocked by SAL. Structure images were prepared using PDB files 1IW7, 1ZYR, 2O5J, 4G7O, 4MEX, 4OIP, 4XSX, and 4ZH2 in PyMol v1.7.4 (Schrödinger, LLC).

terial RNAP (59, 100, 101). However, the information obtained from studies of STL binding to RNAP may help with the development of RNAP inhibitors targeting the nucleotide addition cycle.

Salinamide

Salinamides A to E are a series of bicyclic polypeptidic molecules isolated from marine and soil *Streptomyces* spp. (102–104). Salinamides A and B (Fig. 2) displayed good inhibition of transcription in both Gram-positive and -negative organisms and with moderate antibacterial activity, which was probably due to the hydrophilic peptidic structure having poor membrane-crossing properties (104). The cocrystal structure of salinamide A with *E. coli* RNAP holoenzyme demonstrated that it bound to a region between the BH and secondary channel (Fig. 4D) and involved interaction with β′ residues R738, R744, L746, M747, A748, S775, and K781 and β residues D675 and N677. These amino acids are not conserved in eukaryotic RNAP, accounting for the specificity of this antibiotic against bacteria (105). A mutagenesis study to identify amino acid substitutions conferring salinamide A resistance correlated well with the information obtained from the cocrystal structure. Substitutions in β′ BH residues R738, A779, and G782 and β residues D675 and N677 result in resistance to salinamide A.

During the nucleotide addition cycle, salinamide A may also interact with the TL due to conformational changes in RNAP (105). Although the binding site of salinamide A was different from that of STL and did not show cross-resistance with other RNAP inhibitors, functionally salinamide A was similar to STL, as it did not prevent the formation of a transcription open complex but was able to inhibit transcription initiation and elongation, probably by prevention of TL refolding due to steric interference (Fig. 4D, dashed line).

Although salinamide A is not suitable for further clinical development due to poor membrane permeativity, the binding site close to the secondary channel and nucleotide addition site represents an interesting validated target for new drug design.

CBR Compounds

The CBR series (Fig. 2) consists of small molecules comprising two linked aromatic rings, isolated by screening a chemical compound library to inhibit *E. coli* transcription (106). CBR703, together with CBR9379 and CBR9393, demonstrated the ability to inhibit transcription elongation by stabilizing elongation complex isomerization and slowing translocation (107). The inhibitory activity of these compounds is proposed to be due to an allosteric effect that prevents TL folding, mediated via the F-loop, and the inhibition of BH movement at its N-terminal hinge (108). An interesting aspect of their antibacterial activity is their ability to inhibit biofilm formation, which is especially important in a clinical setting as biofilms are a major source of nosocomial infection (109).

Recent X-ray crystal structures show that CBR703 and several derivatives bind to *E. coli* RNAP between β lobes 1 and 2 to regions linking the β flap loop II and βDII motifs, the β′ F-loop, and the N-terminal portion of the β′ BH (Fig. 4D) (108, 110). Specifically, CBR703 forms interactions with β residues V550, H551, P552, Y555, R637, G640, E641, and S642 and β′ residues K749, P750, I755 (part of the F-loop), P770, F773, I744, and H777 (BH N terminus) (108). Most of these interactions are hydrophobic, with only β S642 making a polar interaction with the amidoxime moiety of CBR7903 (108, 110). The structural information agrees well with data inferring the binding site from analysis of resistance mutants (at β P560, R637 and S642) (106), and this was further elaborated by saturation mutagenesis experiments that identified a further 11 resistance alleles (110). Despite resistance arising at 14 separate loci, the rate of resistance due to spontaneous mutation in one tested strain was extremely low at $<1 \times 10^{-12}$, which is 10^2- to 10^4-fold lower than those for other antibiotics, suggesting that the CBR binding site may have potential as a useful resistance-refractory binding site (110). Interestingly, two mutations (β′ P750L and β′ F773V) make cells dependent on CBR compounds for growth as well as conferring resistance, although the mechanism for this is currently unknown (108). The predicted structure of CBR703 bound to RNAP determined by *in silico* docking was very similar to what was found in the X-ray crystal structures, except it was inserted into the two hydrophobic pockets the wrong way around (107, 108, 110). This is not surprising since CBR703 is highly symmetrical and brominated derivatives were needed to unambiguously determine the orientation of the compound in the structural experiments (108, 110).

Despite the promise of the CBR family, significant hurdles still need to be overcome before they can be considered bona fide antibiotic leads. Although these compounds have good activity against RNAPs from problem Gram-negative organisms, including *Klebsiella pneumoniae*, and are reported to have activity against Gram-positive *Staphylococcus aureus*, despite the presence of N rather than S at the β642 position that is essential for binding and specificity, they have antibacterial activity only against strains in which the TolC outer membrane drug efflux pump has been deleted (53, 108, 110). In addition, despite not inhibiting human RNAP II activity *in vitro*, the compounds are cytotoxic (53). The fact that they are hydrophobic compounds and bind serum components may have affected assays that suggest that CBR703 has no effect on human tissue culture cell lines at concentrations as high as 100 μM (53, 110). The inhibitory effect on biofilm formation may also be an artifact, as it occurs at high concentrations (2 to 400 μM) when CBR703 starts to precipitate at 100 μM, and the hydrophobic aggregates may have prevented biofilm formation (53). Nevertheless, the information gained from the CBR compounds may provide approaches for development of allosteric inhibitors of transcription that bind the same site. The close proximity (~8 Å between the closest atoms) of the CBR703 and salinamide binding sites may also permit fragment-based development of bipartite inhibitors.

SECONDARY-CHANNEL INHIBITORS

During transcription, NTP substrates need to be made available for the growing RNA chain, but the active site is buried deep inside RNAP at the apex of the primary channel (Fig. 1A). The funnel-shaped secondary channel is formed by the β′ BH spanning the DNA binding clamp immediately downstream of the active site (36). The secondary channel is about ~12 Å in diameter, which is too narrow for double-stranded nucleic acids and allows the passage of only one NTP at a time, and the negative electrostatic potential that predominates in the secondary channel imposes a further constraint on NTP diffusion (55). Several transcription factors regulate RNAP activity through interaction with the secondary channel, such as the Gre factors DksA and Rnk (111–114). For example, in a backtracked elongation complex, the 3′ end of the RNA transcript slides into the secondary channel, and the acidic tips of the Gre factors penetrate into the enzyme close to the active site and facilitate an intrinsic RNA cleavage activity of RNAP (115). The backtracked RNA transcript is then released from the secondary channel, allowing the stalled transcription elongation complex to be rescued (116).

Tagetitoxin

Tagetitoxin (Fig. 2) was isolated from a culture of the plant pathogen *Pseudomonas syringae* pv. *tagetis* and was expected to have broad-spectrum activity on RNAP (117). A series of studies showed that it targeted chloroplast RNAP and RNAP III (but not RNAPs I and II) and could also target bacterial RNAP by inhibition of transcription elongation (118, 119). Despite this *in vitro* activity, tagetitoxin has no antibacterial activity against live cells due to low cell permeativity (120).

The structure of tagetitoxin in complex with *T. thermophilus* RNAP (Fig. 5A) shows that it binds in the secondary channel mainly via polar interactions due to its high oxygen content, which also results in poor membrane permeativity of the compound (120). β′ residues N458, Q504, S733, and Q736 are involved in binding to the bicyclic ring of tagetitoxin, while β residues R678 and R1106 and β′ residue R731 interact with the phosphate group, which also binds to a noncatalytic Mg^{2+} ion (121). It has also been proposed that tagetitoxin is capable of interaction with the TL region (Fig. 5A, closed form, green cartoon), which could account for its inhibitory effect on transcription, but this is a currently unresolved issue (121–123). While tagetitoxin is not an antibiotic, further studies to resolve its mechanism of inhibition may provide important information for the development of new leads that have a similar binding site. The structure of tagetitoxin has also been revised recently (124) and is the structure presented in Fig. 2, which may help with the development of new

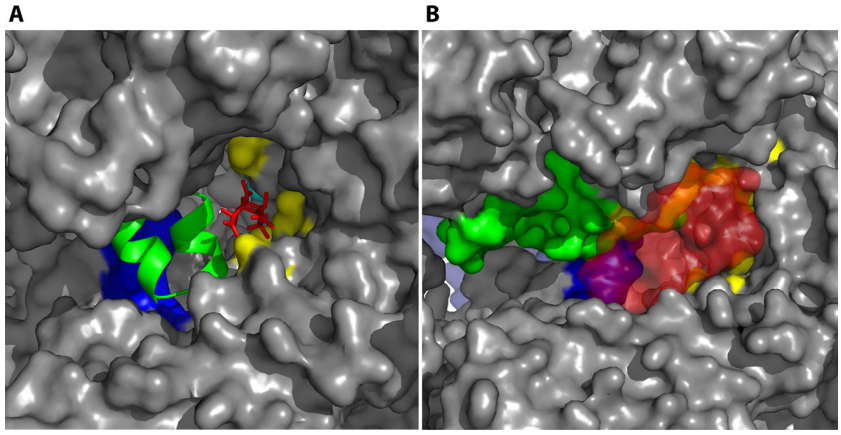

FIG 5 Secondary-channel binding compounds. (A) Tagetitoxin bound inside the secondary channel. The binding site is shown in yellow with the TL (green) in the closed conformation as a cartoon to aid visualization of tagetitoxin, and the BH is shown in blue. (B) Binding site for microcin J25 in yellow, with the TL surface rendered in the open conformation. No structure of microcin J25 in complex with RNAP is available, but modeling indicates that it would block the secondary channel, preventing NTP entry (rendered as a semitransparent red surface). Structure images were prepared using PDB files 1PP5 and 2BE5 in PyMol v1.7.4 (Schrödinger, LLC).

leads through reexamination of electron density maps of the RNAP-tagetitoxin structure along with structure-activity relationship analysis.

Microcin J25

Microcin J25 (Fig. 2) is a 21-mer peptide produced by *E. coli* AY25 that is active against Gram-negative bacteria (125, 126). It has a very unusual lassoed tail structure comprising a ring with the C-terminal end passing through the center (127–129). It inhibits abortive initiation and elongation by competitively preventing NTP uptake or binding (130). Saturation mutagenesis identified substitutions on 51 residues in the regions of the β and β′ subunits that form the secondary channel, which result in resistance to microcin J25. The binding site was proposed to comprise β′ residues 498 to 504, 732 to 733, 922 to 926, and 1244 to 1248 (Fig. 5B) (130). The inhibitory constant (K_i) of microcin J25 was also measured to be approximately 20 μM (131), but due to the relatively low potency, narrow spectrum, and very large number of substitutions that result in resistance to the compound, it is not particularly amenable for further development.

SWITCH REGION INHIBITORS

The overall architecture of the RNAP crab claw undergoes a series of conformational changes to allow template DNA to access the catalytic center. One of the pincers of the claw formed by part of the β′ subunit (termed the clamp region) has been shown to exhibit significant structural flexibility (26, 74) and is hinged, which enables widening of the claw, helping to load DNA into the active site. The clamp then swings back to close the active channel in order to retain the DNA template in the transcription bubble during transcription elongation. The swing motion of the clamp is regulated by the switch region, which is located at the base of the clamp domain (15, 76) (Fig. 1C and D). There are five segments of the switch region, termed "switch 1" to "switch 5." In particular, switches 1 and 2 undergo relatively large conformational changes to mediate clamp movement (132). Important residues of the switch region form direct interactions with the DNA template as well as the nascent RNA transcript, serving regulatory roles in DNA loading/unwinding and clamp closure during initiation and elongation (41).

Myxopyronin, Corallopyronin, Ripostatin, and Squaramides

Myxopyronins (Fig. 2) were isolated from cultures of *Myxococcus fulvus* and displayed broad-spectrum antibacterial activity against Gram-positive and -negative bacteria by inhibiting transcription initiation (133, 134). Mechanistic studies showed that myxopyronins bind to the switch region and lock the clamp and switch regions in a partly closed/fully closed conformation. This prevents the clamp from opening and RNAP from binding double-stranded DNA during the transition from a closed to an open complex during transcription initiation (132, 135).

The binding site of myxopyronin A and 8-desmethyl myxopyronin B on *T. thermophilus* RNAP has been determined and shown to comprise a hydrophobic pocket formed by "switch 1," "switch 2," and adjacent segments, including β residues 1270 to 1292 and 1318 to 1328 and β′ residues 330 to 347, 1319 to 1328, and 1346 to 1357 (132, 135). Although the switch region is conserved across prokaryotic and eukaryotic RNAPs, the adjacent segments are conserved only in bacterial RNAPs, which may explain the specificity of myxopyronins against bacteria (132, 133). It was shown that β E1272, E1279, and S1322 and β′ G344, K345, and K1348 were involved in polar interactions with both myxopyronin A and 8-desmethyl myxopyronin B. Mutagenesis studies demonstrated that amino acid substitutions at β residues E1279 and S1322 and β′ residue K345 generated resistance against 8-desmethyl myxopyronin B, while changes at β V1275 and L1291 and β′ K345 resulted in resistance to myxopyronin A (132, 135).

It is worth noting that corallopyronin, which is structurally similar to the myxopyronins, as well as the structurally distinct ripostatin, demonstrated the same inhibitory activity on transcription initiation, and the amino acid substitutions that resulted in resistance to these molecules also overlapped (136–138). These results indicated that corallopyronin and ripostatin also bind to the switch region (132).

Squaramides (Fig. 2) are a series of synthetic small molecules identified in a high-throughput screen using a bacterial transcrip-

tion-coupled translation assay with an *in vitro* 50% inhibitory concentration (IC_{50}) as low as 0.3 μM (51). It was then demonstrated that these compounds were inactive against the translation process by substituting mRNA for plasmid DNA. The compounds had no activity against representatives of the ESKAPE pathogen group (139) but did show weak activity (MIC values of 100 to 200 μM) in wild-type *Haemophilus influenzae* that could be improved on deletion of the *acrB* gene, which encodes a component of the drug efflux system.

These squaramide derivatives comprise an isoxazole containing a sulfonamide substitution on a four-member squarate ring, as well as a benzylamine moiety and a piperidine as its linker to the squarate (Fig. 2, circles 3, 4, and 5, respectively). Cocrystal structures of two squaramides with *E. coli* RNAP holoenzyme have recently been published, which showed that their major binding site was broadly similar to that of the myxopyronins within the switch 1 and 2 regions, with less substantial interaction with switch regions 3 and 4 (140). The cocrystal structures also demonstrated that the binding of squaramide compounds to RNAP pushed switch 2 into the DNA binding main channel of RNAP, which would prevent the correct positioning of the melted template DNA. Mutagenesis studies using *H. influenzae* RNAP confirmed that β′ residues A1323, L1332, and K1348 and β residue L1326 (*E. coli* numbering) interacted directly with squaramides, while mutations at β residues Q1257, E1279, D1296, and P1320 may also affect squaramide binding (51). Squaramides have potential for drug development, as they are small synthetic molecules and have plenty of options for structural elaboration and because the cocrystal structures with RNAP showed that they did not completely occupy the area encompassing the switch region.

Fidaxomicin and Lipiarmycin

Fidaxomicin (tiacumicin B) (Fig. 2) is a derivative from fermentation products of *Dactylosporangium aurantiacum* and is structurally very similar to lipiarmycin, a metabolite from *Actinoplanes deccanensis* (141–143). Both of these compounds target bacterial RNAP and exhibit antibacterial activity selectively against Gram-positive bacteria, and fidaxomicin is now used for the treatment of *Clostridium difficile*-associated diarrhea (CDAD), with efficacy similar to but relapse rates lower than those for vancomycin (144).

There is currently no published structure of fidaxomicin or lipiarmycin in complex with RNAP, but biochemical experiments indicated that both of these molecules bind to the switch region (136, 145, 146). Lipiarmycin did not prevent promoter binding but could inhibit promoter DNA melting and σ-dependent transcription, as well as template strand DNA binding to RNAP. A mutagenesis study demonstrated that deletion of σ^{70} subunit region 3.2 residues 513 to 519 of *E. coli* RNAP and mutation at switch 2 residue R337 resulted in resistance to lipiarmycin, suggesting that it might target both the σ factor and the switch region to prevent formation of a transcription open complex (145). However, despite having analogous structures and the fact that amino acid substitutions at appropriate sites result in cross-resistance between lipiarmycin and fidaxomicin, fidaxomicin is probably a more promising compound, as deletion of the σ_3 loop does not result in resistance to the drug (146). These results indicate that the way in which these molecules function to inhibit transcription is yet to be fully elucidated.

INHIBITORS WITH UNKNOWN TARGETS

Ureidothiophene was discovered though chemical library screening against *S. aureus* RNAP holoenzyme and demonstrated good antibacterial activity against Gram-positive bacteria, including *S. aureus* and *S. epidermidis* (147). However, mammalian albumin binds tightly to ureidothiophene, eliminating its *in vivo* activity.

Thiolutin and holomycin are types of pyrrothine natural products isolated from *Streptomyces luteosporeus* and *Streptomyces clavuligerus*, respectively (148, 149). Both compounds demonstrated broad activity against bacterial and fungal RNAPs, and functional analysis showed that that they could inhibit bacterial transcription elongation and yeast transcription initiation (149–151).

Etnangien is a natural antibiotic isolated from the Gram-negative soil bacterium *Sorangium cellulosum*, a member of the myxobacteria with a particularly large (>13-Mb) genome that produces many secondary metabolites with a wide range of potential medical applications. Etnangien displayed activity against Gram-positive bacteria with MICs as low as 0.06 μg/ml but with no effect against Gram-negative organisms (152). Although *E. coli* was shown to be resistant to etnangien, *in vitro* inhibitory activity against *E. coli* DNA polymerase and RNAP and low cytotoxicity against a mammalian cell line was observed.

TRANSCRIPTION FACTORS AND RNAP BINDING SITES AS TARGETS

Transcription Factor Overview

Each step of the transcription cycle is highly regulated by transcription factors during normal cell growth and in response to environmental signals. To start transcription at the appropriate site, RNAP must be associated with an initiation factor. Most bacteria have one primary σ factor for the transcription of housekeeping genes (σ^{70} in Gram-negative bacteria and σ^{A} in Gram-positive bacteria) and one or more alternative σ factors for transcription of specific subsets of genes in response to environmental stimuli (153, 154). In the elongation phase, the σ factor dissociates from RNAP while elongation factors become associated (155). Elongation factors represent a diverse range of proteins that regulate transcription activity, such as modulating transcription rates, the response to pause and termination signals, rescue of stalled/backtracked complexes, and removal of protein roadblocks (156). In addition, the compositions of transcription complexes (TCs) involved in mRNA and rRNA synthesis are quite different (157, 158). TCs involved in rRNA synthesis are resistant to pausing signals and transcribe at higher rates than those involved in mRNA synthesis. The difference in responses of RNAP to pause signals, etc., during mRNA and rRNA synthesis is because of its association with different suites of elongation factors (157, 159, 160). Many of these transcription factors are essential for bacterial viability and are not highly conserved in eukaryotic cells. Therefore, the factors as well as their binding sites on RNAP represent potential novel drug targets (Fig. 6).

Termination Factor Rho

The bacterial transcription termination factor Rho is a ring-shaped homohexameric RNA translocase. Each Rho protomer has two distinct functional domains: the N-terminal oligonucleotide/oligosaccharide binding (OB) domain, which constitutes Rho's primary RNA binding site, and the C-terminal domain with a secondary RNA binding and ATP binding site (29, 161). The Rho

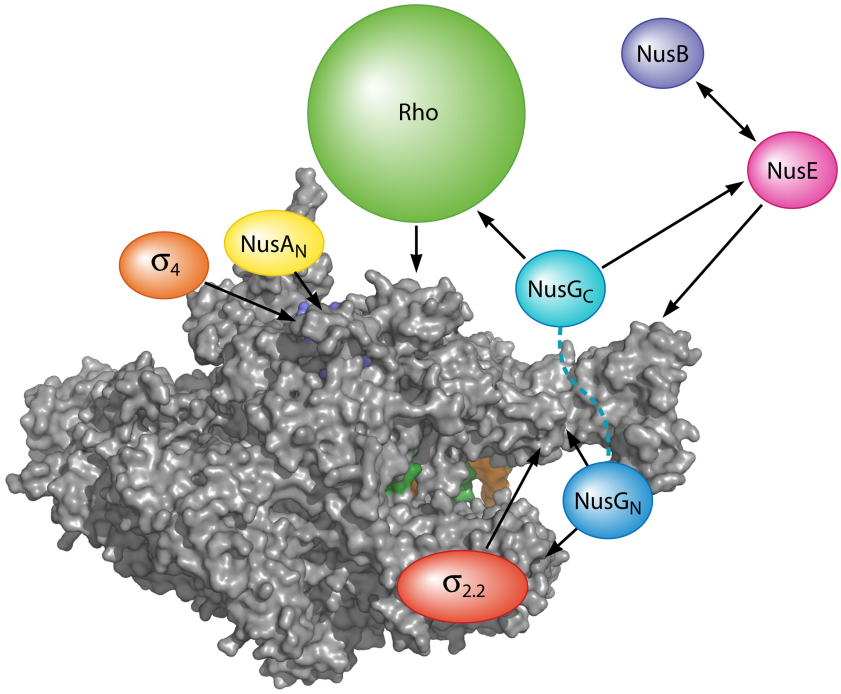

FIG 6 Transcription factor interaction network. The space-filled RNAP elongation complex is shown in gray, with template-strand DNA in green and nontemplate-strand DNA in orange. Arrows indicate known interactions, although the binding sites for Rho and NusE on RNAP are not known. Subscript N and C indicate N- and C-terminal protein domains. NusG$_N$ binds to the CH region of RNAP and across the active channel and has an overlapping binding site with region 2.2 of initiation factor σ. NusG$_C$ can partner switch between Rho and NusE as appropriate. NusE forms a heterodimer with NusB and binds RNAP. NusA$_N$ and region 4 of σ have overlapping binding sites on the β flap tip helix at the top of the RNA exit channel. All of these interactions could potentially be exploited in the development of new antimicrobial compounds. Structure images were prepared using PDB file 2O5I in PyMol v1.7.4, Schrödinger, LLC.

hexamer exists in two forms, as an open or a closed ring. Open-form Rho binds to a specific Rho utilization site (*rut* site) on the RNA transcript via its N-terminal primary RNA binding site, and the newly synthesized RNA is then guided through the primary binding site into the central hole of the hexamer, where the ring closes and RNA binding triggers ATP hydrolysis (29). It is still unclear whether Rho binds to RNAP throughout/for extended periods of the transcription cycle or only during the final stages of termination, but direct interaction with RNAP appears to be required to induce allosteric changes in the active site of RNAP to terminate transcription (162).

Bicyclomycin. Bicyclomycin (Fig. 2) was isolated from *Streptomyces sapporonensis* and is active predominately against Gram-negative bacteria (163), although it is reported to be more effective against problematic nosocomial infections such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* when used in combination with inhibitors of translation (164). While Rho is essential for viability in many Gram-negative organisms, Gram-positive organisms appear to be less dependent on it, although it is important in preventing transcription of noncoding RNA in the latter and has activity against *Micrococcus luteus* (165, 166). Bicyclomycin is the only known antibiotic to target Rho, and it does not affect RNA or ATP binding to Rho but does inhibit Rho-dependent transcription termination and ATP hydrolysis (167–169). Substitutions in *E. coli* Rho residues L208, M219, S266, and G337 conferred resistance to bicyclomycin, suggesting that Rho was the binding target of bicyclomycin (170, 171).

A cocrystal structure of *E. coli* open-form Rho and bicyclomycin indicated that the binding site was between two C-terminal domains of Rho protomers, close to the ATP binding site (Fig. 7A) (172). The bicyclomycin binding pocket is formed by residues E211, R212, D265, S266, and R269 of one protomer and G337 of the adjacent protomer, while bicyclomycin also interacts with the associated Mg^{2+} ion that is required for ATP hydrolysis (Fig. 7B).

The RNAP-σ Interaction

σ factors are required for initiation of transcription. In all bacteria, the general housekeeping σ factor (σ^{70} in Gram-negative bacteria and σ^{A} in Gram-positive bacteria) is absolutely essential for cell viability (173, 174). σ factors are flexible elongated proteins that make multiple contacts with RNAP when forming a transcription initiation complex. However, only one region of the σ factor, called $\sigma_{2.2}$, is absolutely necessary for initiation complex formation, and it binds to a region of the β′ subunit of RNAP called the β′ clamp helix (CH) region (175–177) (Fig. 1B). The $\sigma_{2.2}$-CH interaction involves β′ residues R275, R278, R281, L282, I291, N294, and M298 and σ^{70} residues Q403, Q406, E407, I410, and M413 (37, 38, 176, 177), but this is a nontraditional drug target as it lacks any deep clefts, which are considered desirable binding sites for small molecules. Nevertheless, a peptide-based study showed that peptides mimicking $\sigma_{2.2}$ could inhibit RNAP-σ binding to prevent *in vitro* transcription initiation, and prevention of σ production by antisense peptide nucleic acids could inhibit the growth of clinically relevant multidrug-resistant *S. aureus* (178, 179).

The GKL series. The GKL series (Fig. 2) is a class of bis-indole-

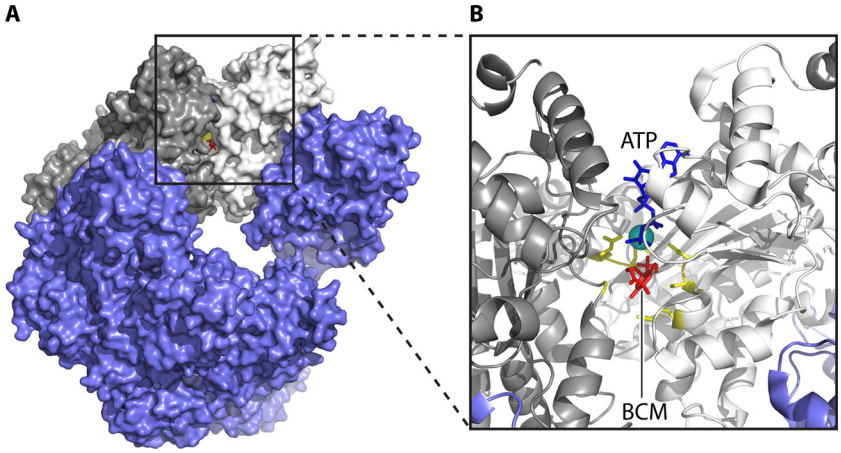

FIG 7 Inhibition of transcription termination factor Rho activity. (A) The Rho hexamer in its open-ring conformation. Four subunits are shown in slate blue with the remaining subunits in gray and white, sandwiching the Rho inhibitor bicyclomycin (red sticks). The bicyclomycin binding site is boxed. (B) Expanded view of bicyclomycin binding site area, with protein shown as ribbons. The bicyclomycin binding site is highlighted in yellow, with contributing amino acids shown as sticks. ATP is shown in blue in stick format and Mg^{2+} as a cyan sphere. Structure images were prepared using PDB file 1XPO in PyMol v1.7.4 (Schrödinger, LLC).

derived compounds targeting bacterial transcription based on the strategy of structure-based drug design (177). Detailed binding studies enabled the characterization of the precise contributions of individual amino acids to the RNAP-σ interaction (176, 177). This resulted in the construction of a pharmacophore model which was used to screen an in-house compound library. Careful selection of potential hit compounds resulted in ~1/3 having potent *in vitro* inhibitory activity against the formation of RNAP-σ complexes (176, 177, 180).

GKL003 was shown to specifically bind to the β′ subunit CH region and had a K_i of ~6 nM as determined from *in vitro* transcription assays (177). Compared to fidaxomicin, which functions only when added prior to RNAP-σ interaction, GKL003 is a competitive inhibitor which can also inhibit transcription initiation after the holoenzyme has formed (177). Despite having excellent activity *in vitro*, GKL003 had poor activity against live cultures of bacteria, probably due to its low solubility in aqueous media and inability to cross bacterial membranes. Nevertheless, it did exhibit broad-spectrum antibacterial activity at high concentrations and has been extensively derivatized to establish structure-activity relationships (181–183). Determination of the structures of GKL compounds in complex with RNAP will be important for their continued development.

DSHS00507. Using structure-based drug design, an *in silico* screen of a publically available drug-like compound library led to seven hits, with one, DSHS00507 (Fig. 2), showing excellent inhibitory activity against RNAP holoenzyme formation (184). This compound, comprising a steroid-like ABC tricyclic carboxylic acid and an indolone (Fig. 2, circles 6 and 7, respectively), binds to the β′ subunit CH region of RNAP with great specificity and inhibits *in vitro* transcription by preventing σ^A binding. Antibacterial assays indicated that the compound was effective against Gram-positive bacteria, including community-acquired methicillin-resistant *Staphylococcus aureus*, and microscopic analysis gave results consistent with RNAP being the target for this compound in live cells. Although eukaryotic RNAPs also contain a CH structure, it shows no sequence similarity to the bacterial CH, and DSHS00507 showed no activity against eukaryotic RNAP in *in vitro* binding assays or in cell culture, suggesting that this compound will be a potentially useful lead for future development.

The SB series. The SB series (Fig. 2) was discovered by compound library screening against *E. coli* RNAP holoenzyme formation using an enzyme-linked immunosorbent assay (ELISA)-based assay (185). These compounds could inhibit *E. coli* σ^{70} binding to RNAP with an IC_{50} of 2 to 15 μM, although their binding site was unknown. The σ-independent transcription inhibition activity of the SB series suggested that the compound binding site was on RNAP rather than σ (186).

A modified SB series of furanyl rhodanine compounds demonstrated activity against Gram-positive bacteria and inhibited formation of biofilm (109). However, it was shown that the SB series could target multiple targets on the membrane and within bacteria instead of acting as a specific inhibitor of transcription (52), making any further development complicated until comprehensive structure-activity relationship studies are performed.

Potential Targets

NusA. As an essential transcription factor, NusA is tightly associated with RNAP throughout the elongation phase of transcription (187) and is important for the regulation of several aspects of transcription elongation, including pausing, termination, and the formation of termination-resistant (antitermination) complexes essential for the production of rRNA (157, 188). Recent biochemical work found that NusA is involved in the "immune system" in *E. coli* host cells to suppress the toxic activity of foreign genes (189). NusA has also been shown to interact with translesion DNA synthesis polymerases and to play a role along with the UvrD helicase in nucleotide excision repair (190, 191). Residues F59, R61, E94, and Q96 of the N-terminal domain of NusA are required for interaction with L895, E899, K900, R903, and E908 on a conserved region of RNAP called the β-flap tip helix (Fig. 1D), which forms one side of the RNA exit channel, although the structure of the complex at high resolution is yet to be elucidated (44, 192, 193). NusA is highly conserved among the eubacteria, is

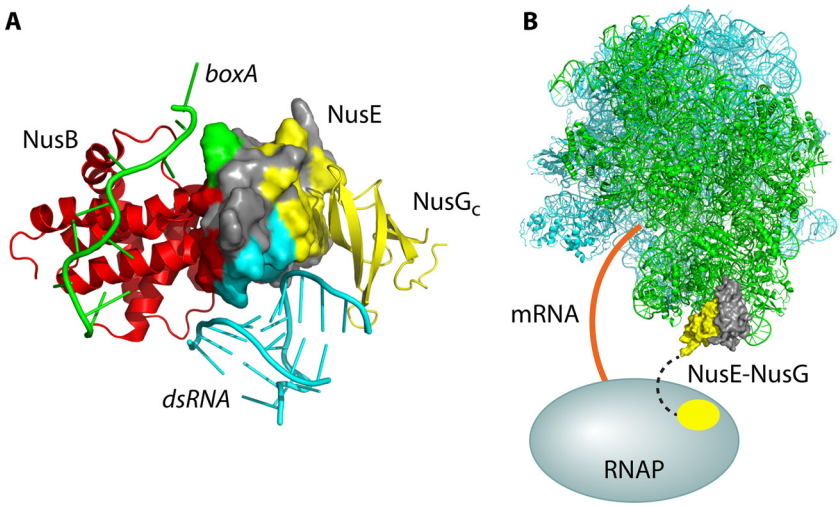

FIG 8 NusE is an "interaction hub" that links transcription and translation. (A) Nonoverlapping interactions that NusE makes with NusB (red), NusG$_C$ (yellow), *boxA* RNA from an rRNA operon leader (green), and double-stranded RNA (dsRNA) (cyan), representative of the *boxB* hairpin from an rRNA operon leader. (B) Location of NusE (gray) (also known as ribosomal protein S10) in the context of a translating ribosome. The 30S subunit is shown in green and the 50S subunit in cyan. NusG$_C$ (yellow) would not be sterically inhibited from interacting with NusE integrated into the 30S ribosomal subunit and so could be important in linking transcription with translation. Structure images were prepared using PDB files 2JVV, 2KVQ, 3CXC, 3D3B, 3R2C, and 3R2D in PyMol v1.7.4 (Schrödinger, LLC).

unique to this kingdom, and is essential for viability in all organisms tested (173, 174, 194, 195), indicating its importance in transcription and making it an ideal target for the development of new antitranscription compounds that prevent its interaction with RNAP.

NusB/E. In bacteria, rRNA is the most abundant RNA species in the cell and the major component of the ribosomes, and its rate of synthesis is growth rate limiting. NusB and NusE are small transcription factors required for the formation of antitermination complexes that carry out rRNA synthesis (158, 188, 196). NusE is ribosomal protein S10 and so has dual functions in the cell as both a transcription factor and a component of the small ribosomal subunit. Antitermination complex assembly initiates when NusB binds to a newly transcribed conserved RNA sequence in the rRNA operon leader sequence called *boxA*. Next, a NusB-NusE heterodimer is formed to strengthen the interaction with *boxA* and facilitate binding of other regulatory factors, and the complex interacts with RNAP via NusE (188, 197). Therefore, formation of the NusB-NusE-*boxA* complex represents a regulatory step in antitermination complex assembly (198). The NusB-NusE heterodimer is also able to bind double-stranded RNA (199) and *in vivo* may bind the RNA hairpin *boxB*, which is also part of the leader sequence of rRNA operons, although it does not appear to be important for viability (200). Since rapid cell proliferation is a characteristic of bacterial infection, small molecules that disrupt NusB-NusE heterodimer formation or interaction with *boxA* or that inhibit NusB-NusE-*boxA* interaction with RNAP have potential as lead compounds for novel antibiotics (Fig. 6). While the structure and interactions between NusB, NusE, and RNA (both *boxA* and dsRNA) have been determined (199, 201) and NusE is known to bind to the β subunit of RNAP (202), its precise binding site on RNAP remains to be determined. NusE also interacts with the C-terminal domain of NusG (NusG$_C$) (see below). This small protein is therefore able to interact with a wide range of proteins and nucleic acids, with very little overlap between the binding sites (Fig. 8A). Indeed, the context of NusE/S10 within the small ribosomal subunit leaves the NusG$_C$ binding site available for interaction and has been suggested to be important in linking transcription and translation in bacteria (Fig. 8B) (203).

NusG. As the only conserved transcription factor across all three domains of life (204), bacterial NusG exerts diverse, or even opposite, functions in the regulation of gene expression. NusG is an essential protein in Gram-negative bacteria (173, 195) and is involved in enhancing factor-dependent termination by termination factors such as Rho and bacteriophage HK022 Nun (205, 206). Together with Rho, NusG is responsible for limiting the expression of toxic foreign genes on cryptic phages integrated in the *E. coli* chromosome (189). On the other hand, NusG has been shown to increase the transcription elongation rate both *in vitro* and *in vivo* by preventing transcription pauses incurred by RNAP backtracking (207–209). In conjunction with other Nus factors (NusA and NusB/E; see above), NusG participates in the formation of antitermination complexes required for the rapid and efficient transcription of rRNA operons (188). NusG has also been suggested to have a role in coupling transcription and translation (Fig. 8B) (203). NusG consists of an amino-terminal domain (NusG$_N$) joined by a flexible linker to the carboxyl-terminal domain (NusG$_C$) (210). As illustrated in Fig. 6, NusG$_N$ competes with σ factors for RNAP binding (211), while NusG$_C$ binds mutually exclusively to either Rho or NusE factors (203). By analogy to the interaction between the Spt4/5 in complex with the CH region from the archaeon *Pyrococcus furiosus* (212), the tip of the β′ CH region of RNAP binds to a hydrophobic cavity on NusG$_N$ comprising F65, P66, Y68, L70, I93, I103, E107, V108, and I111. Analysis of a NusB-NusE-NusG$_C$ tripartite complex by nuclear magnetic resonance (NMR) indicates that residues G139, P140, F141, S161, V162, I164, F165, G166, R167, A168, T169, E172, and D174 are involved in interaction with NusE

(203), whereas mutagenic studies imply a role for G146, V148, L158, V160, and I164 in interaction with Rho (213). While the residues involved in interaction with NusE are all solvent exposed, those implicated in binding to Rho are buried within the hydrophobic core of $NusG_C$. NusG is closely related to another specialized transcription factor, RfaH, whose C-terminal domain undergoes drastic rearrangement from a β-barrel form to a $NusG_C$-like conformation (214), and it is possible that $NusG_C$ undergoes conformational change upon binding to Rho, although this seems unlikely as circular dichroism spectra of wild-type and mutant NusG are all similar (213). The $NusG_C$ binding site on Rho is solvent exposed and is within two small depressions on the outer surface of the hexameric ring comprising hydrophobic residues 203 to 208 (VLMVLL), V227 and V228 (213). Small molecules targeting the $NusG_N$-RNAP interaction (with the possibility of also inhibiting σ-RNAP binding), as well as the $NusG_C$-Rho/NusE binding surface, would have the potential to inhibit the cellular function of the essential factor NusG in bacteria, although the apparent hydrophobic nature of the regions involved in the RNAP β′ CH-$NusG_N$ and Rho-$NusG_C$ interactions may prove difficult to target with specificity.

$NusG^{SP}$ factors. While essentially all microbes contain a gene encoding a NusG transcription elongation factor, many also contain paralogous NusG-like proteins that control transcription of small subsets of genes, and these have been called specialized NusGs ($NusG^{SP}$s) (215). The best characterized $NusG^{SP}$ is RfaH, which is involved in the control of transcription of genes required for extracytoplasmic functions such as lipopolysaccharide (LPS) synthesis, toxin production, and phage sensitivity (216). $NusG^{SP}$s bind to the same region of RNAP as NusG via their structurally conserved N-terminal domains (217), and their C-terminal domain (as determined for RfaH) undergoes a dramatic structural transformation into a structure analogous to the C-terminal domain of NusG, which permits interaction with NusE/S10, ensuring efficient translation (214). $NusG^{SP}$s are not essential, but since they are required for the expression of genes associated with virulence (e.g., LPS and toxin production), compounds that target their interaction with RNAP and/or NusE may be useful as antiinfective agents. However, given the similarity of $NusG^{SP}$ binding sites with NusG, the same problems with producing compounds of sufficient specificity may be encountered.

Helicases. Helicases are enzymes that catalyze the unwinding of DNA · DNA, RNA · RNA, and RNA · DNA hybrid molecules into their component single strands using the energy produced by the hydrolysis of a nucleoside 5′-triphosphate (218). Helicases are required for all aspects of nucleic acid metabolism/genome maintenance in both prokaryotic and eukaryotic cells, including transcription, RNA splicing, and translation initiation, as well as DNA replication, repair, and recombination (219). Although the role of helicases in eukaryotic transcription has been studied for many years (reviewed in reference 219), other than Rho, transcription in bacteria is not a process traditionally thought to require the activity of helicases to unwind or rewind DNA/RNA or DNA-RNA hybrids. In *E. coli*, UvrD is a superfamily 1 (SF 1) helicase involved primarily in nucleotide excision repair, a process whereby various DNA lesions are located and removed (220, 221). More recently, it has been shown in *E. coli* that UvrD is able to bind directly to transcribing RNAP to a level comparable to that for common transcription factors (such as NusA [191]). This is likely to be a highly conserved interaction, as the essential UvrD ortholog PcrA from *Bacillus subtilis* has also been shown to interact with its cognate RNAP (222–224). Once the elongation complex is stalled at DNA lesion sites, UvrD is able to pull RNAP backwards (in the direction opposite to that of transcription), exposing the damaged DNA for repair (191). *E. coli* RapA (HepA), a Swi2/Snf2 helicase (225), has been shown to be involved in RNAP recycling and RNA polyadenylation (226, 227), and in *B. subtilis*, HelD is required for efficient recycling of RNAP, which is achieved synergistically with the δ subunit (228). As our understanding of the roles of helicases in transcription increases, these important enzymes could also represent potential targets for the design of transcription inhibitors.

CONCLUSIONS AND PERSPECTIVES

The need for new antibiotics is pressing, and it is essential that new targets are identified, validated, and developed to ensure that alternative compounds are available to combat organisms that are resistant to currently available antibiotics. Transcription represents an excellent target for the continued development of important drugs, as it can be targeted through direct and allosteric inhibition of enzymatic activity or through inhibition of protein-protein/protein-nucleic acid interactions that are essential for viability. Historically, high-throughput screens of natural product libraries have been used in the discovery of potent antitranscription inhibitors, and this approach is still yielding compounds that have great promise as stand-alone compounds or in combination with existing inhibitors as novel chimeric antibiotic leads (see, e.g., reference 86).

More directed approaches are now also possible due to the rapid increase in high-resolution structural information on RNAP and transcription complexes that is becoming available. Chemical space can be explored for the design and development of new inhibitor compounds using more rational approaches that require the careful construction of templates (pharmacophores) around which inhibitors are constructed. Such approaches can be used in the design/modification of compounds that target the enzymatic activity of RNAP as well as protein-protein interactions.

There are advantages and disadvantages to both approaches. Through the process of natural selection, most natural products tend to have very good activity (low MICs) but are also subject to the coevolution of resistance mechanisms that can result in a short effective lifetime in the clinic. Furthermore, since current screens have tended to focus on discovery of compounds isolated from soil samples, which are dominated by Gram-positive organisms, few new compounds that target the Gram-negative organisms that are starting to dominate antibiotic-resistant infections have been identified. This is exacerbated by the fact that many high-throughput screens use strains of Gram-negative bacteria carrying *tolC* deletions (TolC is a component of Gram-negative efflux systems) or mutations that result in defective outer membranes, thus allowing easier access of compounds to the cytoplasm. Screening natural product libraries from other environments may well provide leads effective against both Gram-positive and -negative bacteria.

Compounds that are rationally designed, screened, and identified based on the production of a pharmacophore have the advantage that the target site is known, enabling the rapid development of compounds with high specificity. While such inhibitors often have excellent properties when used *in vitro*, they may require substantial modification in order to be developed into useful clinical compounds; the medicinal chemist is required to perform

in months/years the tasks that evolution has carried out on natural products over millennia.

History shows that there is a place for antibiotics derived from natural products (e.g., penicillin) as well as synthetic compounds (e.g., fluoroquinones). What is most important is the commitment and investment from government and industry to ensure that all avenues can be exhaustively explored to ensure the continued discovery and development of essential medicines. Targeting bacterial transcription is one area that offers a wealth of untapped opportunity.

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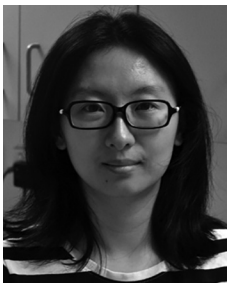

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